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Differentiation specific functions of the Bin1 SH3 domain

by

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A thesis submitted to the School of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Masters of Science in Growth and Development

Department of Cellular Molecular Medicine

Faculty of Medicine

University of Ottawa

May 13, 2002

Abstract

A significant percentage of breast and prostate tumours have a missing or aberrantly expressed Bin1 protein. However, preliminary work in skeletal muscle has shown that Bin1 may also be involved in regulating differentiation. Based on the variable roles attributed to Bin1, we proposed a hypothesis in which distinct domains of Bin1 regulate different functions of the protein. This study examines one particular domain of Bin1, the SH3 domain, through a dominant negative approach in a mouse model. Overexpression of the SH3 domain using a broadly expressed promoter resulted in a skeletal muscle phenotype that included large myofibre diameter *in vivo* and an inability to complete the differentiation process *in vitro*. Importantly, these deficits were not accompanied by disruptions in apoptosis, increased cell proliferation or tumorigenesis. These results suggest that Bin1 has distinct functions that are mediated by the modular SH3 domain and separate from its role in cell cycle regulation.

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aa	Amino acid
Amph	Amphiphysin
BAR	Bin1/Amphiphysin/RVS
bHLH	Basic helix-loop-helix
Bin	Bridging integrator
cdk	Cyclin dependent kinase
CKI	Cyclin dependent kinase inhibitor
CTD	C-terminal domain
CMV	cytomegalovirus
CNS	Central nervous system
DN	Dominant-negative
DNA	Deoxyribonucleic acid
dpc	Days post coitum
FBS	Fetal bovine serum
GTPase	Guanosine triphosphatase
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
Hob3	Homologue of Bin3
HPV	Human papilloma virus
HS	Horse serum

Inr	Initiator (element)
MB1, MB2	Myc box 1, 2
MBD	Myc binding domain
MCK	Muscle creatine kinase
MEF2	Myocyte enhancer factor 2
MHC	Myosin heavy chain
MRF(s)	Muscle regulatory factor(s)
NTD	N-terminal domain
NLS	Nuclear localization signal
PCD	Programmed cell death
PI	Propidium iodide
PRD	Proline rich domain
Rb	Retinoblastoma
RNA	Ribonucleic acid
RVS	Reduced Viability upon nutrient Starvation
SH3	Src homology 3
SMS	Stiff-Man Syndrome
SRF	Serum response factor
SV40	Simian virus 40
U1, U2, U3	Unique-1, 2, 3 (domains)

Acknowledgements

I wish to acknowledge and thank those people who contributed to this thesis:

Dr. Lynn Megeney, my supervisor, for his 'sink or swim' philosophy that forced me to swim, and the confidence he had to put a pipette in a PHE grad's hand.

Kim Balazsi, for being my lifeline, especially in my first few months, for her patience, untiring willingness to help, and for her ability to make sure we were laughing through all of it.

All the other 'Megeney's': Pasan Fernando, Andrée Hierlihy, Jennifer Knudson, Steve Kolodziejczyk, Julie Lalonde, Beata Pekalska, Andrea Rowan and Simon St. Pierre, for making the lab the best one on the floor, for ensuring humour was a predictable constant, for engaging in the requisite science discussions, and for not getting mad at me during troubleshooting sessions when "I knew that for a long time". A special thanks to Pasan who was the other half of Bin1, for starting our lab in the primary culture business, for his expertise in protein work, and for the Bin1 discussions that were instrumental in getting my head around what Bin1 bin doing.

All the post docs, Céline Boudreau-Larivière, Patrice Côté, Christine DiDonato, Pasan Fernando, Steve Kolodziejczyk and Ron Saulnier for doing the part of supervising that supervisors can't do.

All of the 'Kothary's' for bailing me out when I needed a reagent and for putting up with the 'Megeney's' in general...and of course, Yves De Repentigny for having the patience and perseverance to inject countless numbers of pronuclei for the Bin1 transgenic.

Dr. Bernard Jasmin and Dr. Rashmi Kothary for being the questioning, yet supportive advisors a student needs.

Jaime Sanchez-Dardon for running the flow cytometer.

Everyone else at the Ottawa Health Research Institute and University of Ottawa for helping to make this possible.

Dr. Bruce Hutcheon for his much needed and immensely appreciated insight into statistics. Similarly to Dr. Gitanjali Adlakha-Hutcheon for her encouragement and scientific wisdom, especially when it came to giving me confidence before a presentation.

Dr. Peter McPherson and Dr. George Prendergast for their generous donations of Bin1 antibody and for their collaborative support in the field of Bin1.

The running community at the Ottawa Lions Track Club for giving me the excuse I needed to leave the lab and for making the stamina needed in research seem easy compared to the workouts we were assigned.

My friends and family in Ottawa and beyond for their blind faith support in what I was doing.

My parents for creating an environment in which following this path seemed so natural.

And last, but not least, Brian, for the incredible amount of patience he had with me, while managing not to show his certain frustration at times...and especially for the support when the going was really tough. It's time to start doing the list of things we promised we'd do 'after I'm done my thesis'.

Bin there, done that.

CHAPTER 1

1.0 Introduction

1.1 Bin1 characteristics

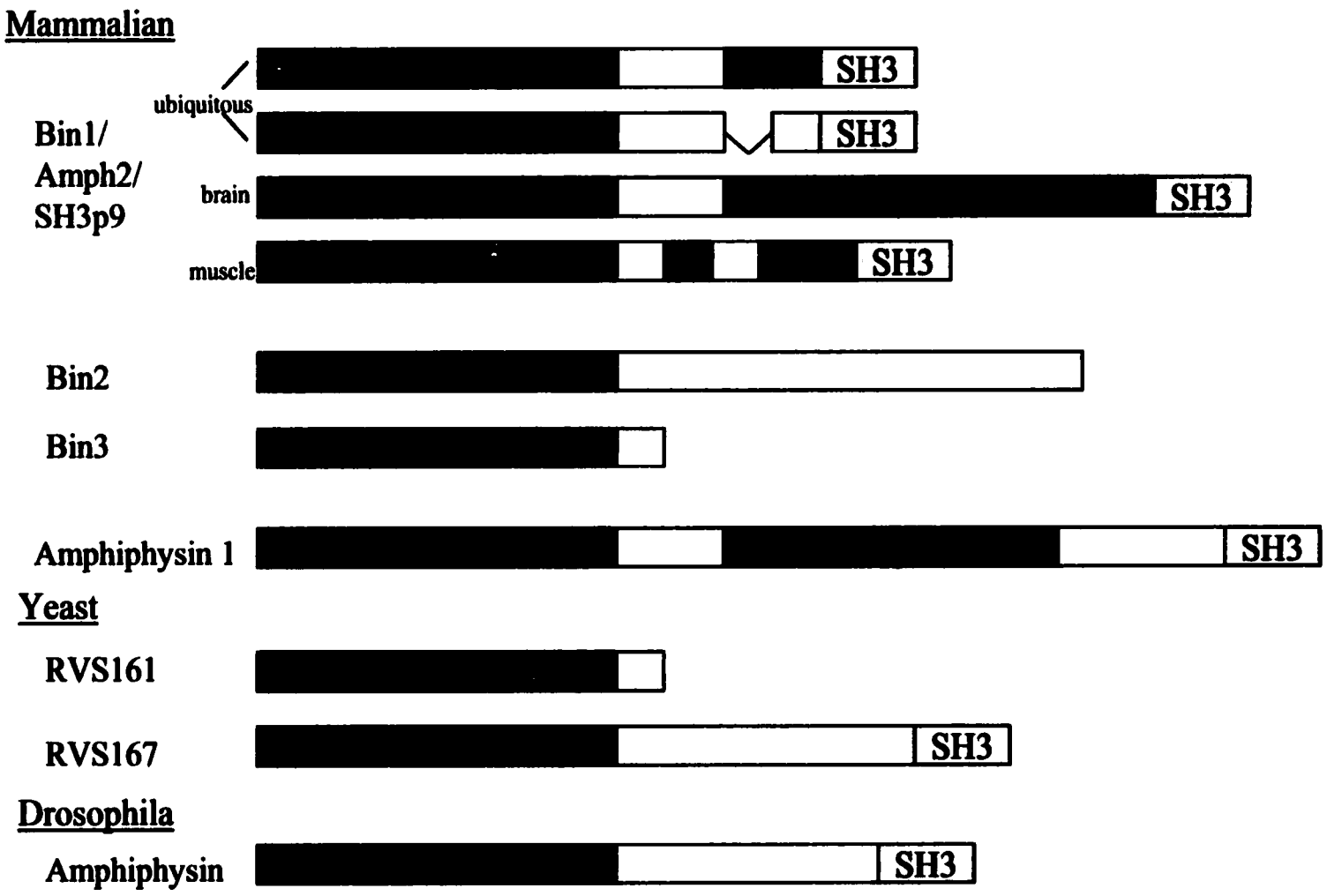
Bin1 was discovered in a yeast 2-hybrid screen for myc-interacting proteins (Sakamuro et al., 1996). The goal was to identify proteins that interacted with the N-terminus of c-myc, specifically the myc-box 1 domain (MB1), a region frequently mutated in cancer (Sakamuro et al., 1996). A large body of evidence suggests that c-myc is a transcription factor with central roles in the regulation of cell proliferation, differentiation, apoptosis, and tumorigenesis (Dang, 1999; Eisenman, 2001; Sakamuro and Prendergast, 1999). Bin1 is a ~70 kDa protein and has been shown to functionally interact with c-myc and inhibit c-myc activation, as well as inhibit malignant cell transformation (Elliott et al., 1999; Hogarty et al., 2000; Sakamuro et al., 1996).

Although the evidence for Bin1 as a c-myc-interacting protein is compelling, the presence of tissue specific isoforms in the brain and skeletal muscle suggests a broader range of function for Bin1. Skeletal muscle and brain have robust Bin1 expression (Sakamuro et al., 1996), yet they are composed of highly differentiated tissues where c-myc expression is downregulated (Hanson et al., 1994; Heikkila et al., 1987; Holt et al., 1988; Sawyers et al., 1992; Sklar et al., 1991). In addition, Bin1 distribution is nuclear and cytoplasmic, which argues for an interaction with non-nuclear proteins (Sakamuro et al., 1996). Indeed, the closest family member to Bin1 is Amphiphysin 1 (Amph1), a cytosolic protein that functions in endocytosis with a brain enriched expression pattern (David et al., 1996; Shupliakov et al., 1997; Volchuk et al., 1998). Amph1 and Bin1 are both members of the BAR (Bin1/Amphiphysin/RVS) domain protein family that includes the Amphiphysin and RVS proteins (Sakamuro et al., 1996). These proteins share a

highly conserved N-terminal region referred to as the BAR domain (Figure 1). In addition, Bin1 contains a C-terminal SH3 domain and a central region unique to Bin1 (Mao et al., 1999; Wechsler-Reya et al., 1997b).

Figure 1. BAR family members.

Schematic depicting known mammalian, yeast and drosophila BAR family proteins. The N-terminal BAR fold is shown in *orange*. Domains that interact with c-myc are in *green*, while those implicated in endocytosis are in *red*. SH3 domains are depicted in *yellow*. The U3 domain is in *blue*. Note the similarity in structure between Bin1, RVS167, and drosophila amphiphysin and between Bin3 and RVS 161. Drawings are not precisely to scale.



adapted from Routhier et al., 2001; Zelhof et al., 2001

1.2 BAR family proteins

1.2.1 Bridging integrator proteins (Bin)

The Bin sub-family of BAR domain proteins includes Bin1, Bin2 and Bin3. Bin1 was the first Bin family member discovered and characterized. However, Bin2 appears to have separate, non-redundant roles (Ge and Prendergast, 2000). On Western blot analysis, the predominant Bin2 protein is ~85 kDa and is expressed at high levels in tissues that are rich in hematopoietic cell lineages such as spleen, peripheral blood leukocytes, thymus, colon, and placenta. Consistent with its expression pattern, Bin2 is induced during granulocyte differentiation of HL60 cells, a promyelocytic leukemia cell line (Ge and Prendergast, 2000). While Bin1 has a putative role in differentiation, it appears to differ from Bin2 in that it functions in receptor-mediated endocytosis, displays antiproliferative activity, and has tumour-suppressor properties (Elliott et al., 1999; Ge et al., 1999; Ge et al., 2000a; Ge et al., 2000b; Sakamuro et al., 1996; Wigge et al., 1997a). Overexpression of Bin2 is unable to inhibit transferrin endocytosis and re-introduction of Bin2 into Bin2-null malignant cell lines does not affect cell proliferation (Ge and Prendergast, 2000). As with other BAR proteins, Bin2 contains an N-terminal BAR domain through which it associates with Bin1. However, Bin2 appears unable to form a stable complex with other BAR proteins, such as amphiphysin. In addition, Bin2 has an extended C-terminal region that lacks an SH3 domain (Ge and Prendergast, 2000).

The most recently identified Bin family member is Bin3 (Routhier et al., 2001). The Bin3 protein is 253 aa long and similar to RVS161, is comprised solely of a BAR domain. Bin3 has only 27% identity to the Bin1 BAR domain, suggesting a distinct role

for this protein. Indeed, unlike Bin1 and Bin2, Bin3 expression is not altered in malignant cell lines (Routhier et al., 2001). Experiments testing the function of the homologue of Bin3 (hob3) in *Schizosaccharomyces pombe* also support the concept that Bin3 has Bin1 independent roles (Routhier et al., 2001). Hob3Δ mutants revealed cell division defects where a fraction of cells were longer and contained more than two nuclei when compared to normal cells. In addition, F-actin localization was defective in hob3Δ, which disrupts septation, cytokinesis, and ultimately, cell separation. This phenotype is also present in RVS161 mutants in *S. cerevisiae*. Unlike RVS161 mutants, hob3Δ mutants responded normally to nutrient and osmotic stress and displayed normal fluid-phase endocytosis (see section 1.2.3 RVS). Strikingly, the hob3Δ mutant phenotype was completely rescued by the ectopic expression of human Bin3, while RVS161 could only partially rescue it and RVS167 had no effect (Routhier et al., 2001). These results suggest yet another BAR family member with non-redundant roles in cell fission.

1.2.2 Amphiphysin

The other mammalian BAR proteins are the amphiphysins: amphiphysin I (Amph1) and amphiphysin II (Amph2, the brain-specific isoform of Bin1). Amph1 is a 125 kDa, nerve terminal enriched protein involved in clathrin-mediated endocytosis (David et al., 1996; Lichte et al., 1992; McPherson et al., 1996; Owen et al., 1998; Ramjaun et al., 1997; Wigge et al., 1997a; Wigge et al., 1997b). Amph1 shares 49% amino acid sequence identity with Bin1/Amph2 (Wigge et al., 1997a), with an enriched neural expression pattern (Butler et al., 1997; Ramjaun et al., 1997; Razzaq et al., 2001; Watanabe et al., 2001; Wigge et al., 1997a). Amph1 is also distinctly expressed in nonneural tissues such

as testes (Ramjaun et al., 1997; Watanabe et al., 2001) lungs, adrenal glands and mammary tissue (De Camilli et al., 1993; Ramjaun et al., 1997; Watanabe et al., 2001).

To date, most of the work relating to endocytosis and amphiphysins has concentrated on a role in clathrin-mediated endocytosis in the brain. Amph1 and Bin1/Amph2 are present in the same molar ratio in the brain (Wigge et al., 1997a) and together as heterodimers, bind synaptojanin and dynamin in an SH3 domain-dependent manner (Ramjaun et al., 1999). This binding is regulated by the state of amphiphysin phosphorylation. The dephosphorylated form represents an active state that binds clathrin, a protein involved in endocytosis, and AP2, a component of clathrin-coated pits (Bauerfeind et al., 1997; Ramjaun and McPherson, 1998; Slepnev et al., 1998; Wigge et al., 1997a). The central region between the N-terminal and SH3 domain of amphiphysin contains a clathrin and AP2 binding motif that is spliced out of the skeletal muscle Amph2/Bin1 isoforms (Owen et al., 1998; Ramjaun and McPherson, 1998; Wechsler-Reya et al., 1997b). Recently, gene targeting of Amph1 in mice has demonstrated brain specific defects in the binding of synaptojanin, clathrin and AP2 in the lipid bilayer and a decreased ability for synaptic vesicle recycling (Di Paolo et al., 2002). Interestingly, the absence of Amph1 protein also results in a post-translational decrease of Bin1/Amph2, presumably from an inability of these proteins to dimerize. In addition, the mice suffer from cognitive defects and decreased viability (Di Paolo et al., 2002). These results suggest that amphiphysin proteins regulate synaptic vesicle endocytosis by targeting a variety of endocytic proteins to the clathrin-coated pits on the plasma membrane (Wigge et al., 1997a).

Amph1 has also been studied in some detail as the autoantingen in the breast cancer associated paraneoplastic disease of Stiff-Man Sydrome (SMS) (David et al., 1994; De Camilli et al., 1993). SMS is a rare disease of the central nervous system (CNS) characterized by progressive rigidity of the body musculature with superimposed painful spasms. As in the case of autoantigens of other autoimmune paraneoplastic diseases of the CNS, amphiphysin is an intracellular protein and the link between humoral autoimmunity directed against the autoantigen and the clinical symptoms remains to be explained (David et al., 1994; De Camilli et al., 1993). However, the neurological symptoms of SMS improve after removal of the cancer, suggesting an interesting link between breast cancer, the course of SMS and amphiphysin expression (David et al., 1994).

1.2.3 RVS

The BAR family also extends to a yeast protein sub-group, termed the RVS (Reduced Viability upon nutrient Starvation) factors. These proteins were originally identified in a selection for mutant *Saccharomyces cerevisiae* strains showing loss of viability with deletion of essential nutrients from growth media (Crouzet et al., 1991). RVS161 contains only the N-terminal BAR domain and thus, is likely a Bin3 homologue (Routhier et al., 2001). RVS167 is weakly homologous to Bin1 and Amph1 and contains the BAR domain and an SH3 domain, with a shorter central region than observed in Bin1 and Amph1 proteins (Routhier et al., 2001). Although RVS161 lacks the GPA-rich and SH3 domains, mutations in either RVS161 or RVS167 cause a decrease in cell viability and an increased budding ratio upon nitrogen or carbon exhaustion compared to wild-

type *S. cerevisiae* strains. This phenotype is associated with actin delocalisation, a random budding pattern in diploid cells, and heterogeneity in cell morphology that differs markedly from the normally homogenous yeast cells (Bauer et al., 1993; Sivadon et al., 1995). Both mutations also affect cell fusion and result in a reduction in endocytosis (Brizzio et al., 1998; Munn et al., 1995). Intuitively, it is possible to imagine that the actin delocalisation is related to the disruption in cell shape, cell budding, fusion and endocytosis seen in the mutants. However, it is not clear whether the actin delocalisation is directly related to the decreased viability phenotype, or whether these are two separate phenomena. In addition, a distinct or specific role for the SH3 domain of RVS167 remains undetermined. Considering that an SH3 domain is absent in RVS161, it appears unlikely this domain is involved in the shared mutant phenotype.

1.3 Bin1 structure

The highly conserved N-terminal or BAR domain identified Bin1 as a BAR family member, along with the Amphiphysin and RVS proteins. The terminal regions of Bin1 are structurally similar to Amph1. Bin1 is also structurally similar to RVS167 and RVS161, which are negative regulators of the cell cycle in yeast and are also implicated in endocytosis and karyogamy (see previous section). The N-terminal or **BAR** domain of Bin1 functions in dimerization, plasma membrane targeting in the brain, and is necessary for anti-transformation activity (Cestra et al., 1999; Ge and Prendergast, 2000; Ramjaun et al., 1999; Wigge et al., 1997a). Bin1 is able to form homodimers and also heterodimers with Amph1 and Bin2, but the interaction with other BAR family members is unknown (Ge and Prendergast, 2000; Ramjaun et al., 1999; Wigge et al., 1997a).

The brain specific functions of Bin1/Amph2 are coordinated by exons 12A-12D. The **neural-specific** domain of Bin1/Amph2 is important for interaction with clathrin and AP2 (Ramjaun and McPherson, 1998; Slepnev et al., 1998). The other central region of Bin1 contains the **unique** domain (exons 9-11) present only in Bin1. The unique 1 domain (U1) encoded by exon 9 is necessary to suppress malignant cell transformation through E1A and mutant p53 (Elliott et al., 1999). U3 (exon 10) was originally believed to encode a nuclear localization signal (NLS) (Sakamuro et al., 1996). However, the presence of this coding region alters the subcellular distribution of Bin1 from the nucleus to the cytoplasm, thus, arguing against a role as an NLS (Wechsler-Reya et al., 1998). Similarly, the function of U2 (exon 11) remains unknown (Elliott et al., 1999).

While the N-terminal identifies Bin1 as a BAR family member, the **C-terminal domain** suggests it also has features of an adaptor protein. This C-terminal domain contains the myc-binding domain (MBD) and the src homology 3 domain (SH3), which suggest a potential role in signalling cascades (Elliott et al., 1999; Owen et al., 1998; Sakamuro et al., 1996; Wechsler-Reya et al., 1997b). Together, the BAR domain and MBD are necessary for the interaction of Bin1 with c-myc (Elliott et al., 1999). However, c-myc is the only protein identified to date that appears to interact through the MBD of Bin1 (Sakamuro et al., 1996). In the brain, the SH3 domain binds the endocytotic proteins synaptojanin and dynamin. However, the Bin1 SH3 domain interacting proteins/obligate binding partners remain undefined in non-neural tissue (Cestra et al., 1999; de Heuvel et al., 1997; Volchuk et al., 1998; Wigge et al., 1997b). Therefore, the two modular

domains of Bin1, the MBD and the SH3 domains, may coordinate unique activities for the Bin1 protein.

1.4 Src homology domains

Src homology 3 (SH3) domains consist of approximately 60 amino acids and are commonly found in signal transduction enzymes such as non-receptor tyrosine kinases and phospholipase Cγ, and cytoskeletal proteins such as myosin I and the α chain of spectrin (Mayer and Eck, 1995). The overall structure of an idealized SH3 domain includes two small β sheets that are packed against each other and three variable loops: the RT, N-Src, and distal loops (Mayer and Eck, 1995) (Figure 2a, b). The RT and N-Src loops are the ligand binding regions, and the distal loop is on the opposite face and may interact with other regions of interacting proteins. The presence of SH3 domains in a large variety of proteins suggests that the motif confers a modular function, such as protein binding. Indeed, the role of SH3 domains in the formation and dissociation of intra- and intermolecular protein-protein interactions has been confirmed by high affinity binding of the specific SH3 domains to bacterially expressed peptides (Cicchetti et al., 1992). For example, the adaptor protein Grb2 is comprised of repetitive SH2 and SH3 domains. It binds via its SH3 domains to a proline-rich region of the Ras activator protein Sos in nematodes, fruit flies and humans (McCormick, 1993). In comparison, SH2 domains in general bind phosphotyrosine-containing sequences (Koch et al., 1991).

SH3 domains bind the specific proline-rich sequence P-X-X-P (where P is proline, and X is an amino acid, most commonly prolines or other hydrophobic residues). Often an

arginine (R) is also present a few residues either amino-terminal or carboxy-terminal to the P-X-X-P motif. However, specificity of binding is also dependent on other factors such as slight structural differences, van der Waals forces and hydrogen-bonding to backbone atoms, multiple binding sites, and multiple SH3 domains in a protein. In fact, many SH3-binding proteins contain relatively long proline-rich domains (PRD) with multiple potential SH3 interaction sites (Cestra et al., 1999).

1.4.1 Bin1 SH3 domain structure and specificity

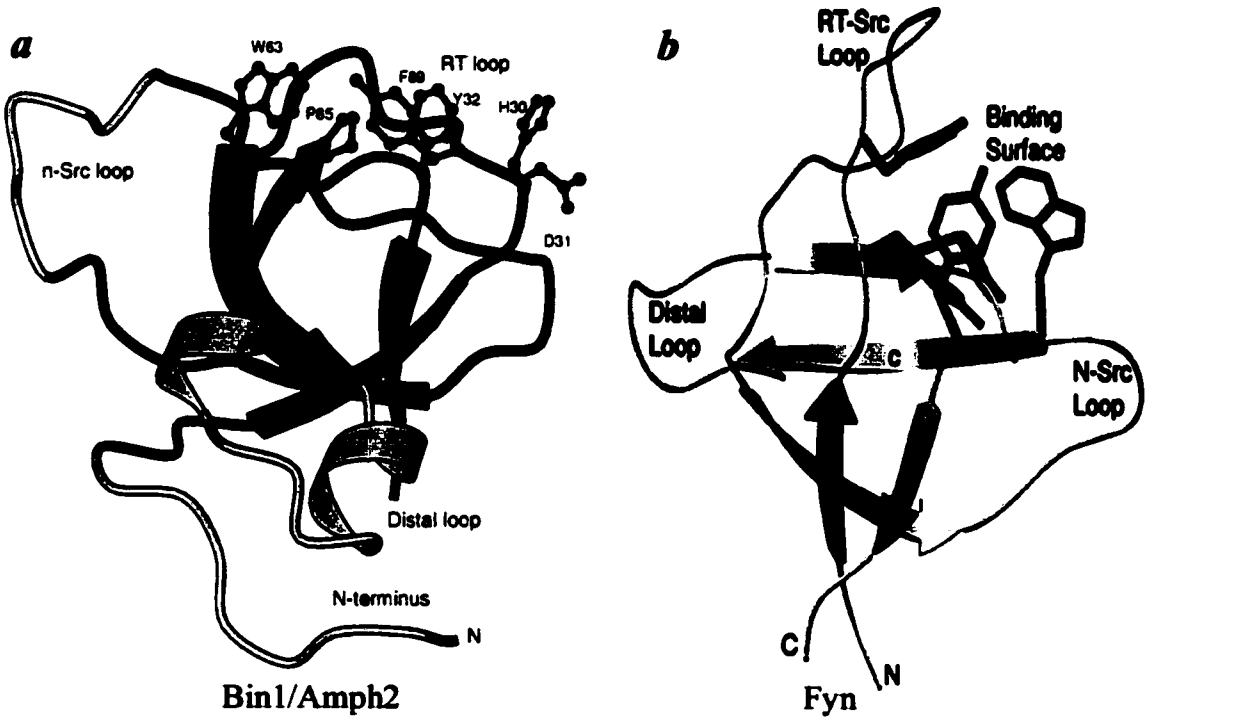
Bin1 has a single SH3 domain that exhibits a highly unique structure and thus, may provide a useful function for analyzing domain specific attributes of the protein (Cestra et al., 1999; Onofri et al., 2000; Owen et al., 1998; Volchuk et al., 1998; Wigge et al., 1997b). Although the Bin1 SH3 domain shares features common to SH3 domains in general, this region of Bin1 may have a more discriminatory binding preference. For example, it possesses an extensive patch of amino acids with negative electrostatic potential that is not present in other SH3 domains (Figure 2d, right). This patch accounts for the specific requirement of Bin1/Amph2 for two arginines in the proline-rich binding motif to which it binds on dynamin (Owen et al., 1998). In addition, it has an unusually large n-src loop, which may obstruct certain interactions with other proteins (Figure 2c). Finally, experiments have verified that the Bin1 SH3 docking domain in dynamin conforms to the PXXP motif and the requirement for two arginines, i.e. PSRPNR (Owen et al., 1998) (Figure 3).

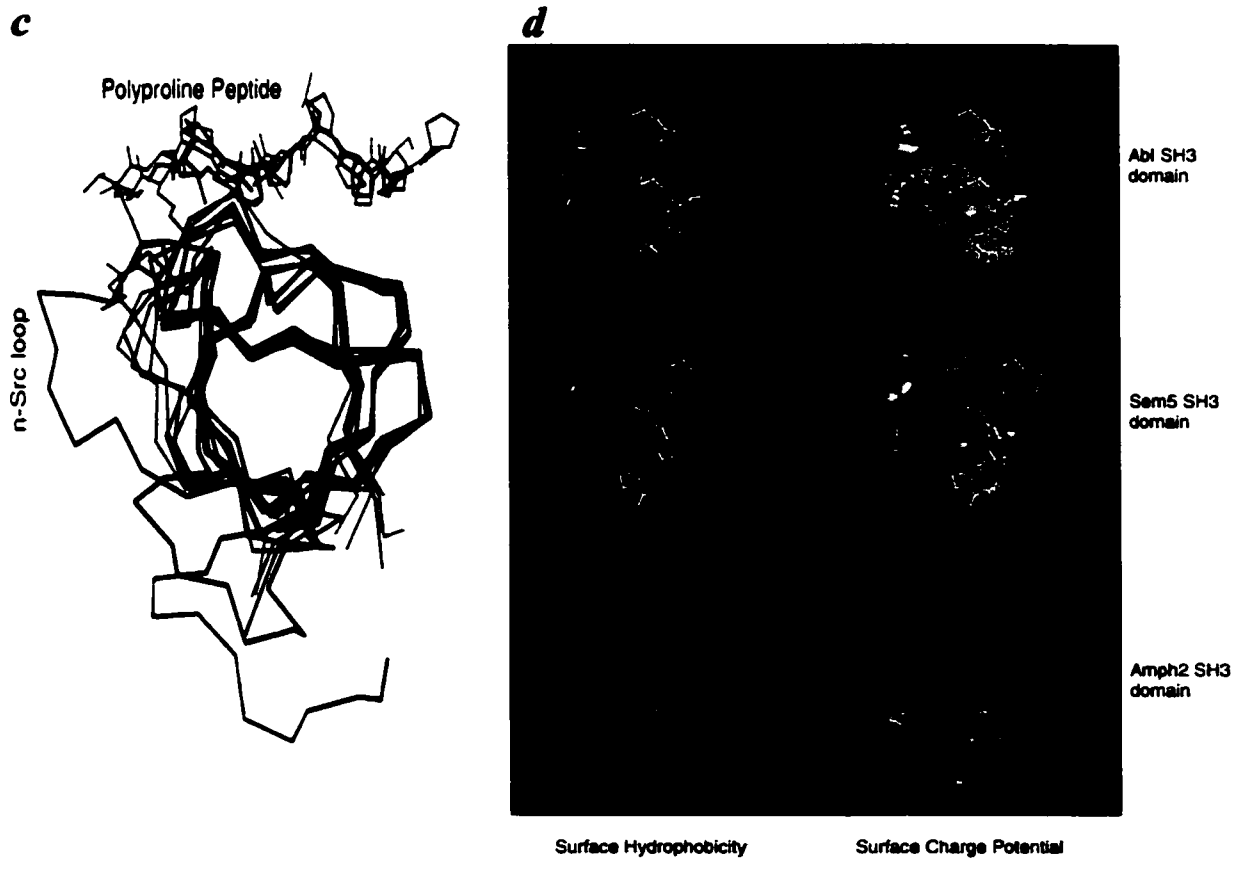
Of interest, numerous experiments have established a high degree of specificity for individual SH3 domains. For example, injection of a tagged Amph1-SH3 domain into COS cells, 3T3-L1 adipocytes and the lamprey giant reticulospinal synapse inhibits clathrin-mediated endocytosis (Shupliakov et al., 1997; Volchuk et al., 1998; Wigge et al., 1997b). Following action potential stimulation to promote exocytosis and subsequent endocytosis, an accumulation of clathrin-coated pits on the plasma membrane is observed in Amph1-SH3 injected cells (Shupliakov et al., 1997). At this stage of endocytosis, dynamin usually forms an electron dense-collar around the neck of clathrin-coated pits; however, this collar is absent in injected cells (Shupliakov et al., 1997). Furthermore, transferrin uptake, a marker of receptor-mediated endocytosis, is dramatically reduced in Amph1-SH3 injected cells (Volchuk et al., 1998; Wigge et al., 1997b) and is rescued by the overexpression of dynamin in a dose-dependent manner (Wigge et al., 1997b). Other dynamin interacting SH3 domain proteins (Grb2, specrin and PLCγ) are unable to affect transferring uptake, indicating that this Amph1-SH3 effect is not a generic SH3 domain interaction but specific to the Amph1 SH3 domain. The accumulation of clathrin-coated, endocytotic pits, and the specific affinity for the Amph1 SH3 domain for dynamin, suggest that dynamin binds to the SH3 domain of Amph1, which then recruits dynamin to the site of endocytosis. Once in position, Amph1 also binds to the clathrin adaptor protein AP1 (Wigge et al., 1997b), allowing dynamin to proceed with ring formation and fission at the neck of the endocytotic pit. The SH3 domain of Bin1/Amph2 displays 51% amino acid identity to that of Amph1 (Owen et al., 1998) indicating that these two SH3 domains may have similar binding partners. However, the limited expression of dynamin

(brain and testis) suggests that Bin1 SH3 dependent functions may be dependent on the interactions of additional proteins (Wigge et al., 1997b).

Figure 2. SH3 domain comparison.

a) Ribbon diagram of the SH3 domain of Bin1/Amphiphysin 2. The peptide backbone of the SH3 domain core is shown in *dark blue*. Inserts relative to other SH3 domains are shown in *gold* and labelled. Conserved hydrophobic residues involved in binding proline residues are shown in *pale blue* (Owen et al., 1998). *b)* Ribbon diagram of a representative SH3 domain from the Fyn tyrosine kinase. The β-strands are labelled a-e, and the loops and residues involved in binding proline residues are labelled for comparison to Bin1 (Larson and Davidson, 2000). *c)* Superposition of carbon traces of SH3 domains from Amph2 (*dark blue*), Sem5 (*red*), Fyn (*magenta*), c-Crk (*cyan*), spectrin (*pink*) and Abl (*green*). The structures of Sem5, c-Crk and Abl are shown with their bound peptides (Owen et al., 1998). *d)* Surface representations of SH3 domains from Abl, Sem5 and Bin1/Amph2. The pictures on the left depict surface accessible hydrophobic regions coloured green to yellow for increasing hydrophobicity (M. Noble, X objects, unpublished). Bin1/Amph2 SH3 shows only two hydrophobic patches as opposed to the three present in Abl and Sem5. On the right are representations of electrostatic potential (created using GRASP) showing the large negatively charged patch in *red* on the peptide binding surface (the peptide is shown in the case of Abl and Sem5). The representations show the peptide binding surfaces of the SH3 domains with the n-Src loops pointing toward the top of the page. Coordinates for the SH3 domain of Abl and the N-terminal SH3 domain of sem5 were obtained from the Protein Data Bank (Owen et al., 1998).

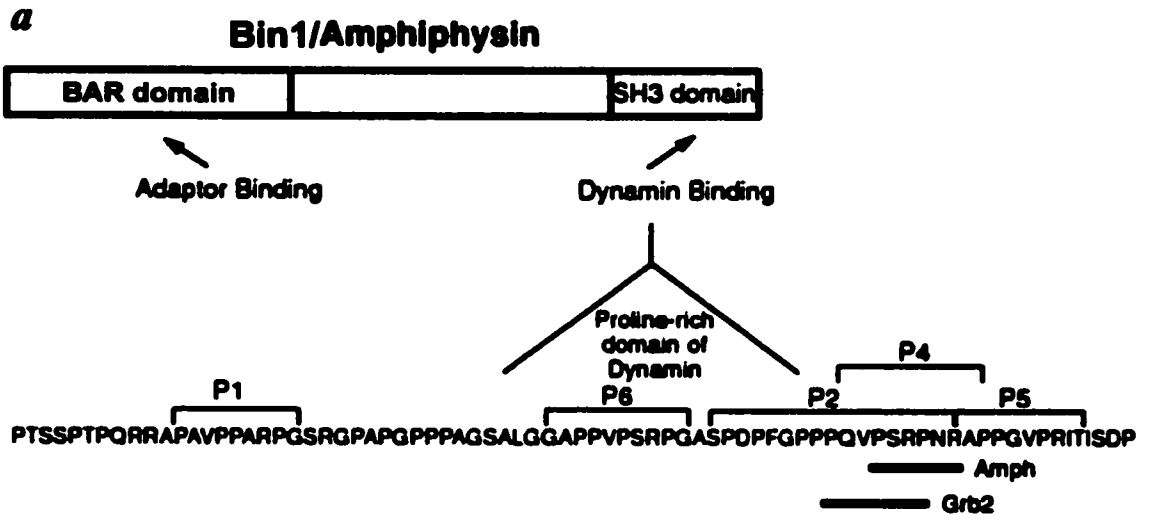


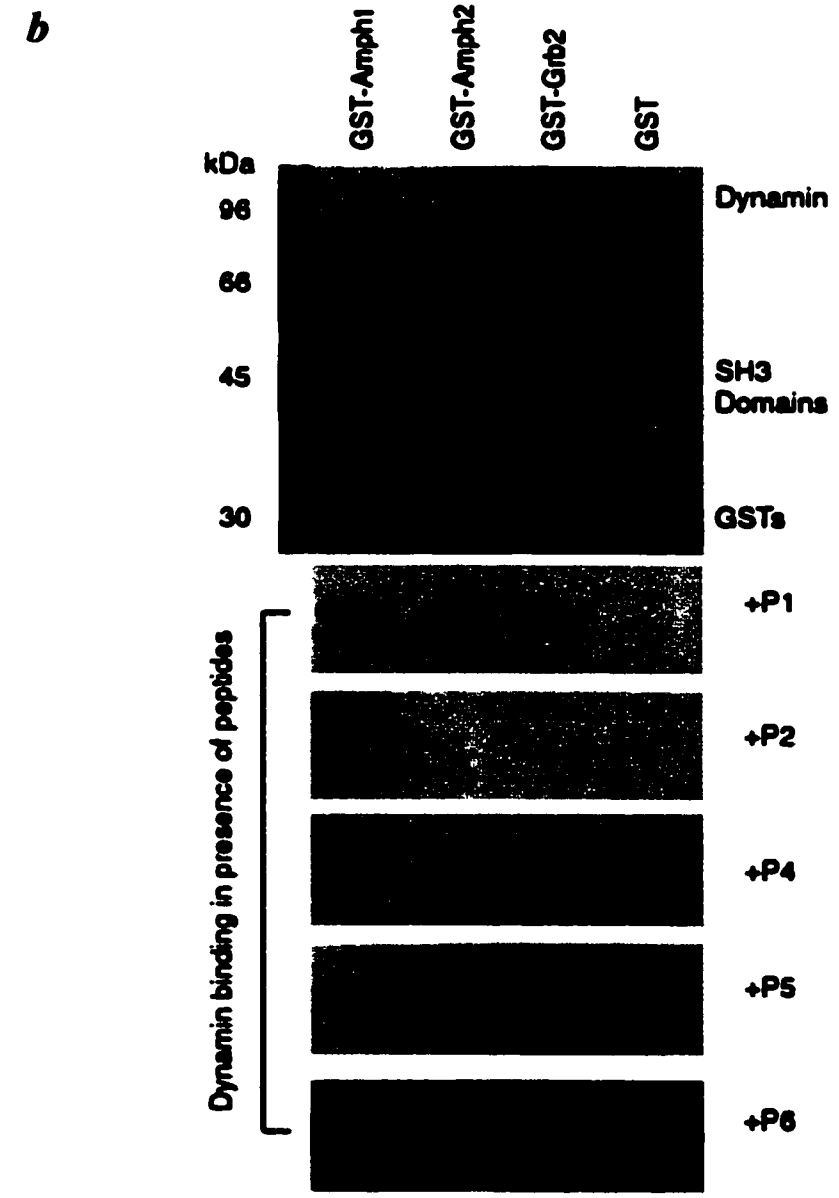


adapted from Larson et al., 2000; Owen et al., 1998

Figure 3. Bin1/Amph2 SH3 domain peptide binding specificity.

a) Schematic representation of the common domain structure of Amph1 and Amph2, along with the sequence of the polyproline region of dynamin with which both isoforms interact. Sequences P1, P2, P4, P5 and P6 indicate the five peptides used for mapping the binding regions, and the solid bars indicate Grb2 N-terminal SH3 and amphiphysin SH3 domain binding sites. *b)* The SH3 domains of both Amph1 and Amph2 target the same site on dynamin, QVPSRPNRAP. Recombinant SH3 domains, prepared as GST fusion proteins immobilized on glutathione agarose, were incubated with brain extract in the absence of peptide (top panel) or in the presence of 200 μM of each of the peptides P1, P2, P4, P5 and P6. The extent of dynamin binding was assayed by separation of bound proteins by SDS-PAGE, followed by staining with Coomassie Blue (Owen et al., 1998). Both P2 and P4 are able to bind to GST-Amph1 and GST-Amph2 SH3 domains and specifically inhibit dynamin binding.





Owen et al., 1998

1.5 Bin1 in cell cycle regulation and apoptosis

A role for Bin1 in cell cycle and apoptosis derives from the discovery of Bin1 as a c-myc binding protein. c-Myc is amplified or overexpressed in many tumours including lung, breast, and colon carcinoma (Dang, 1999). It is through the myc binding domain (MBD) that Bin1 interacts with c-myc to moderate cell cycle progression (Sakamuro et al., 1996). A functional interaction between the myc box 1 domain (MB1) and the myc box 2 domain (MB2) of c-myc, and the MBD of Bin1 has been demonstrated to inhibit transformation of cells by c-myc (Elliott et al., 1999; Hogarty et al., 2000; Sakamuro et al., 1996). Indeed, the MB1 is a major site for phosphorylation events that regulate transcription and transformation and is commonly mutated in human cancers (Sakamuro and Prendergast, 1999). Structurally, c-myc contains an N-terminal transcription activation domain and resides in the nucleus, implying that it has a role as a transcription factor (Eisenman, 2001; Sakamuro et al., 1996). Nevertheless, classifying c-myc as a transcription factor has been problematic. For example, the transactivating activity of c-myc is weak and variable compared with most other activators (2-5 fold weaker). c-Myc can also act as a transcriptional repressor through the initiator or Inr element. Finally, mutations in the c-myc transactivating region (MB2) have given inconsistent results, with different studies reporting varying effects on activation and repression (Eisenman, 2001; Sakamuro and Prendergast, 1999).

Interestingly, the biological roles of c-myc in promoting proliferation, apoptosis, and oncogenesis are better understood. c-Myc expression is rapidly induced in proliferating cells and remains elevated, suggesting that it is required for continuous cell growth

(Dang, 1999; Eisenman, 2001). Induction of c-myc is sufficient to drive quiescent cells into the cell cycle and its inhibition can block mitogenic signals and facilitate cell differentiation (Hanson et al., 1994; Heikkila et al., 1987; Holt et al., 1988; Sawyers et al., 1992; Sklar et al., 1991). Investigations into the manner by which Bin1 regulates cell cycle and apoptotic functions suggest that Bin1 has evolved c-myc-dependent and c-myc-independent roles. For example, Bin1 can also inhibit E1A-dependent transformation through the U1 domain, and mutant p53-dependent transformation through the U1 and SH3 domains (Elliott et al., 1999). Bin1 does not require the MBD, and by extension c-myc interaction, to suppress E1A- or p53-dependent transformation (Elliott et al., 1999; Sakamuro et al., 1996).

Further support for Bin1 as a cell cycle regulating protein comes from the observation that Bin1 expression is absent in many breast, liver, lung, cervix, and prostate tumours, and primary tumour cell lines (Ge et al., 2000a; Ge et al., 2000b; Sakamuro et al., 1996). The re-introduction of Bin1 into cell lines that lack Bin1 expression inhibits colony formation, suggesting a functional loss of Bin1 in tumour cell lines (Sakamuro et al., 1996). However, not all tumours and tumour cell lines are deficient for Bin1 expression. Malignant melanocytes and some prostate tumours display elevated Bin1 expression (Ge et al., 1999; Ge et al., 2000b). Closer examination reveals a misspliced Bin1 isoform that includes the brain specific exon 12A (Ge et al., 1999; Ge et al., 2000b). Importantly, this isoform lacks the antitransforming activity of the ubiquitous Bin1 isoform (Ge et al., 1999). Re-introduction of the ubiquitous Bin1 isoform into a variety of transformed cell lines and primary tumour cell lines, including malignant melanocytes, triggers a specific

programmed cell death response (DuHadaway et al., 2001; Elliott et al., 2000; Elliott et al., 1999; Galderisi et al., 1999; Ge et al., 1999; Ge et al., 2000a; Sakamuro et al., 1996).

The ability of c-myc and other oncogenes to successfully accelerate proliferation/ malignant transformation must either be accompanied by dysfunctional apoptotic signalling, or the cells must evolve a resistance to apoptosis that would normally restrict inappropriate cell division. In non-transformed cells, an increase in proliferation is accompanied by an increase in apoptosis. In contrast, malignant cells elude cell death; yet the conditions that allow for this shirking of the death penalty remain undetermined. Mutant p53, as well as increased expression of telomerase represent two alterations that are believed to contribute to the immortality of tumour cells through avoidance of the death penalty (Dang, 1999). It has been suggested that Bin1 may also facilitate transformation without apoptotic penalty, as evidenced in the functional absence of Bin1 in tumours and malignant cell lines (Ge et al., 1999).

1.5.1 The role of Bin1 in cell cycle control

As mentioned in the previous section, the MBD of Bin1 binds the MB1 and MB2 of c-myc. Both the MBD and BAR domain of Bin1 are necessary for the protein's antitransforming activity (DuHadaway et al., 2001; Elliott et al., 1999). This has been illustrated by deletion constructs lacking either the BAR domain or the MBD domain, neither of which is able to suppress c-myc transformation in primary cells (Elliott et al., 1999). The antitransforming activity of the BAR domain has been reduced to 6 aa within this domain. When deleted, this Bin1 construct (Bin1 DN) is unable to decrease the rate

of proliferation in primary cells transformed with c-myc and, as such, lacks antitransforming activity (DuHadaway et al., 2001). An overexpressed MBD domain is similarly unable to suppress c-myc transformation, underscoring the presence of c-myc-independent growth suppression by Bin1 (DuHadaway et al., 2001; Elliott et al., 1999). Interestingly, the BAR domain alone is sufficient to suppress transformation in HepG2 cells, a cell line that does not express endogenous Bin1 protein (Elliott et al., 1999).

Bin1 suppression of cell growth has also been linked to an inherent ability of this protein to induce apoptosis. For example, full-length Bin1, when reintroduced into transformed cell lines that lack endogenous Bin1, activates a caspase-independent cell death program (Elliott et al., 2000). In addition, transformed cells transfected with Bin1 DN or the MBD exhibit less apoptosis, suggesting that c-myc overexpression normally triggers Bin1-dependent apoptosis (DuHadaway et al., 2001). Similarly, HepG2 cells do not undergo apoptosis when transfected with a truncated Bin1 that lacks the BAR domain (Elliott et al., 2000). Notably, Bin1 induced apoptosis is independent of caspase, p53 and Rb pathways (DuHadaway et al., 2001; Elliott et al., 2000). Indeed, p53 repression is not concurrent with the Bin1 mutations that are present in breast cancer and melanoma cell lines (Ge et al., 1999; Ge et al., 2000a). Rather, Bin1 initiates apoptosis through the activation of serine dependent proteases (Elliott et al., 2000). Furthermore, a link between apoptosis and Bin1 is suggested by a highly conserved NF-κB consensus sequence in the Bin1 promoter, a protein that has important roles in oncogene-mediated cell transformation and apoptosis (Mao et al., 1999).

The N-terminal BAR domain of Bin1 may also regulate cell cycle progression through protein interaction with non c-myc cell cycle regulators. For example, Amph1 is phosphorylated by cdk5 and bound by the cdk5 regulatory subunit p35 in the BAR domain (Floyd et al., 2001). Cdk5 functions in cellular growth control, endocytosis and actin function (Floyd et al., 2001). In addition, the cdc2/cyclin B1 complex phosphorylates serine residues in the BAR domain of Amph1 during the mitotic phase of the cell cycle (Floyd et al., 2001). Although it is apparent that Bin1 is involved in cell cycle progression, the detailed mechanisms by which it accomplishes this function remain elusive.

1.5.2 c-Myc independent roles for Bin1

The elevated expression of Bin1 in differentiated skeletal muscle suggests that Bin1 has a function beyond cell cycle regulation and c-myc-interaction. In addition, a quantitative comparison of Bin1 versus c-myc expression reveals that Bin1 exists in stoichiometric excess of c-myc in proliferating myoblasts (Wechsler-Reya et al., 1998). This disparity is further accentuated during differentiation, i.e. Bin1 expression increases while c-myc expression is eliminated (Eisenman, 2001). The presence of a Bin1 isoform that lacks the MBD also supports a c-myc-independent function for Bin1. Indeed, several different Bin1 isoforms appear in both a tissue-specific and ubiquitous expression pattern (Kim et al., 2001; Sakamuro et al., 1996; Tsutsui et al., 1997; Wechsler-Reya et al., 1998). Brain specific isoforms that lack anti-transforming activity are involved in endocytosis and are localized exclusively to the cytosol (Ramjaun and McPherson, 1998). In contrast, ubiquitous and skeletal muscle specific isoforms are expressed in the nucleus and the

cytosol (Kadlec and Pendergast, 1997; Wechsler-Reya et al., 1997a). However, during skeletal muscle differentiation, a new Bin1 isoform (that includes exon 10) is expressed and localized to the cytosol (Butler et al., 1997; Kadlec and Pendergast, 1997; Mao et al., 1999; Wechsler-Reya et al., 1998). The presence of tissue-specific isoforms and isoforms that lack the MBD strongly suggests that Bin1 proteins mediate c-myc independent functions.

1.6 Myogenic differentiation

During myogenesis, committed myoblasts originate from pluripotent mesodermal cells in the somatic mesoderm. Myoblasts within the somites then migrate and differentiate, populating areas throughout the developing embryo, ultimately forming multinucleate myofibres (Buckingham, 2001). The basic helix-loop-helix (bHLH) regulatory proteins MyoD, Myf5, myogenin, and MRF4 are the key muscle-specific transcription factors that regulate myogenesis. Individually, they are each able to initiate the muscle differentiation program when introduced into nonmuscle tissue culture cells (Olson and Klein, 1994; Perry and Rudnicki, 2000; Rudnicki and Jaenisch, 1995). These proteins have been demonstrated to form positive regulatory loops in which the expression of one bHLH protein promotes its own and other family member expression (Weintraub, 1993). In turn, the bHLH proteins coordinate with the myocyte enhancer factor 2 (MEF2) family of MADS box transcription factors to activate terminal differentiation markers, such as myosin heavy chain (MHC) and muscle creatine kinase (MCK) (Weintraub, 1993). Using gene-knockout technology, several laboratories have created mice lacking functional myogenic bHLH factors that are now providing useful models for studying

skeletal muscle development. From these and other experiments, a genetic hierarchy emerges in which MyoD and Myf5 determine a myogenic fate, whereas myogenin and MRF4 are involved in the differentiation process and maintain the terminally differentiated state (Megeney and Rudnicki, 1995; Perry and Rudnicki, 2000).

1.6.1 Myogenesis and Bin1

Investigations of the murine (Mao et al., 1999) and human (Wechsler-Reya et al., 1997b) Bin1 promoter regions have identified potential muscle regulatory modules. The human Bin1 promoter contains consensus sequences for myogenin/MyoD and SRF (Mao et al., 1999). The murine promoter contains SRF, Mef2 and Tef consensus sequences, but not myogenin/MyoD, suggesting differences in Bin1 regulation (Mao et al., 1999). SRF (serum response factor) is crucial for regulating skeletal muscle- and smooth muscle-specific gene expression (Duprey and Lesens, 1994). Tef is a transcription factor important in the control of cardiac muscle, skeletal muscle, and placental gene regulation and has been shown to bind to the M-CAT element found in the regulatory region of several cardiac and skeletal muscle genes, such as MHC (Farrance and Ordahl, 1996).

As suggested by the presence of muscle-specific consensus sequences in the Bin1 promoter, Bin1 expression is dramatically upregulated during C2C12 (Wechsler-Reya et al., 1998) and primary myoblast (Megeney et al., unpublished observations) differentiation. Furthermore, overexpression of Bin1 in C2C12 myoblasts causes a more rapid and pronounced differentiation than control transfected, anti-sense transfected, or wild type C2C12 myoblasts (Wechsler-Reya et al., 1998). During C2C12 differentiation,

Bin1 is alternately spliced into isoforms that contain the U3 region (Wechsler-Reya et al., 1998). It appears as though the U3 region allows for the translocation of Bin1 into the cytosol during differentiation. However, the significance of this observation is unknown, as U3 deleted isoforms and U3 containng isoforms are equally capable of accelerating differentiation (Wechsler-Reya et al., 1998).

In vivo, Bin1 is present early in myogenesis (by 10.5 dpc) in the somites (Mao et al., 1999), and is localized to the T-tubules in adult murine (Butler et al., 1997) and drosophila (Razzaq et al., 2001) muscle. In particular, flies lacking the Bin1 homologue, amphiphysin, are flightless and have severely disorganized excitation-contraction coupling machinery in muscle. However, no concurrent endocytosis abnormalities are apparent (Razzaq et al., 2001). Consequently, mammalian Bin1 protein may have a critically conserved function in differentiated skeletal muscle.

1.7 Experimental rationale

The tissue-specific functions for Bin1 in the brain are well documented and represent one function of Bin1 that is separable from its roles in cell cycle regulation and c-myc interaction. Indeed, deletion constructs of Bin1 that consist of only the SH3 domain are not able to inhibit malignant cell transformation (Elliott et al., 1999). In contrast, overexpression of the SH3 domain in neural cells is sufficient to inhibit endocytosis in a specific manner (Shupliakov et al., 1997; Volchuk et al., 1998; Wigge et al., 1997b). Therefore, the activities of Bin1 in the brain occur primarily through the neural-specific and SH3 domains, signifying that a similar regulation may occur in skeletal muscle.

Taken together, these observations suggest that the SH3 domain may represent the Bin1 module/subdomain which regulates the differentiation specific role for this protein.

1.8 Hypothesis and Objectives

My prevailing hypothesis is that Bin1 has tissue specific functions that are regulated by the SH3 domain. Within this context, I further hypothesize that

1. The Bin1 SH3 domain mediates skeletal muscle differentiation
2. The Bin1 SH3 domain does not affect cell growth or proliferation
3. The Bin1 SH3 domain does not affect apoptosis

To address these questions, I propose to study the function of the Bin1 SH3 domain in a dominant negative mouse model.

CHAPTER 2

2.0 Materials and Methods

2.1 Generation of Bin1 SH3 construct

Nucleic acids 1262-1480, which represent the SH3 domain from murine Bin1/SH3P9 (see appendix for SH3P9 sequence) were obtained from Megeney et al. (unpublished data). *ECO*RI and *HIND*III sites were added at the 5' end using BIN1SH3 primer 1: CGGAATTCAAGCTTTTCATGTTCAAGGTTCAAGCCCAG and an *ECO*RI site was added at the 3' end using BIN1SH3 primer 2: CCGGAATTCTCACTGTACCCG CTCTGTAAAATTCTC. The PCR conditions were as follows: 94°C, 2 min, followed by 30 cycles of 94°C, 30 sec, 57°C, 30 sec, and 72°C, 45 sec, and a final elongation of 5 minutes at 72°C. DNA was electrophoresed on 2%/1xTAE (40 mM tris-acetate, 1 mM EDTA) agarose gel, purified using the QIAquick gel extraction kit (Qiagen) and its sequence was verified by Cortec. The insert was ligated into the *ECO*RI site of the mammalian expression vector pCAGGS (Niwa et al., 1991), which has a cytomegalovirus (CMV) enhancer and a chicken β-actin promoter in the 5'→3' orientation. Orientation was confirmed by the presence of an 800 bp band upon digestion with *HIND*III. The sequence of the final construct was also verified by Cortec.

2.2 Generation of Bin1SH3 transgenic line

In preparation for injection, the Bin1 SH3/pCAGGS construct was linearized with *Sal*I and the digested DNA was electrophoresed through 0.8%/1xTAE agarose gels and a 4.7 kb fragment was excised and purified using the Qiaex II gel extraction kit (Qiagen). This DNA was quantitated and diluted to a final concentration of 3 ng/μl or 1.5 ng/μl in filtered TE buffer (10 mM Tris-HCl pH 7.4, O.1 mM EDTA) and injected into the pronuclei of 0.5 days post coitum (dpc) zygotes from B6C3F1 mice. Viable embryos

were implanted into pseudopregnant females using standard methods (Hogan and Tilly, 1977). Transgenic animals were either studied as embryos at 12.5 days or 15.5 days, or stable lines were established for breeding. PCR was used to identify transgenic animals by the presence of a 320 bp band resolved on 1.2%/1xTAE agarose gels from either yolk sac or tail DNA. Primers 'topbin' and 'bottombin' were used as described below with the same reaction and cycling conditions.

2.3 PCR

Specific primers for the identification of the Bin1 SH3 transgene were 'Topbin': TTCAAGGTTCAAGCCCAGCATG, embedded in the 3' end of the SH3 domain, and 'Bottombin': CAGAAGTCAGATGCTCAAGGGG, embedded in the rabbit β-globin poly A. Conditions for all Bin1 transgenic identification amplifications were 95°C, 1 min, followed by 25 cycles of 95°C, 1 min, 60°C, 1 min, 72°C, 3 min, and a final elongation of 10 min at 72°C. Primers for the identification of GAPDH used as a loading control were 3': TGACTCCACTCACGCCAAATTCAA and 5': TGCCTGCTTCACCACCTTCTTGAT. Conditions for all GAPDH amplification reactions were 95°C, 1 min, followed by 22 cycles of 95°C, 1 min, 52°C, 1 min, 72°C, 3 min, and a final elongation of 10 min at 72°C. Samples were analyzed on 1.2-2% agarose/1xTAE gels and visualized by ethidium bromide staining.

2.4 Primary myoblast culture

The hindlimb muscles from 8-12 week-old female B6C3F1 mice were harvested for primary myoblast cells as in (Sabourin et al., 1999). Primary myoblasts lines were

prepared from 7 wild type and 7 Bin1 SH3 individual mice from line 869. Briefly, muscles were digested with dispase (2U/ml, Roche)–collagenase (10mg/ml, Roche) solution for 30-40 min with gentle pipetting every 15 minutes. After filtration and subsequent centrifugation at 1000 rpm for 5 minutes to eliminate fragments, the cell pellet was reconstituted and pre-plated overnight to enrich for primary cells in Ham's nutrient mixture F-10 (Gibco) supplemented with 20% fetal bovine serum (FBS), penicillin/streptomycin (100 U/100 μg/ml, Sigma), fungizone (400ng/ml, Gibco), heparin (5ng/ml, Sigma) and human hepatocyte growth factor (HGF) (2.5ng/ml, Gibco). The following day, the cells were transferred onto and subsequently maintained on collagen type 1-coated Petri dishes in a CO_2-enriched (5%) humid atmosphere at 37°C in Ham's F-10 nutrient mixture (Gibco) as above, with the following difference: basic fibroblast growth factor (human recombinant, 2.5ng/ml, Pharma Biotechnology) was substituted for human HGF. Differentiation was induced by incubating cultures in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Gibco) containing 5% horse serum (HS).

2.5 Fusion index

Fusion index was calculated as the number of nuclei in myotubes divided by the number of total nuclei. A myotube was defined as any multinucleate cell. Three separate fields were counted at growth, 12, 24 and 48 hours differentiation in wild type and Bin1 SH3 myoblasts. The procedure was repeated on two separate occasions with distinct myoblast lines from line 869. Nuclei were stained with Harris Modified Hematoxylin (Fischer) as described below and cells were visualized using a Zeiss inverted microscope.

2.6 RNA preparation

Tissue from 3-5 month old Bin1SH3 and wild type mice was harvested and immediately placed in Trizol Reagent (Gibco) on ice. The tissue was homogenized in Trizol Reagent (Gibco) and RNA was isolated according to the manufacturer's instructions. Similarly, cells grown in monolayer were treated with Trizol Reagent (Gibco) according to the manufacturer's instructions. RNA was resuspended in Dep-C treated water and stored at –70°C until further use.

2.7 Southern and Northern blot analysis

DNA samples were resolved on 0.8% agarose/1xTAE gels. RNA samples were resolved on 1% agarose/1xMOPS formaldehyde gels according to pages 7.32-7.34 in (Sambrook and Russel, 2001). Transfer and hybridization to nylon membranes was performed according to the Hybond-N+ nylon membrane manual (Amersham Pharmacia Biotech). Membranes were hybridized using the following cDNA probes which were labelled by Rediprime II random prime labelling system (Amersham Pharmacia Biotech): 1) rabbit β globin poly-A from the pcAGGS vector (Niwa et al., 1991) and 2) trap3.1 (an exon near the dystonin gene locus, obtained from Dr. R. Kothary's laboratory and used as a loading control). Expression levels were quantified on InstantImager Electronic Autoradiography (Packard).

2.8 Reverse transcriptase

Total RNA samples from mouse heart, skeletal muscle, lung, prostate, liver, brain, kidney, small intestine, testis, and uterus (5 μg) were simultaneously reverse transcribed into cDNA in buffer containing 1μl Oligo $(dT)_{15}$ (500μg/ml, Invitrogen), 1 μl of 10mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP), 1x First-Strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 2 μl DTT (01. M, Invitrogen), RNaseOUT Recombinant Ribonuclease Inhibitor (40U, Invitrogen), and SUPERSCRIPT II RNase H-Reverse Transcriptase (200U, Invitrogen). Total reaction volume was 20 μl. PCR was performed on 5 μl of the RT samples according to the conditions mentioned previously. Samples containing no reverse transcriptase (RT) were used as a control for DNA contamination and GAPDH primers were used as controls to verify RNA presence and integrity.

2.9 Whole mount *in situ* hybridization

Embryos were collected by caesarean section between 8.5 and 12.5 dpc. They were fixed overnight at 4°C in PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4) containing 4% paraformaldehyde. Embryos were then methanol dehydrated and stored at -20°C until use. For *in situ* detection of the pCAGGS transgene, a riboprobe was generated to the full length of the rabbit β-globin poly A region. The myogenin riboprobe used was as previously described (Sassoon et al., 1989). Digoxigenin-labeled sense and antisense riboprobes were generated according to the manufacturer's instructions (Boehringer Mannheim). Whole mount *in situ* hybridization was performed according to the methods described by (Wilkinson and Nieto, 1993). Intact embryos

were photographed in 80% glycerol/PBS using a Zeiss dissecting microscope. For sectioning, embryos were immersed in O.C.T. Compound (Tissue-Tek) at 4°C overnight and then frozen in iso-amyl alcohol cooled in liquid nitrogen for cryosections at a thickness of 10 μm.

2.10 Whole mount tunel assay

Embryos were dissected between 8.5-11.5 dpc in cold PBS containing 2.5 mM EGTA and fixed for two hours in 4% paraformaldehyde/2mM EGTA/2.5 mM EDTA in PBS. Whole mount tunel assay was based on the original description (Gavrieli et al., 1992). Briefly, embryos were nick end labelled with digoxigenin-11-dUTP (Roche) introduced by terminal deoxy-transferase (Roche), and then stained using horse radish peroxidase-conjugated anti-digoxigenin antibody (Roche). Intact embryos were photographed in 80% glycerol/PBS using a Zeiss dissecting microscope.

2.11 Protein isolation

Cells from primary cultures were washed twice with ice-cold PBS conatining 10 mM NaF and lysed on ice for 45 minutes in modified –RIPA buffer (50 mM Tris HCl, pH 7.4, 1% NP40, 150 mM NaCl, 1 mM EDTA, 1% glycerol) 10 ug/ml each of aprotinin, leupeptin, pepstatin and PMSF added on day of use. Total protein was measured using the BCA protein assay (Pierce).

2.12 Western blot analysis

Antibodies used for the detection of Amphiphysin II, MEF2C, myogenin, MyoD and tubulin were: Amphiphysin II antibody (obtained from Dr. Peter McPherson, Montreal Neurological Institute and Hospital) diluted 1:1500, polyclonal MEF2C antibody (Cell Signaling Technology) diluted 1:1000, F5D supernatant anti-myogenin (Developmental Studies Hybridoma Bank) diluted 1:20, polyclonal MyoD antibody (Santa Cruz) diluted 1:500, and E7 supernatant anti-tubulin (Developmental Studies Hybridoma Bank) diluted 1:20. All dilutions were done in 1x TBST/5% skim milk. Proper transfer of proteins and equivalence of loading was verified by Ponceau S, Coomassie Blue or tubulin. Membranes were washed in TBST and incubated for 45 min with a peroxidase-conjugated secondary antibody (Amersham, Les Ulis, France) at a dilution of 1:7500 in 1xTBST/5% skim milk. After several washes in TBST, membranes were incubated with chemiluminescence reagents (Amersham Pharmacia Biotech) and exposed to X-ray film (Kodak).

2.13 FACS analysis

Adherent primary myoblasts were collected and stained with Annexin V-FITC and propidium iodide (PI) as described in the annexin V-FITC Apoptosis Detection Kit (Pharmingen). Briefly, cells were washed twice with cold PBS, removed from the plate using a cell scraper, pelleted at 1000 x g at 4°C, and resuspended in 1 x Binding Buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). ~$1x10^5$ cells were incubated with Annexin V-FITC and PI for 15 minutes in the dark. Of these, $2x10^4$ cells were promptly analyzed by flow cytometry (within one hour). Samples were analyzed on a

Beckman Coulter-Ultra flow cytometer using an argon laser with an excitation wavelength of 488 nm and a FITC and PI detection wavelength of 525 nm and 610 nm respectively. Distributions were graphed and visualized using Expo Cytometer Software, Version 1 (Applied Cytometry Systems). The same cytosettings were maintained throughout the entire experimental time-course.

2.14 Immunocytochemistry

Primary myoblasts were fixed in 90% methanol for 5 minutes and then rinsed three times with cold PBS. Cells were incubated for 1 h in 5% skim milk powder, 1 h in 1:40 anti-MF20 (Developmental Sgudies Hybridoma Bank)/5% skim milk powder, and washed three times in PBS. Nuclei were detected by incubating cells for 30 seconds in concentrated Harris Modified Hematoxylin (Fischer) and washing three times in PBS. Cells were visualized and photographed under a Zeiss inverted light microscopy.

2.15 Histological analysis

Tissues were removed and fixed in 10% formalin for 4-5 days (skeletal msucle, heart, mammary gland, prostate, skin, liver, lung, brain). Fixed tissues were then embedded in paraffin, sectioned at 10 μm and counter-stained with haematoxylin/eosin. Muscle fibre diameters were assessed on hindlimb muscle groups (gastrocnemius, rectus femoris, soleus, tibialis anterior) from line 869, with a minimum of 5 individual muscles measured per group. Fibre diameter, area, and perimeter measurements were calculated using Scion Image for Windows (Version Beta 4.0.2, Scion Corporation) or Adobe Photoshop (Version 5.0, Adobe Systems).

CHAPTER 3

3.0 Results

3.1 Transgenic viability is negatively affected by overexpression of the Bin1 SH3 domain.

The high expression of Bin1 in developing somites and in adult brain and skeletal muscle suggest a tissue specific role for this protein. However, Bin1 also appears to have a ubiquitous role governed by its interaction with c-myc, and expressed by its functional loss in numerous malignant tissue and cell lines. Such a paradox can be resolved by constructing a model whereby the distinct domains of Bin1 have independent roles. As c-Myc binds the MBD portion of Bin1 (Sakamuro et al., 1996), the other defined protein-protein interaction domain, the SH3 domain, may regulate tissue specific functions. To test this model, a putative Bin1 dominant-negative construct (overexpression of the Bin1 SH3 domain) was engineered to disrupt endogenous Bin1 activity. The SH3 domain has reliable specificity and its function in protein-protein interactions is well-described (see section 1.4; Cestra et al., 1999; Mayer and Eck, 1995; Owen et al., 1998; Volchuk et al., 1998). Therefore, such a truncated protein would be hypothesized to sequester the endogenous Bin1 SH3 binding partners, rendering endogenous Bin1 unable to complete its SH3-specific functions. The SH3 domain was cloned into the pCAGGs vector containing a rabbit β-globin poly A under the regulation of a cytomegalovirus (CMV) enhancer and a modified β-actin promoter to mediate a broad expression pattern (Niwa et al., 1991) (Figure 4). The Bin1 SH3 transgene was injected into the pronuclei of murine zygotes. Four injections were performed in total. Two of these injection experiments (3 ng/μl) were harvested during development at 12.5 days post coitum (dpc) and 15.5 dpc (Table 1). The third injection (3ng/μl) resulted in one viable founder. The fourth injection (1.5ng/μl) resulted in 11 founders. Interestingly, the number of transgenics

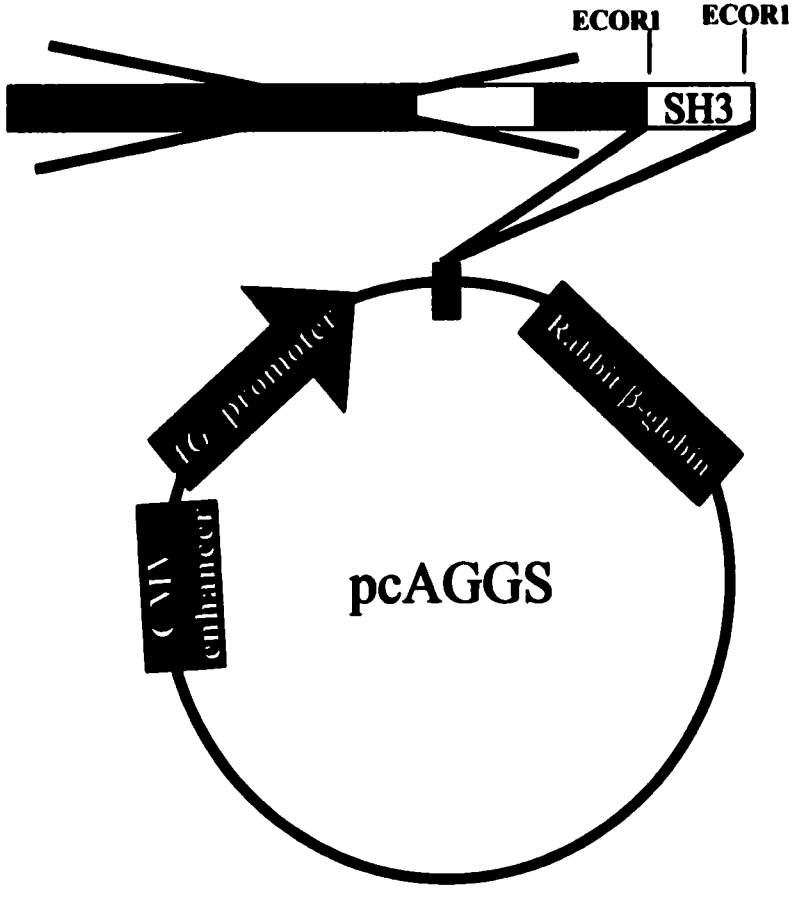
retrieved from successive time points decreased from 40% at 12.5 dpc to 18% at birth for the first three injections. When the concentration of DNA was reduced by 50%, the percentage of founders born increased from 18% to 36%. The decrease in viability over time and across different injection concentrations is suggestive of a dose-dependent effect of the Bin1 transgene. In other words, Bin1 may negatively affect viability when it is present in higher copy number or at a higher expression level. However, confirmation of such an effect will require further experimentation.

Table 1: Summary of injection data

Injection Number (concentration)		Number of transgenics (percent)	Phenotype of transgenics
1 (3ng/μl)	12.5dpc embryos	14/35 (40%)	Smaller heads and limbs Myogenin whole mount *in situ* hybridization – decreased expression in 5/8 transgenics
2 (3ng/μl)	15.5dpc embryos	5/17 (29%)	No apparent phenotype
3 (3ng/μl)	Founders	2/11 (18%) – one founder line 869	Viable – large myofibre diameter
4 (1.5ng/μl)	Founders	12/33 (36%) – 11 founders	Varying phenotypes and levels of expression

Figure 4. Transgene map for mouse model.

Schematic representation of the pcAGGS vector containing the Bin1 SH3 domain. The pronuclei of 0.5 dpc zygotes were injected with the pcAGGS construct. The CMV enhancer is shown in *purple,* while the AG promoter is shown in *blue*, and the rabbit β-globin poly A is shown in *red*.


ECOR1
ECOR1
SH3
Rabbit β-globin
pcAGGS

3.2 Founder expression profile demonstrates transgene message in many tissues.

As has been shown in other transgenic models (Dalpe et al. 1999; Lobe et al. 1999; (Figure 5c), the pCAGGs vector provided significant levels of expression across a variety of tissues, although heart and skeletal muscle showed the highest expression of our Bin1 SH3 transgene. The third round of injections resulted in one founder, line 869. A transgenic littermate died at birth, although the cause of death was undetermined. Northern blot analysis directed against the rabbit β-globin poly A revealed that line 869 had high expression in skeletal muscle and heart compared to other tissues (Fig 5c). The genotyping and expression profiles of lines 508, 501, 487, 482 and 502 from the fourth injection also showed high expression in heart and skeletal muscle (Figure 5c). Other tissues with robust expression were lung, kidney, small intestine, liver, prostate and skin. The Bin1 SH3 transgene displayed sporadic and low expression in the brain with no observed defects. Expression during embryonic development for all expressing lines was also confirmed using whole mount *in situ* hybridization with the rabbit β-globin poly A from the pCAGGs vector (Figure 5a, b).

While the Bin1 SH3 transgene was expressed in similar tissues between different lines, the overall level of expression in each line varied. Comparison of expression levels was carried out using InstantImager Electronic Autoradiography (Packard). Compared to the medium-high expressor (line 508), line 501 had the highest expression in skeletal muscle, with 7-fold higher expression than line 508 (Figure 6, Table 2). Line 869 had 50% the expression of line 508, and lines 482 and 502 had similar expression levels when compared to line 508. Line 487 had only 20% the expression level of line 508. Lines

that showed the highest levels of transgene message during development were also expressing the highest levels of message as adult mice (compare Figure 5a, b and Figure 6). Line 869 was characterized as a medium expresser and was the line chosen for most experiments, as it was also the first founder available. While most lines were healthy and fertile, only one transgenic from line 512 was born. It experienced pronounced muscle atrophy and its development was severely delayed. In addition, its muscles, though small, showed large fibre diameter compared to wild type controls (Figure 9a, refer to section 3.5).

Table 2: Founder lines summary

Line	Level of expression (% compared to 508)	Female trans-genics	Male trans-genics	Total trans-genics	Female wild types	Male wild types	Total wild types	Total females	Total males	Ave. litter size	Total offspring[§]
869	44	56*	57*	113	21	21	42	77	78	9.6	155
482	112	6	6	12	26	24	50	32	30	8.9	62
501	726	11	13	24	11	14	25	22	27	8.2	49
487	20	19	7	26	9	14	23	28	21	8.2	49
502	121	3	0	3	5	11	16	8	11	9.5	19
508	100	4	2	6	7	6	13	11	8	6.3	19
485	Not expresssing	9	11	20	7	4	11	16	15	7.7	31

*inflated number of trangenic offspring reflects attempts to breed the Bin1 SH3 transgene to homozygosity

[§]these numbers do not include harvested embryos

Figure 5. Bin1 SH3 transgene expression pattern.

a) Bin1 SH3 transgene expression during embryonic development in line 501, 10.5 dpc. *b)* Bin1 SH3 transgene expression during embryonic development in line 869, 10.5 dpc. A rabbit β globin poly A cDNA was used as a DIG-labelled probe for whole mount *in situ* hybridization on transgenic (n=5) and wild type (n=5) embryos. Bin1 SH3 transgene expression is ubiquitously high in the transgenic embryo (left) and absent in the wild type embryo (right). *c)* Bin1 SH3 adult transgene expression pattern in lines 869, 508, 512, 501, 487, 482 and 502. Northern blot analysis used a rabbit β globin poly A cDNA as a P^{32} labelled probe. 28S and 18S ribosomal RNA is shown as a loading control above each Northern blot. Bin1 SH3 transgene expression is high in adult heart and skeletal muscle, with expression also observed in lung, liver, skin, prostate and brain.

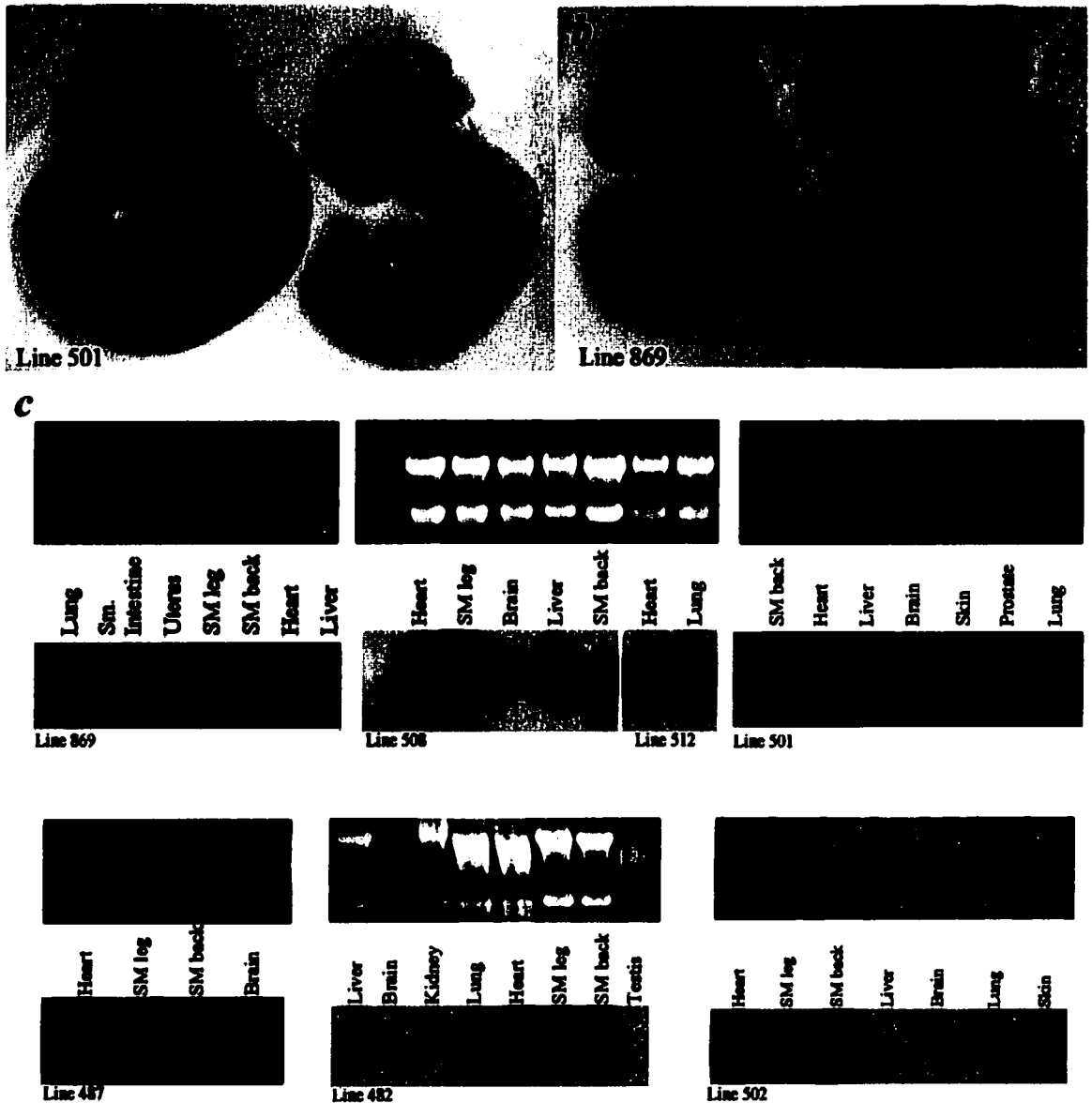
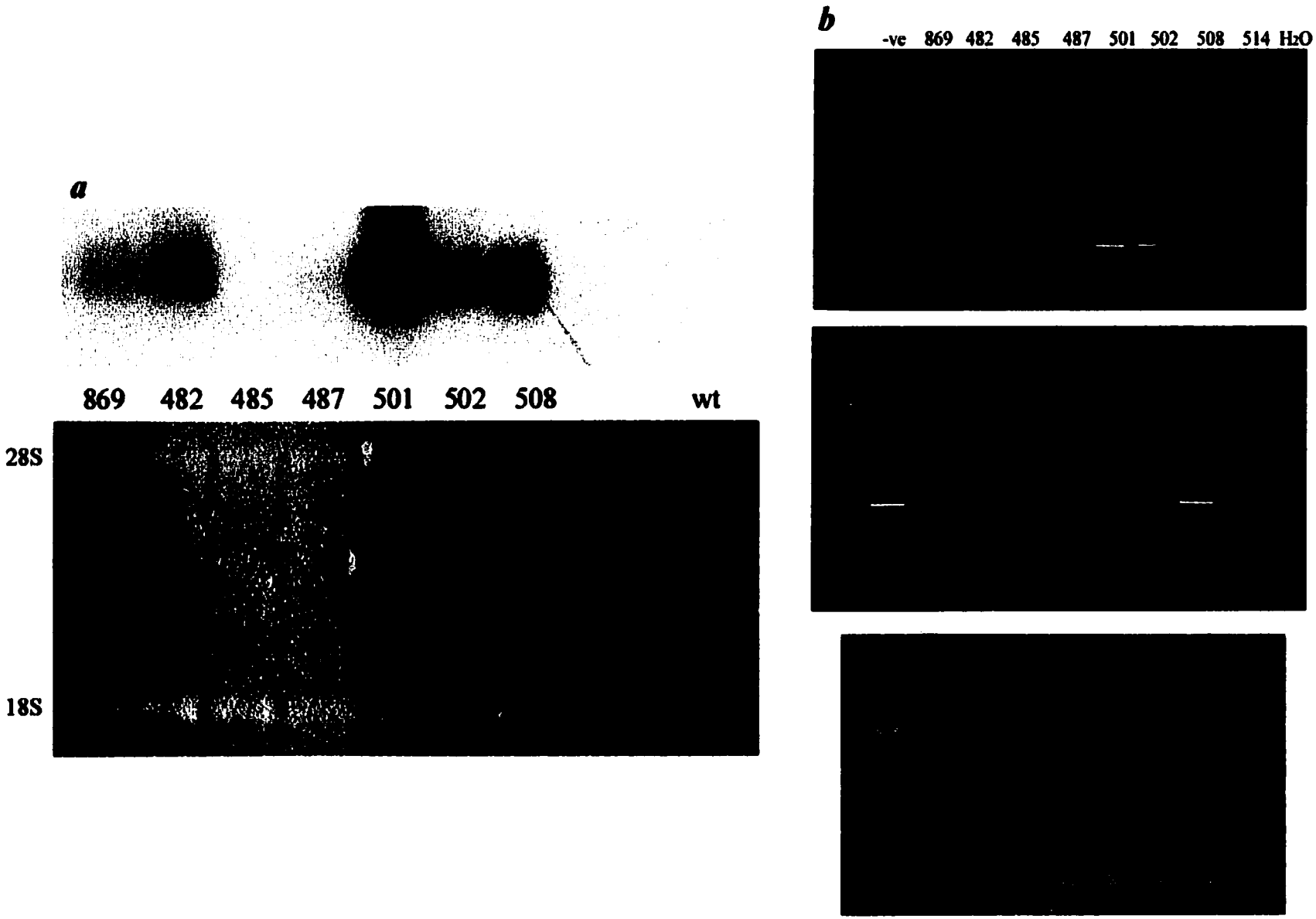

Line 501
Line 869
c
Lung
Sm. Intestine
Uterus
SM leg
SM back
Heart
Liver
Line 869
Heart
SM leg
Brain
Liver
SM back
Heart
Lung
Line 508
Line 512
SM back
Heart
Liver
Brain
Skin
Prostate
Lung
Line 501
Heart
SM leg
SM back
Brain
Line 487
Liver
Brain
Kidney
Lung
Heart
SM leg
SM back
Testis
Line 482
Heart
SM leg
SM back
Liver
Brain
Lung
Skin
Line 502

Figure 6. Comparative Bin1 SH3 transgene expression level.

Bin1 SH3 transgene expression in adult skeletal muscle from lines 869, 482, 487, 501, 508 and a wild type control (wt). 485 is a control that is positive for the Bin1 SH3 transgene, but not expressing detectable levels of RNA. a) Northern blot analysis used the rabbit β globin poly A from the pcAGGS vector as a P^{32} labelled probe. Bin1 SH3 transgene expression is highest in line 501 and lowest in line 487 (refer to Table 2). Quantification of expression levels was calculated using InstantImager Electronic Autoradiography (Packard). The lower panel is a 1% agarose-formaldehyde gel, which illustrates RNA integrity (28S and 18S ribosomal bands) and loading control for the Northern blot. b) RT-PCR from adult skeletal muscle from lines 869, 482, 485, 487, 501, 502, 508, 514, and a wild type control. Note expression in line 487 that is not visible in the Northern blot, but whose radioactivity was detected by electronic autoradiography.


a
869
482
485
487
501
502
508
wt
28S
18S
b
-ve
869
482
485
487
501
502
508
514
H2O

3.3 Myogenin message levels are reduced in founder transgenic embryos.

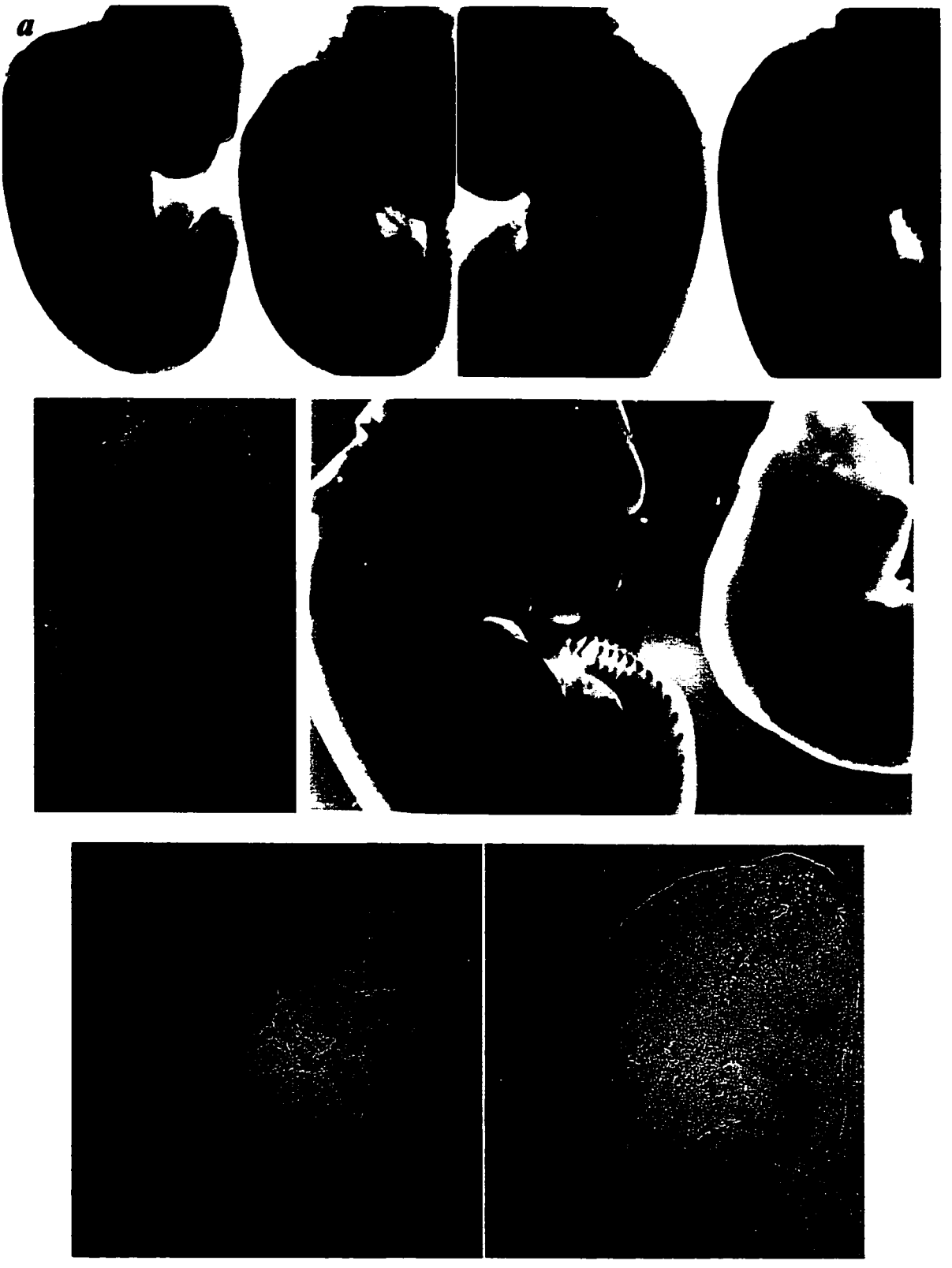
A role for Bin1 during myogenesis is suggested by its ability to be upregulated by MyoD (Mao et al., 1999), its increased expression during myoblast differentiation (Wechsler-Reya et al., 1997b; Wechsler-Reya et al., 1998), and its presence in somites during embryonic development (Megeney and Sandoz, unpublished observations). Therefore, we were interested in examining the role of the Bin1 SH3 domain during myogenesis in the founder embryos. Following cDNA injection and re-implantation at 0.5 dpc, embryos were dissected by caesarean section at 12.5 dpc. To assess the impact of the overexpressed truncated protein on myogenesis, whole mount *in situ* hybridization was performed using an anti-sense myogenin riboprobe. Myogenin, a member of the basic helix-loop-helix family of transcription factors, is a predominant factor during early stages of myogenesis *in utero*, beginning on day 8.5 in the myotome and on day 11.0 in the developing limb buds (Sassoon et al., 1989).

Five of the eight 12.5 dpc founder embryos confirmed by PCR as transgenic also displayed decreased levels of myogenin expression when compared to litter mate controls (Figure 7). In these five embryos that were the same size as controls, myogenin message varied from a slight decrease (Figure 7a) to complete absence (Figure 7b). Decreases in myogenin message were noticeable in the somites and developing forelimb buds (arrows). Cross-sections of these whole mount embryos confirmed the decrease in myogenin message in the somites and forelimb buds (arrows) in the Bin1 SH3 transgenics (Figure 7 d) compared to wild type (Figure 7e). A sixth transgenic embryo was particularly small, in addition to displaying significantly decreased levels of

myogenin message throughout the somites and developing limb buds (Figure 7c). These results strongly suggested that overexpression of the Bin1 SH3 domain disrupted skeletal myogenesis.

Figure 7. Myogenin message in Bin1 SH3 founder embryos.

Reduced (*a*) and absent (*b*) myogenin message at 12 dpc in Bin1 SH3 transgenic founder (left) and wild type founder (right) embryos. *c)* Developmental delay and reduced myogenin message at 12 dpc in Bin1 SH3 founder (left panel) and compared to wild type founder embryo (right panel) Whole mount *in situ* hybridization for myogenin shows diminished expression in those areas of active muscle development as evidenced by decreased staining in the somites and forelimb buds (arrows) of the transgenic embryo (n=6). Myogenin expression patterns coincide both spatially and temporally with endogenous Bin1 expression (Megeney et al., unpublished observations). Reduced myogenin message in somites and limb buds at 12 dpc in transgenic (*d*) and wild type (*e*) embryos. Following whole mount *in situ* hybridization for myogenin, embryos were cryogenically frozen in OCT and sectioned (10 μm). Diminished myogenin message within the somites and limb buds of transgenic versus wild type embryos (arrows) confirmed similar observations in the whole embryos. Bar represents 0.1 mm (*d, e*).

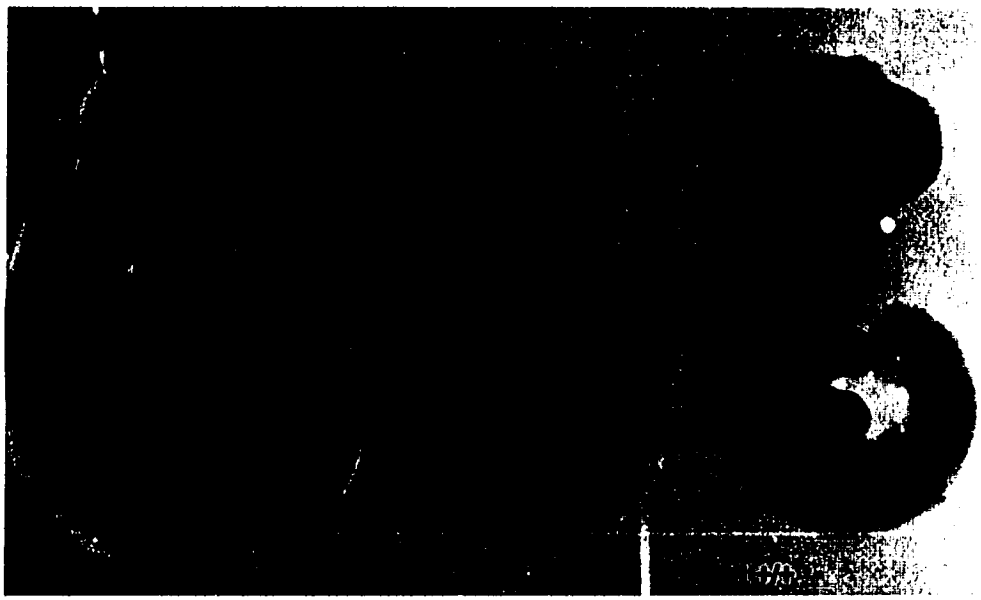

a

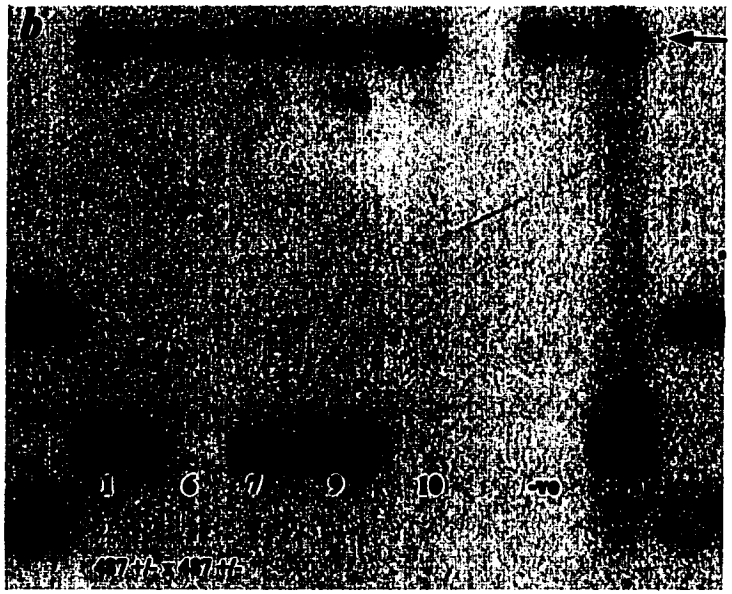
3.4 Myogenin message levels are reduced in F1 transgenic embryos.

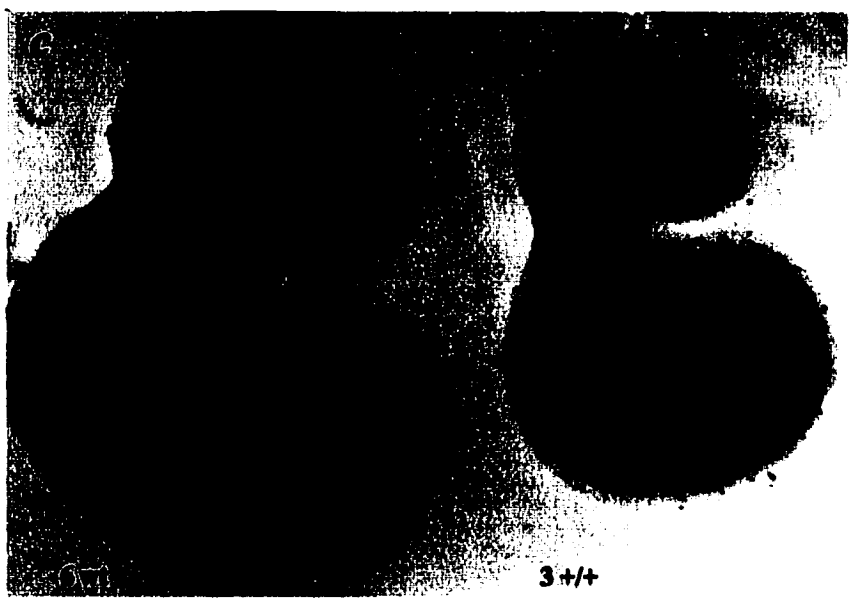
To determine whether the decrease in myogenin expression in the founder embryos also occurred in the established Bin1 SH3 transgenic lines, *in situ* hybridization using the same anti-sense myogenin riboprobe was performed on 10.5 dpc homozygous embryos from line 487 (Figure 8a, c). Three of six of the confirmed homozygous embryos (Figure 8b, d) were significantly smaller than their littermates and displayed dramatically reduced myogenin expression. This result did not occur in any heterozygous embryos or wild type embryos. Myogenin expression was not monitored in all transgenic lines. However, the similar reduction of myogenin expression in the F1 generation from line 487 and in founder embryos suggests that this may be a common effect of overexpression of the Bin1 SH3 transgene.

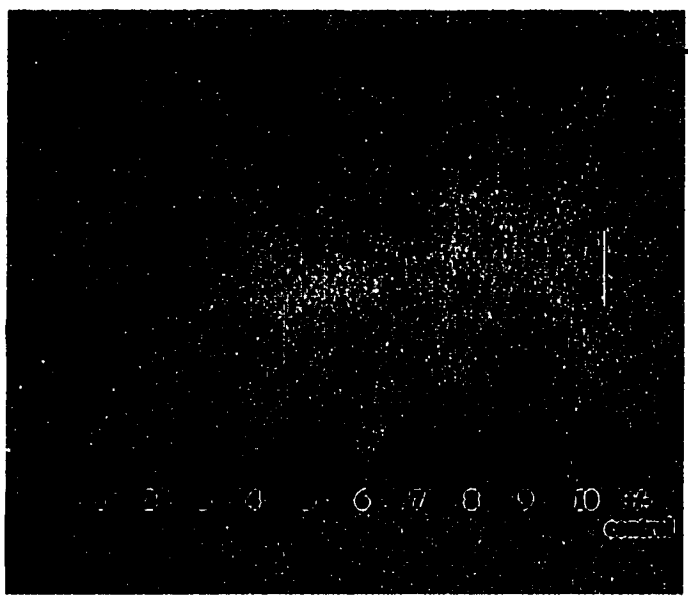
Figure 8. Myogenin message in Bin1 SH3 F1 embryos.

a), c) Myogenin whole mount *in situ* hybridization expression at 10.5 dpc in wild type (wt), heterozygous (+/-, contains one Bin1 SH3 allele), and homozygous (+/+, contains two Bin1 SH3 alleles) embryos from line 487. Heterozygous embryos appear unaffected for myogenin expression (n=7). However, homozygous embryo myogenin expression is severely diminished in the somites (n=3). *b)* Southern blot analysis of the litter shown in *a)*, using the rabbit β globin poly A cDNA as a P^{32} labelled probe. *d)* Southern blot analysis of the litter shown in *c)*. Homozygosity was determined according to band intensity using InstantImager Electronic Autoradiography (Packard). Equivalent loading was ensured using the non-specific hybridization of the rabbit β globin poly A that is present in both wild type and Bin1 SH3 transgenic embryos (*b, d*, top bands, arrows). In addition, trap3.1 (an exon near the dystonin gene locus) was used to confirm this observation (not shown).






3+/+



3.5 Bin1SH3 transgenic mice have large muscle fibre diameter.

A targeted mutation in the myogenin gene in mice results in a marked reduction in muscle tissue throughout the body (Hasty et al., 1993; Nabeshima et al., 1993). The muscle tissue is characterized by increased numbers of mononucleate myoblasts expressing MyoD. The myogenin null myoblasts do not express MHC and as such, appear arrested in the differentiation pathway. The increased number of myoblasts is concurrent to a reduction in the number of myofibres. However, these few myofibres appear normal and express terminal differentiation markers, such as MHC (Venuti et al., 1995). Closer examination of the myogenin mutants during embryogenesis reveals that the initial wave of myogenesis (10.5-14.5 dpc) occurs similar to wild type controls, presumably through a myogenin-independent mechanism. However, during secondary myogenesis (after 15.5 dpc), myotube formation is severely disrupted in the myogenin-mutant mice (Venuti et al., 1995). Therefore, the decreased myogenin transcripts observed in Bin1 SH3 embryos may suggest a similar disruption in secondary myogenesis, i.e. a predominance of the larger, primary myofibres. To address whether a similar predominance of primary myofibres was occurring with Bin1 disruption, six Bin1 SH3 and five wild type tibialis anterior and soleus muscles from 10 day old (p10) pups (line 869) were collected for transverse paraffin sections. Six Bin1 SH3 and five wild type tibialis anterior and soleus muscles from p15 pups (line 869) were also examined. The maximum diameter, perimeter, and cross sectional area of a minimum of 350 haematoxylin/eosin stained myofibres were measured from each comparative group using Scion Image software (Version Beta 4.0.2, Scion Corporation). Myofibre cross sectional area of tibialis anterior ($p<0.001$) and soleus muscle ($p<0.001$) were significantly larger

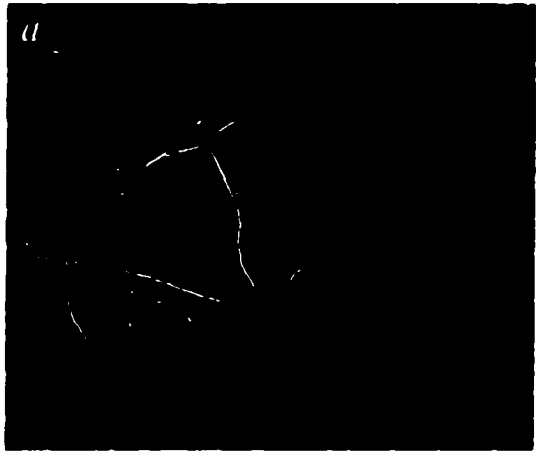
in Bin1 SH3 mice compared to controls (Figure 9a, b, Figure 10a and Table 3). In addition, fibre maximum diameter and perimeter of tibialis anterior and soleus muscle were significantly larger in p10 pups ($p<0.001$), but the difference was no longer apparent by p15 in the tibialis anterior (Figure 10b, c, Table 3). The distribution of fibre diameter in these same transverse sections revealed a broader distribution of fibre diameter in Bin1 SH3 soleus muscle when compared to wild type at p10 and p15 (Figure 11). This pattern of distribution suggests that a significant percentage of the primary myofibre population persists in the Bin1 SH3 transgenic mice. Longitudinal sections through tibialis anterior, gastrocnemius, soleus and rectus femoris muscles were performed in post-natal day mice from day 4 (p4) to day 15 (p15) with similar results (Figure 9c, d, f). This data demonstrates that the downregulation of myogenin during embryogenesis in Bin1 SH3 transgenics may impact on the prevalence of primary (larger) versus secondary (smaller) myofibres.

Table 3: Cross Sectional Analysis of Bin1 SH3 and wild type soleus and tibialis anterior muscles

	Area (sq. μm) (± Standard Error)		Perimeter (μm) (± Standard Error)		Maximum Diameter (μm) (± Standard Error)	
p10 wt Soleus	170.35± 2.61	p<0.001	52.99± 0.41	p<0.001	17.74± 0.17	p<0.001
p10 tg Soleus	316.15± 6.53		68.33 ± 0.71		23.92 ± 0.28	
p15 wt Soleus	352.42±4.06	p<0.001	74.52 ± 0.43	p<0.001	25.74± 0.19	p<0.001
p15 tg Soleus	443.81± 6.00		82.54± 0.56		28.44± 0.24	
p10 wt TA	252.66± 6.51	p<0.001	62.39± 0.83	p<0.001	21.03± 0.30	p<0.001
p10 tg TA	300.84± 5.75		70.14± 0.018		23.36± 0.26	
p15 wt TA	362.85± 9.62	p<0.001	75.03± 1.04	p<0.03	26.15± 0.41	p<1
p15 tg TA	408.64± 10.13		78.20± 0.96		26.11± 0.34	

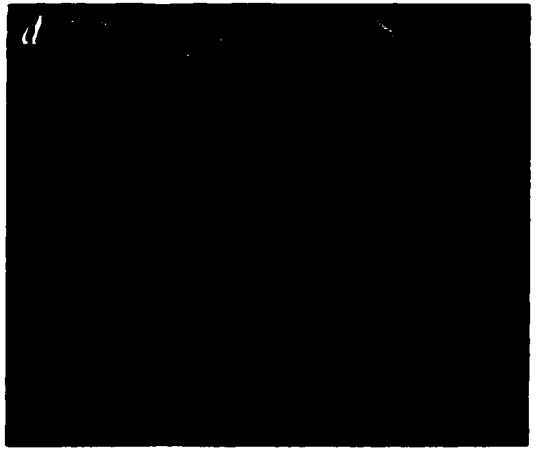
Figure 9. Enlarged myofibre diameter.

Wild type (*a*) and transgenic (*b*) cross sectional haematoxylin/eosin stained paraffin sections (10 μm) from p10 soleus muscle (line 869). Wild type (*c*) and transgenic (*d*) longitudinal haematoxylin/eosin stained paraffin sections (10 μm) from p10 soleus muscle (line 869). Myofibre diameter and cross sectional area is significantly larger in transgenic muscle (double headed arrows). Bar in (*b*) is 0.1 mm. *e)* Littermates from line 512 (p14). Only one transgenic from line 512 was born. It displayed pronounced muscle atrophy and was significantly smaller than its littermates. *f)* Bar graph of longitudinal myofibre diameter in various muscle groups at various ages from line 869. Longitudinal diameter was determined using arbitrary units with Adobe Photoshop (Version 5.0, Adobe Systems). Statistical significance was calculated using the Student's T-test (two tailed distribution, two-sample equal variance). Gastr – gastrocnemius; sol – soleus; rf – rectus femoris; ta – tibialis anterior; +/+ - homozygous; +/- - heterozygous; wt – wild type. Error bars represent standard deviation.


a


c


b


d

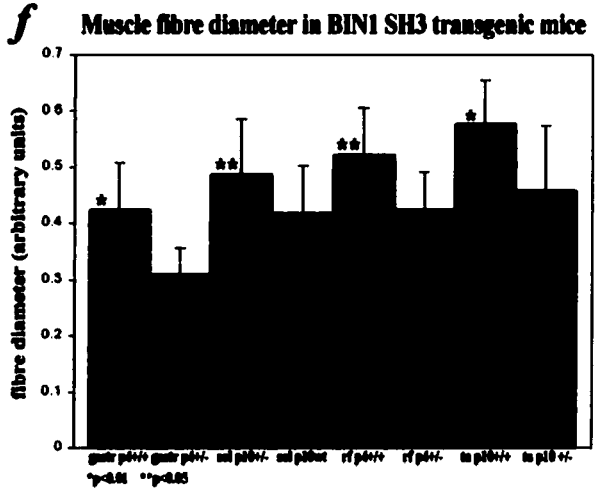
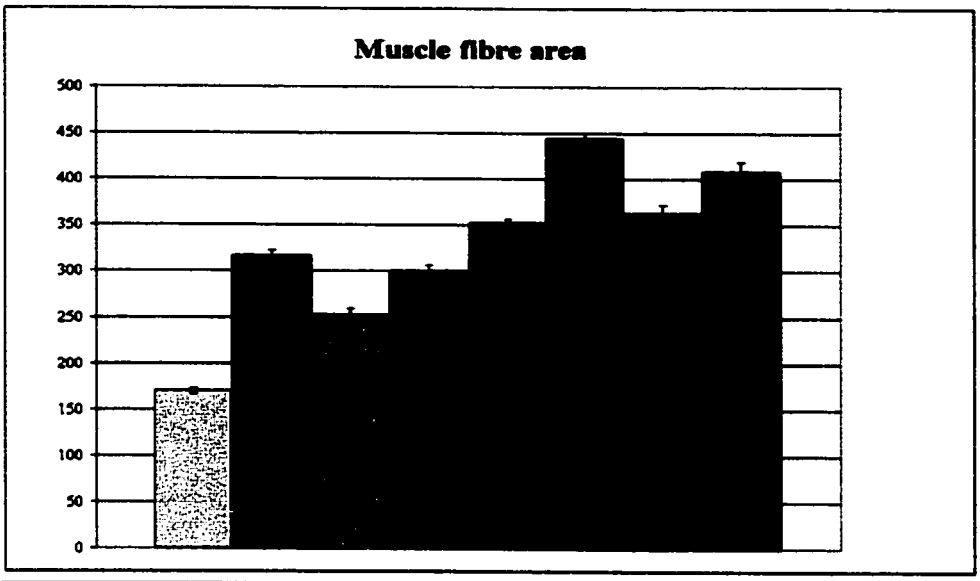

f
Muscle fibre diameter in BIN1 SH3 transgenic mice
fibre diameter (arbitrary units)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
**
**
*

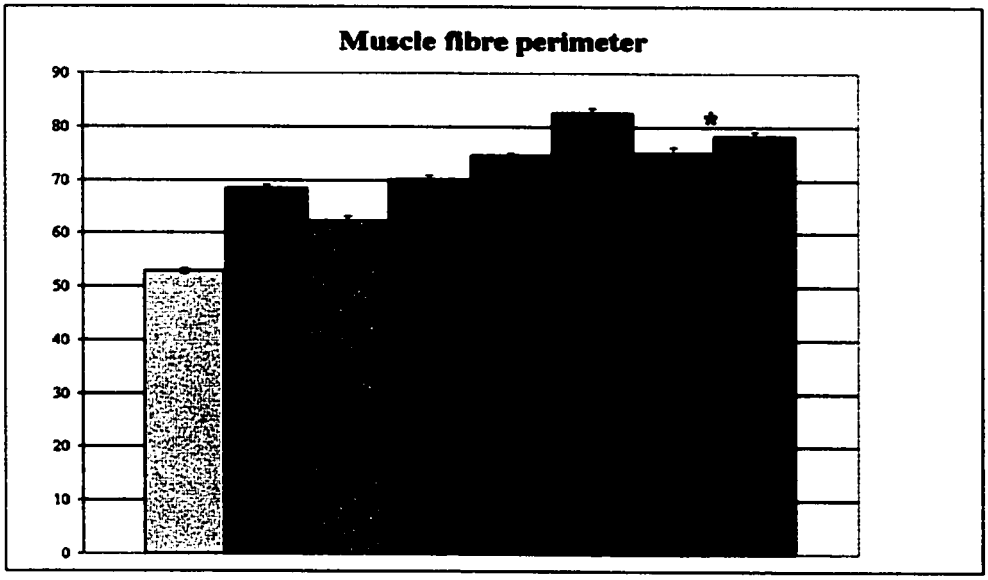
Figure 10. Cross sectional analysis of myofibres.

Bar graphs depicting myofibre area, perimeter and diameter from paraffin cross sections (10 μm) stained with haematoxylin/eosin. Samples were taken from p10 and p15 pups from line 869 and a minimum of 350 fibres (367-473) was counted for each sample group (p10 wt soleus, p10 tg soleus, p10 wt TA, p10 tg TA, p15 wt soleus, p15 tg soleus, p15 wt TA, p15 tg TA). A minimum of 60 fibres was counted from each individual muscle (range 60-100) and each sample group contained 5-6 muscles. Error bars represent standard error. All comparisons between wild type (wt) and Bin1 SH3 transgenic (tg) from each age group and muscle were highly significant ($p<0.001$) except for perimeter in p15 TA (*; $p<0.03$), which was moderately significant, and diameter in p15 TA (**; $p<0.1$), which was not significant. Sections were photographed using a Zeiss dissecting microscope. Area, perimeter and diameter were determined using Scion Image for Windows (Version Beta 4.0.2, Scion Corporation). Statistical significance was calculated using the Student's T-test (two-tailed distribution, two-sample equal variance). wt – wild type; tg – transgenic; TA – tibialis anterior.

a


Muscle fibre area
500
450
400
350
300
250
200
150
100
50
0

b


Muscle fibre perimeter
90
80
70
60
50
40
30
20
10
0
*

c

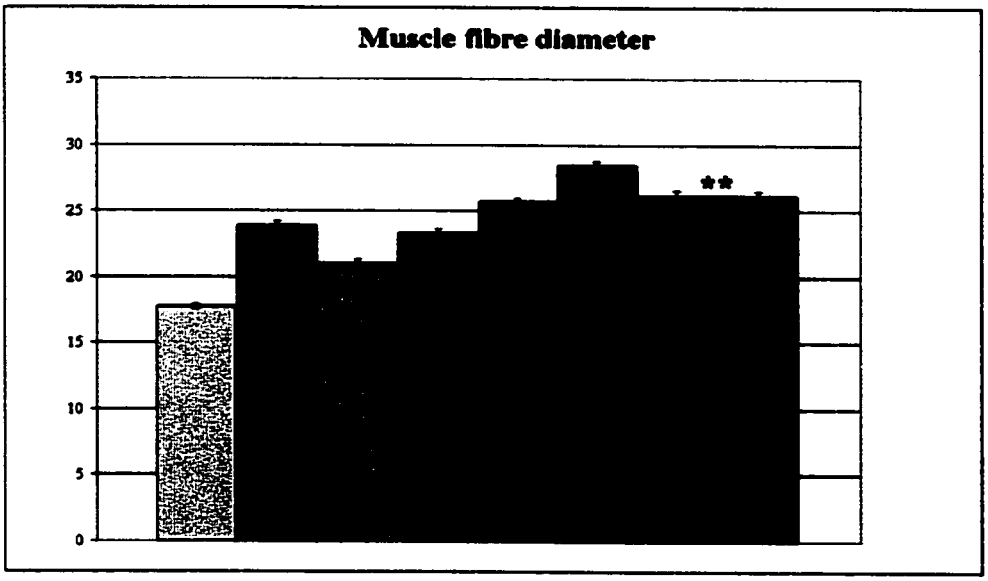
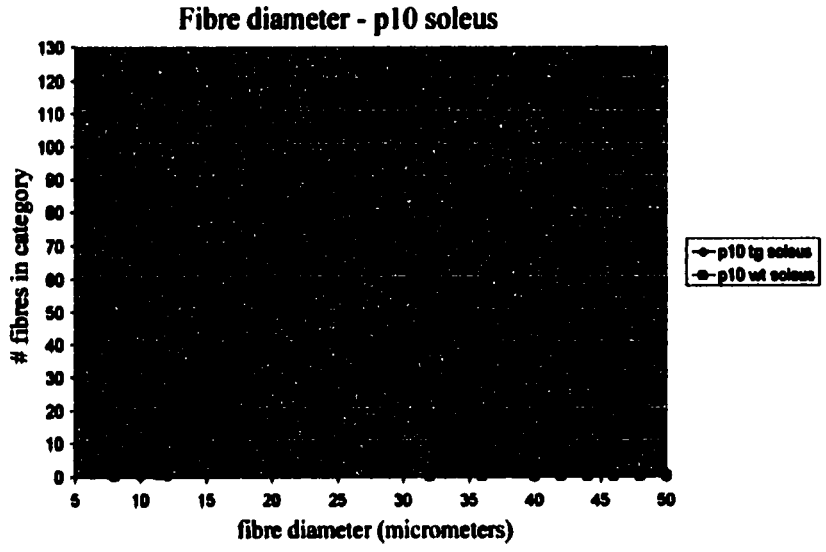
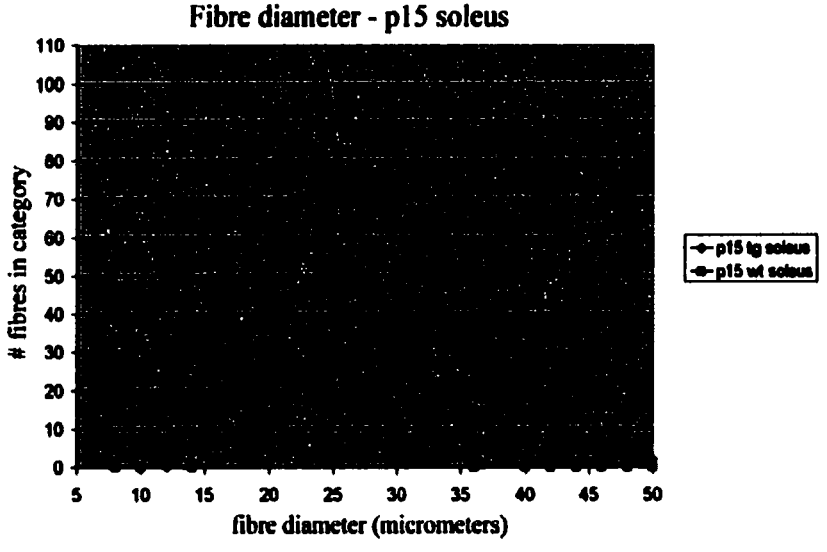
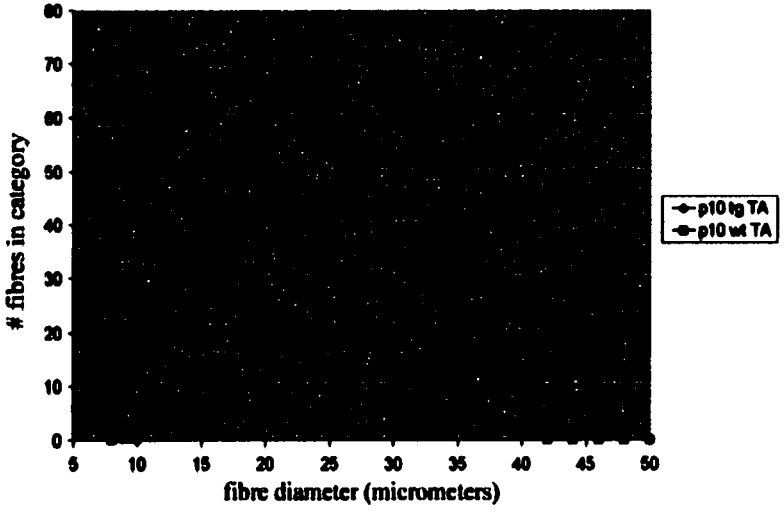

Muscle fibre diameter
35
30
25
20
15
10
5
0
**

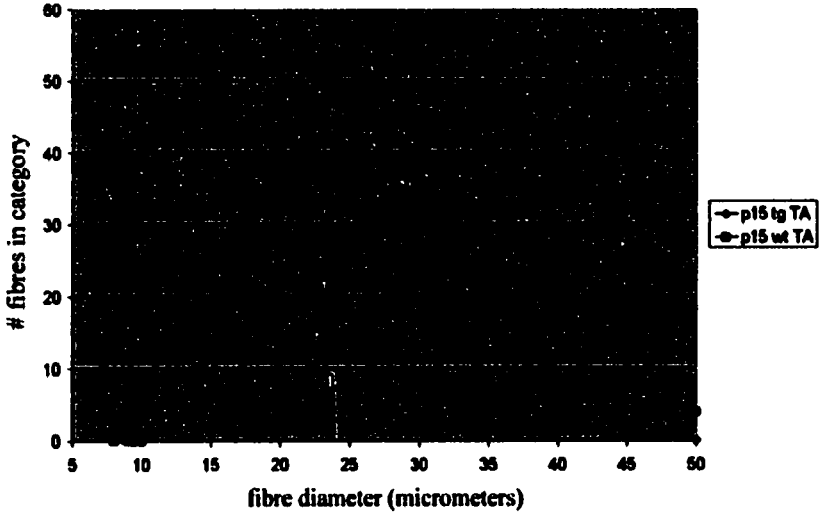
Figure 11. Fibre diameter distribution histograms.

Line graphs depicting myofibre maximum diameter from paraffin cross sections (10 μm) stained with haematoxylin/eosin. Samples were taken from p10 and p15 pups from line 869 and a minimum of 350 fibres (367-473) was counted for each sample group (p10 wt soleus, p10 tg soleus, p10 wt TA, p10 tg TA, p15 wt soleus, p15 tg soleus, p15 wt TA, p15 tg TA). Sections were photographed using a Zeiss dissecting microscope. Myofibre diameter was determined using Scion Image for Windows (Version Beta 4.0.2, Scion Corporation). Microsoft Excel was used to count the number of fibres within a two-micrometer range (i.e between 6 and 8, 10 and 12, 12 and 14, etc.) beginning at 6 μm and ending at 50 μm. Note the narrower distribution of fibre diameter in wild type soleus muscle when compared to Bin1 SH3 soleus muscle at p10 and p15. wt – wild type; tg – transgenic; TA – tibialis anterior.


Fibre diameter - p10 soleus
fibres in category
fibre diameter (micrometers)
p10 tg soleus
p10 wt soleus


Fibre diameter - p15 soleus
fibres in category
fibre diameter (micrometers)
p15 tg soleus
p15 wt soleus


Fibre diameter - p10 tibialis anterior
fibres in category
fibre diameter (micrometers)
p10 tg TA
p10 wt TA


Fibre diameter - p15 tibialis anterior
fibres in category
fibre diameter (micrometers)
p15 tg TA
p15 wt TA

3.6 Bin1 SH3 primary myoblasts display aberrant morphology during differentiation.

To further examine the effect of the Bin1 SH3 domain on myogenesis, primary myoblasts were harvested and cultured from 8-12 week old transgenic and wild type mice from line 869. Transgene expression during growth and differentiation was confirmed with RT-PCR using primers to the rabbit β-globin poly A (Figure 12b). Equal numbers of transgenic and wild type myoblasts were plated and allowed to reach 80% confluence before the differentiation program was triggered by serum reduction from 20% fetal bovine serum to 5% horse serum. Myoblasts were cultured for four days and protein was collected (Fernando and Heikkila, 2000) and quantified (BCA protein assay, Pierce) at growth, 12, 24, 48, 72, and 96 hours post-induction of differentiation. Both homozygous and heterozygous Bin1SH3, but not wild type primary myoblasts, displayed striking deficits in myotube formation (Figure 12a). Although multinucleate cells were present, they were decreased in number and were rarely able to form the long myotubes characteristic of wild type differentiation morphology (Figure 12a, top panels). These Bin1 SH3 multinucleate cells were positive for MHC antibody staining suggesting that the differentiation program was able to proceed and that these cells were not arrested during early stages of differentiation. Fusion index (number of nuclei in myotubes/total number of nuclei) was calculated to quantify the percentage of cells that were able to form multinucleate cells when triggered to differentiate. Three different fields from two separate isolations from line 869 were calculated at growth, 12 hours, 24 hours and 48 hours in Bin1 SH3 and wild type cells. Cells were stained with haemotoxylin to visualize nuclei. At 12 hours post-induction of differentiation, significant differences were

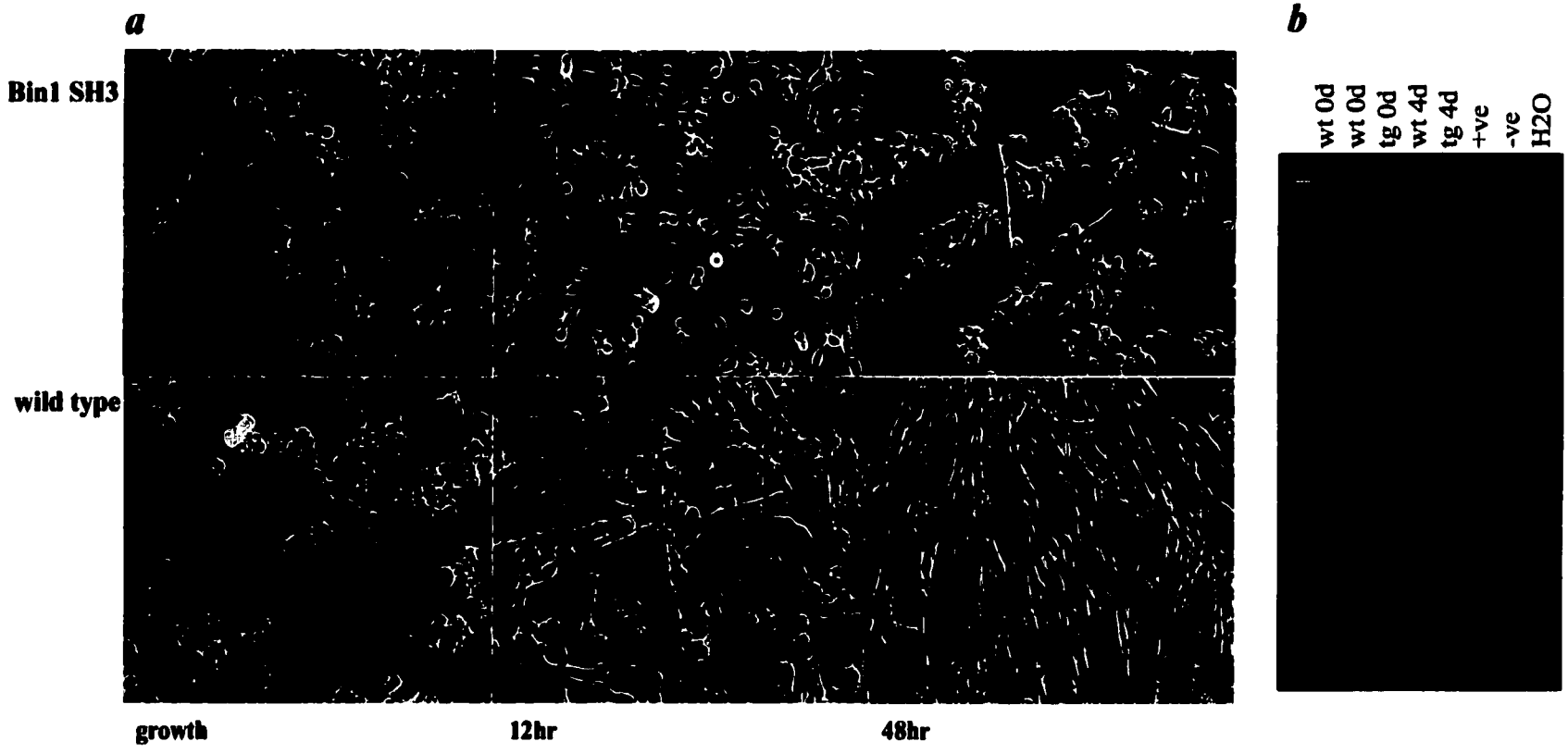
apparent with Bin1 SH3 myoblasts, i.e. the fusion index in Bin1 SH3 myoblasts was 1.56%, compared to wild type myoblasts at 10.85%. At 48 hours, Bin1 SH3 myoblasts were 22.49% while the wild types cells were 68.15% (Table 4). This dramatic reduction in the ability of Bin1 SH3 myoblasts to fuse into multinucleate cells, coupled with their inability to subsequently elongate into healthy myotubes, suggests that overexpression of the Bin1 SH3 domain impairs the *in vitro* differentiation program.

Table 4: Fusion index during differentiation of Bin1 SH3 and wild type primary myoblasts.

Time point	wild type (% ± STDEV)	Bin1 SH3 (% ± STDEV)	TTEST value
12 hours	10.8491 ± 0.05	1.5779 ± 0.03	$p<0.002$
24 hours	54.8186 ± 0.07	10.2849 ± 0.04	$p<0.001$
48 hours	65.4777 ± 0.14	22.4922 ± 0.08	$p<0.001$

Figure 12. Primary myoblast differentiation morphology.

a) Differentiating Bin1 SH3 (top panels) and wild type (bottom panels) primary myoblasts from line 869. Myosin heavy chain antibody (MF20) staining is robust in wild type primary myoblasts. However, myosin heavy chain staining is weaker in Bin1 SH3 myoblasts, which fail to form multi-nucleate, elongated myofibres by 48 hours (third panel). Four separate primary myoblast isolations and numerous differentiations elicited the same results. Bar is 0.1 mm. *b)* RT-PCR on differentiating myoblasts from *a)* confirms transgene expression up to four days in differentiation media. RNA was collected using TRIZOL reagent (Gibco) from wild type and transgenic adherent primary myoblasts at growth and 4 days differentiation. RNA was reverse transcribed to produce cDNA for PCR amplification. RT-GAPDH confirms RNA presence and integrity. RNA-BIN1 is PCR on samples that were not reverse transcribed, confirming that the RNA isolations are not contaminated with DNA. Positive (+ve) and negative (-ve) controls in RT-BIN1 and RT-GAPDH are RNA samples from expressing and non-expressing tissue from line 869, respectively. The positive (+ve) control in RNA-BIN1 contains tail DNA from a known positive mouse.


a
Bin1 SH3
wild type
growth
12hr
48hr
b
wt 0d
wt 0d
tg 0d
wt 4d
tg 4d
+ve
-ve
H2O

3.7 Aberrant bHLH protein expression in differentiating primary myoblasts.

The human Bin1 promoter has a functional regulatory site for myogenin and MyoD and the murine Bin1 has a potential promoter region site for Mef2, suggesting direct regulation of Bin1 by muscle-specific transcription factors (Mao et al., 1999; Wechsler-Reya et al., 1997b). MyoD is involved in the initial determination of the myogenic lineage and was properly downregulated upon differentiation in both wild type and Bin1 SH3 myoblasts (Rudnicki et al., 1993; Sabourin et al., 1999; Sabourin and Rudnicki, 2000) (Figure 13a). This would suggest that the function of the Bin1 protein during differentiation is directed downstream of the primary MRF's (MyoD and Myf5) and that normal MyoD expression is not sufficient to overcome the observed *in vitro* myotube deficiencies and *in vivo* muscle phenotype. In cultured myoblasts, myogenin is preferentially expressed during the differentiation of myoblasts (Andres and Walsh, 1996; Cusella-De Angelis et al., 1992). Western blot analysis with a monoclonal myogenin antibody (F5D, Developmental Studies Hybridoma Bank) revealed a twelve-hour delay in peak expression levels in differentiating Bin1 SH3 myoblasts when compared to wild type controls (Figure 13b). In addition, myogenin expression in the Bin1 SH3 myoblasts is not sustained, and its level declines by the fourth day of differentiation. Likewise, western blot analysis with a polyclonal MEF2C antibody (Cell Signalling Technology) revealed that the normal increase in MEF2C expression during differentiation was delayed and downregulated in transgenic myoblasts. Specifically, differentiating Bin1 SH3 myoblasts are prolonged in increasing the expression of MEF2C, attaining a weaker peak expression level (Figure 13c). These observations demonstrate that expression of the Bin1 SH3 domain can affect the expression of a subset

of muscle-specific transcripts. In addition, these results suggest that Bin1 participates in the differentiation program downstream of MyoD.

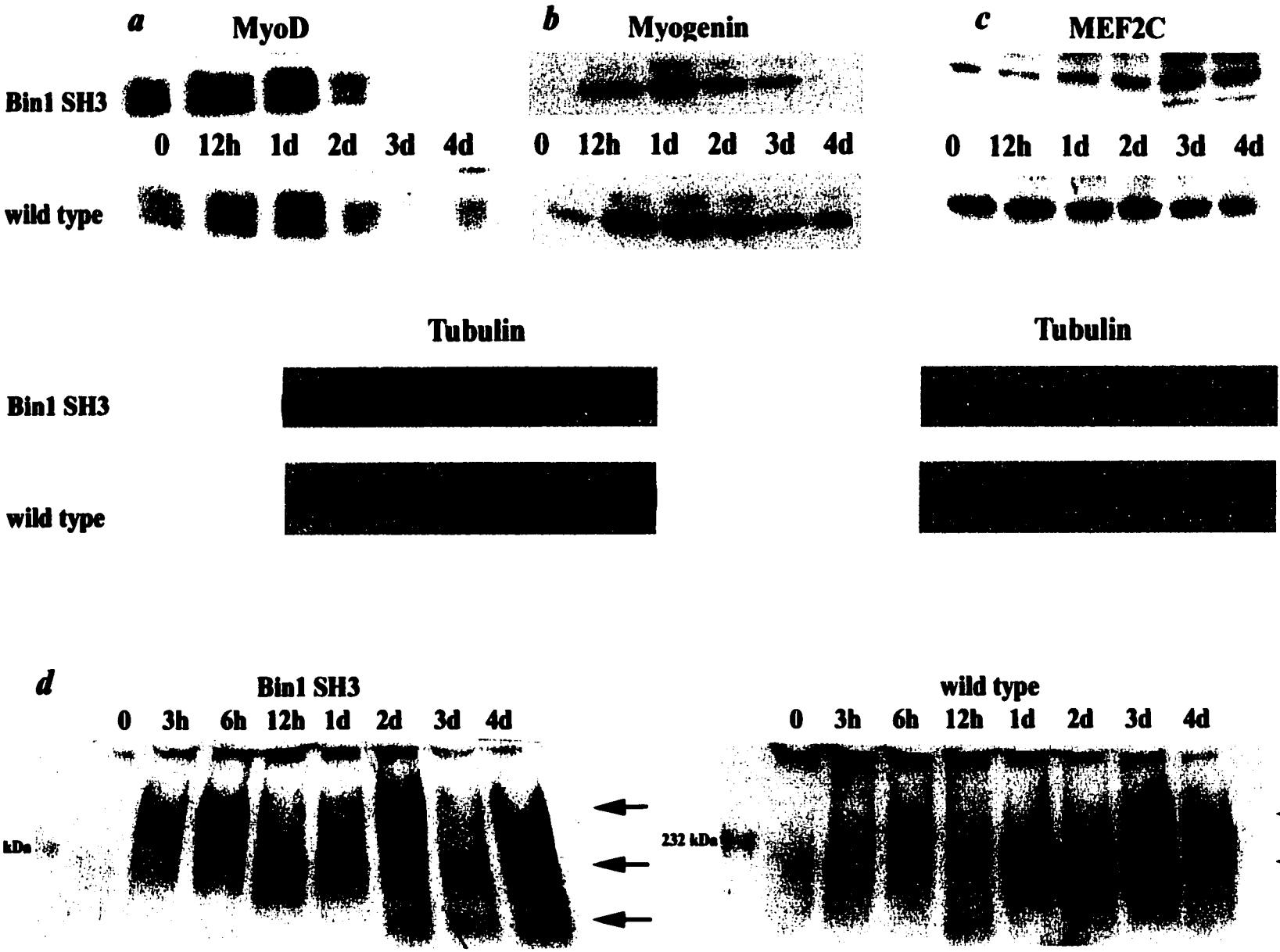
3.8 Disrupted Bin1 complex expression during differentiation of Bin1 SH3 primary myoblasts

Bin1 is an adaptor protein, and as such, is characterized by its protein-protein interactions mediated by its myc-binding and SH3 domains. By overexpressing the SH3 domain of Bin1, we have potentially affected the ability of endogenous Bin1 to form a stable protein complex necessary during differentiation. Therefore, an unstable complex would be incapable of mediating the normal functions of Bin1 during myotube formation. To examine Bin1 complex formation during differentiation, both in wild type and Bin1 SH3 primary myoblasts, protein lysates were separated on PAGE-electrophoresis using a native (SDS-free) gel and transferred onto a nylon membrane. The native gel prevents the dissociation of individual proteins from the Bin1 complex. A polyclonal Amphiphysin II antibody was used to detect full-length Bin1 (obtained from Dr. P. McPherson, Montreal Neurologic Institute and Hospital) in wild type and Bin1 SH3 primary myoblasts. Expression of the Bin1 complex during differentiation was dramatically different between Bin1 SH3 and wild type cells. In the wild type cells, a prominent band around the 230 kDa marker appeared at 24 hours of differentiation (Figure 13d, right panel). Integrity of this complex remained strong until the fourth day of differentiation when the complex was abruptly downregulated. Additionally, a larger Bin1 complex (between 230 kDa and 440 kDa) appeared at 6 hours, disappeared by 12 hours, and began to reappear at three and four days. In contrast, Bin1 complex formation

in Bin1 SH3 primary myoblasts appeared comparatively unstable (Figure 13d, left panel). The larger Bin1 complex is present at 3hr and is highly upregulated by 6 hr, 18 hours earlier than in the wild type cells. By 12 hr, it has almost entirely disappeared and transiently reappears at 2d, disappearing 24 hours later. The band around 230 kDa that is prominent in the wild type cells between 1 and 3 days differentiation is prematurely upregulated in the Bin1 SH3 cells and its expression tapers off early, by 2 days of differentiation. By the third day of differentiation in the Bin1 SH3 myoblasts, Bin1 complex formation appears highly unstable so that by day four, three to four bands are present. The Bin1 SH3 domain may be responsible for the de-stabilization of endogenous Bin1 in complex formation through its ability to sequester the binding partners of Bin1. This would render endogenous Bin1 unable to assemble and conserve the binding partners required for stable complex formation.

Figure 13. Aberrant protein expression in Bin1 SH3 primary myoblasts.

a), b), c) Western blot of differentiating primary myoblasts from line 869. MyoD (*a*) is unchanged between Bin1 SH3 and wild type myoblasts. Myogenin (*b*) and MEF2C (*c*) expression is delayed and diminished in BIN1 SH3 myoblasts. Specifically, myogenin reaches peak expression 12 hours later and subsequently diminishes quickly in BIN1 SH3 as compared to wild type myoblasts (compare expression levels at 12 hours, 1 day, 3 days, 4 days). MEF2C expression is downregulated throughout and is unable to attain peak expression until 3 days. Lower panels (left panel for *a* and *b*, right panel for *c*) show tubulin expression as a protein loading control. *d)* Bin1 protein complex assembly in Bin1 SH3 and wild type myoblasts. A differentiation complex appears within 24 hours in wild type myoblasts and remains strongly expressed until 4 days. The differentiation complex in Bin1 SH3 differentiating myoblasts appears by 12 hours, but is de-stabilized by 2 days. Bin1 SH3 and wild type primary myoblasts were cultured in growth (20% FBS) or differentiation (5% HS) media for four days. 20 μg of protein from cell lysates were separated on a 10% acrylamide gel (*a*-c) or a 2-13% gradient, SDS free, acrylamide gel (*d*) (to prevent protein denaturing). Protein was transferred to PVDF membranes and the membranes were blocked and incubated in the appropriate antibody.


a
MyoD
Bin1 SH3
0 12h 1d 2d 3d 4d
wild type
b
Myogenin
0 12h 1d 2d 3d 4d
c
MEF2C
0 12h 1d 2d 3d 4d
Tubulin
Bin1 SH3
wild type
Tubulin
d
Bin1 SH3
0 3h 6h 12h 1d 2d 3d 4d
232 kDa
wild type
0 3h 6h 12h 1d 2d 3d 4d
232 kDa

3.9 Apoptosis is unaffected in either embryos or transgenic primary myoblasts.

Full length Bin1 has been reported to increase programmed cell death when re-introduced into transformed cells that lack Bin1 (DuHadaway et al., 2001; Elliott et al., 2000; Elliott et al., 1999; Galderisi et al., 1999; Ge et al., 2000a; Ge et al., 2000b; Hogarty et al., 2000; Sakamuro et al., 1996). Indeed, a hallmark of oncogenic activity is not only the ability to increase proliferation, but to do so without the death penalty associated with tumour suppressor activity. These results suggest that a primary function of Bin1 is to induce an apoptotic program, thereby limiting the risk for transformation events. Based on these observations, we investigated the ability of the Bin1 SH3 domain to regulate apoptosis. Whole mount tunel assay was performed on 6 Bin1 SH3 and 15 wild type embryos at 9.5 dpc and 10.5 dpc from line 869. This assay measures a characteristic of apoptosis, sheared nuclear DNA, and can be used to monitor apoptosis in intact embryos (Conlon et al., 1995). However, this assay is limited beyond 10.5 dpc by the size of the embryo. At 9.5 dpc apoptosis was apparent in the otic vesicle in both wild type (Figure 14b, arrow) and Bin1 SH3 (Figure 14a, arrow) embryos. By 10.5 dpc, apoptosis was detectable at low levels within the somites and along the apical ectodermal ridge of the developing forelimb bud (Figure 14c, d, arrows). These areas of apoptosis agree with previous reports that use whole mount Annexin V apoptosis detection (van den Eijnde et al., 1997). Therefore, these results suggest that the Bin1 SH3 domain may not be involved in mediating cell death during embryonic development.

In addition to an *in vivo* examination of apoptosis, Annexin V assays on primary myoblasts were performed. Annexin V recognizes phosphatidyl serine that usually resides on the inner plasma membrane, but is exposed when the plasma membrane inverts

during early apoptosis (Koopman et al., 1994; Vermes et al., 1995). To give a quantitative assessment of the degree of apoptosis between Bin1 SH3 and wild type myoblasts, flow cytometry was performed on Annexin V-FITC immuno-stained cells. Therefore, the analysis was performed during growth conditions and 3, 6, 12, 24, and 48 hours after the addition of differentiation media in primary myoblasts from line 869. During growth conditions, both Bin1 SH3 and wild type myoblast cell populations displayed ~80% viable cells (Table 5). Interestingly, early apoptotic markers (Annexin V positive only) were higher in transgenic cells during early differentiation. However, at 24 hours, apoptosis had equilibrated in both the wild type and Bin1 SH3 myoblasts to 24%, with 64% viability (Figure 15). Although this FACS analysis represents one measurement, our laboratory has repeated this procedure numerous times on wild type myoblasts with similar and consistent results. Therefore, the *in vivo* and *in vitro* results illustrate preliminary evidence that suggest overexpression of the Bin1 SH3 domain does not perturb apoptosis.

Table 5: Apoptosis in Bin1 SH3 and wild type differentiating myoblasts

		Growth (%)	3 hrs (%)	6 hrs (%)	12 hrs (%)	24 hrs (%)	48 hrs (%)
Viable Cells (Annexin, PI negative)	Wt	76.9	91.3	73.1	91.4	64.2	40.6
	Bin1SH3	81.2	91.7	74.7	82.1	64.3	48.7
Early Apoptosis (Annexin positive only)	Wt	2.3	1.0	3.7	2.5	23.6	35.6
	Bin1SH3	1.4	3.0	12.6	7.8	24.1	39.0
Late Apoptosis (Annexin, PI positive)	Wt	19.3	2.2	13.8	3.5	5.2	17.1
	Bin1SH3	14.5	2.4	10.3	3.6	9.1	7.9
Necrosis (PI positive only)	Wt	1.6	5.5	9.4	2.6	7.0	6.7
	Bin1SH3	2.9	2.9	2.4	6.4	2.4	4.4

Figure 14. Whole mount apoptosis detection during development.

Whole mount tunnel assays for apoptosis show no differences in cell death at 9.5 dpc between BIN1 SH3 (*a*) and wild type (*b*) embryos from line 869. Apoptosis in the otic vesicles (arrows) is similar at this stage of development. Apoptosis is also similar at 10.5 dpc between Bin1 SH3 (*c*) and wild type (*d*) embryos from line 869 in the somites and along the apical ectodermal ridge (arrows). Whole mount tunel assay preferentially stains DNA strand breaks in apoptotic nuclei as brown specs.

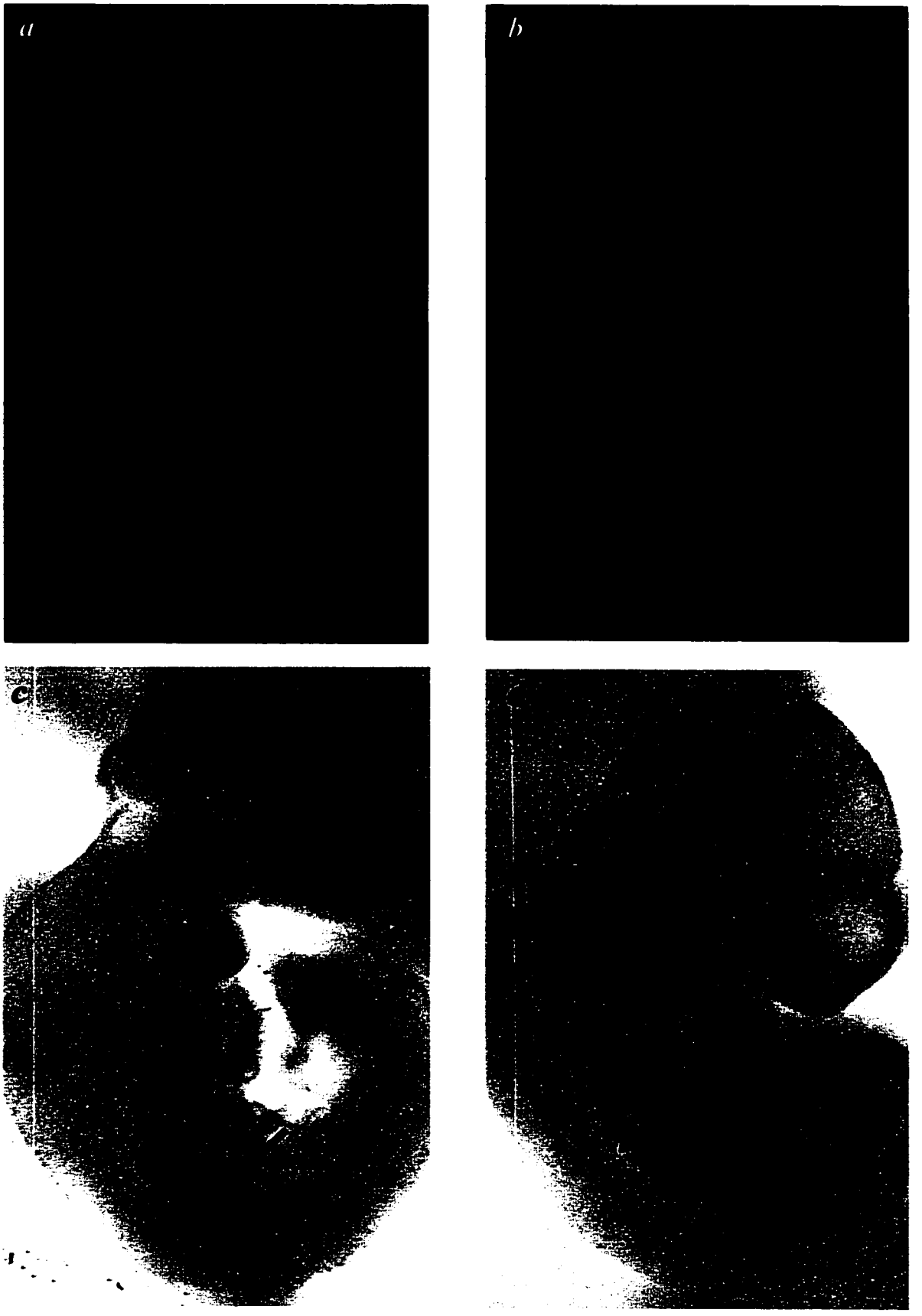
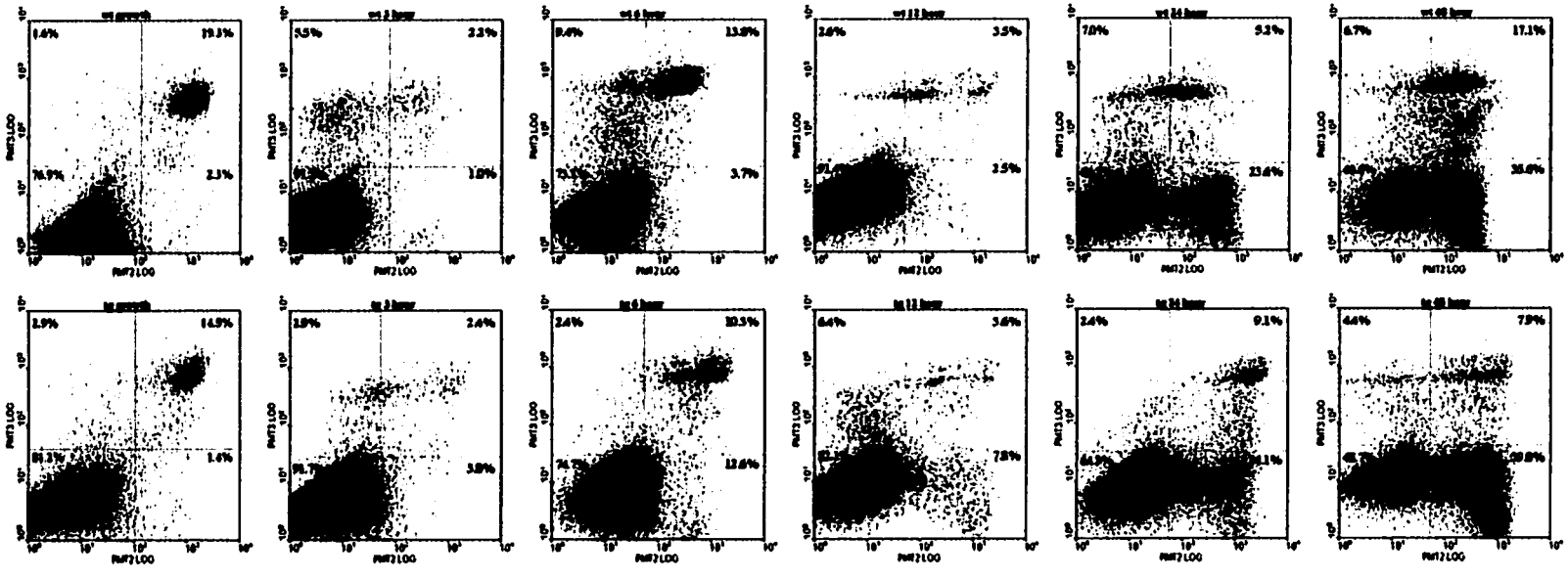

a
b
c

Figure 15. Annexin V flow cytometry of primary myoblasts.

Adherent primary myoblasts (line 869) were differentiated in 5% horse serum and stained for Annexin V and propidium idodide (PI) reactivity. Following incubation with Annexin V and PI, 2 x 10^4 cells were immediately passed through a Beckman Coulter-Ultra flow cytometer and analyzed using Expo Cytometer Software, Version 1 (Applied Cytometry Systems). Annexin V recognizes phosphatidyl serine that becomes exposed to the outer cell surface during the early stages of apoptosis when the plasma membrane inverts. PI is able to permeate the plasma membrane during the later stages of apoptosis and during necrosis, when membrane integrity becomes compromised. Therefore, Annexin-V-FITC staining was used to identify cells undergoing the initial stages of apoptosis (lower right quadrant), while propidium iodide (PI) was used to identify necrotic cells (upper left quadrant). Cells positive for both Annexin-V and PI are in later stages of apoptosis (upper right quadrant). Cells negative for both Annexin-V and PI are viable cells (lower left quadrant). Bin1 SH3 myoblasts appeared slightly more susceptible to apoptosis during the initial stages of differentiation (compare lower right quadrants at 6 hours and 12 hours). At 24 and 48 hours of differentiation, apoptosis was similar in both wild type and transgenic myoblasts.



3.10 Growth rate is unaffected in either transgenic mice or primary myoblasts.

The tumour suppressor characteristics of Bin1 lead to the expectation that alterations in its function could result in a disregulation in cell growth. Therefore, to examine this feature of a tumour suppressor, *in vitro*, growth curves in Bin1 SH3 and wild type primary myoblasts were performed. 1×10^5 cells from three separate Bin1 SH3 and wild type myoblast isolations from line 869 (two of these lines were also used previously in differentiation and FACS analysis) were plated and allowed to proliferate for 24 hours before they were collected and counted over three days. No difference in proliferation was observed (Figure 16a). These growth results, together with the absence of an apoptosis phenotype, suggest that the SH3 domain of BIN1 is an unlikely participant in cell cycle regulation. These observations are coincident with the hypothesis that the SH3 domain of Bin1 acts in a tissue-specific manner in skeletal muscle.

3.11 Lack of non-muscle pathology in adult Bin1 SH3 transgenics

To further examine the potential for growth regulation in the Bin1 SH3 domain, 10 week old Bin1 SH3 (female, n=9; male n=9) and wild type (female, n=9; male n=9) mice from lines 869, 508, and 482 were weighed (Figure 16b). While differences in mass between genders were observed, no differences in overall mass were observed between Bin1 SH3 and wild type mice.

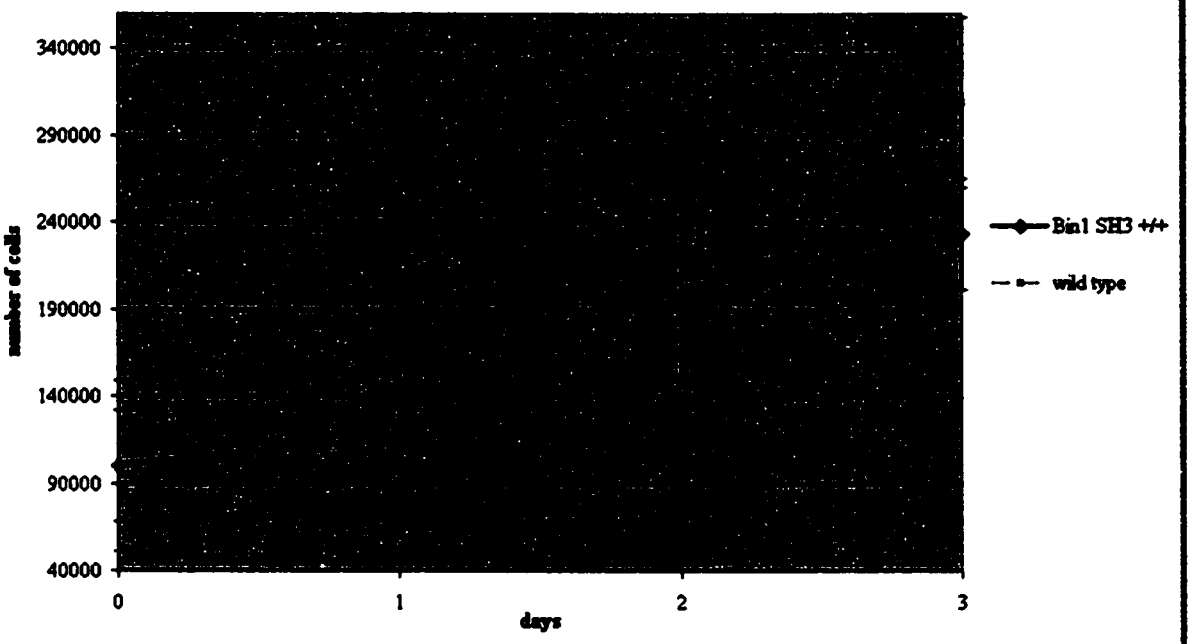
Functional loss of heterozygosity of Bin1 in breast, prostate and lung cancer implicates loss of Bin1 in tumour formation (Ge et al., 1999; Ge et al., 2000a; Ge et al., 2000b; Sakamuro et al., 1996). Therefore, in addition to mass measurements, an organ survey on

transgenic and wild type age-matched controls greater than 8 months old was also performed to search for abnormal growth. Histological sections (10 μm) from lines 501, 502, 487, and 869 from liver, heart, lung, prostate and skin were stained with haematoxylin/eosin (Figure 17). In particular, the exceptionally high expression in the heart warranted a closer examination of the morphology of this organ. All of the sections from all of the mentioned tissues were grossly examined for indications of abnormal growth, aberrant cell morphology, increased cell number, focal necrosis and tumour formation. No obvious irregularities in organ or cell structure were present in the Bin1 SH3 mice from any of the lines investigated. These results suggest that a tissue-specific role for the Bin1 SH3 domain does not lead to alterations in non-skeletal muscle cell morphology and histology.

Figure 16. Body mass comparison and primary myoblast growth curve

a) Rate of growth of Bin1 SH3 and wild type primary myoblasts. Growth curves for low-passage Bin1 SH3 (line 869) and wild type primary myoblasts held in growth media was determined. Data represent an average growth from three separate trials (± SD). At each time point from each trial, cells were counted from three separate cell volumes of 15 μl each and averaged. Both Bin1 SH3 and wild type myoblasts demonstrate similar rates of growth (Day 1 $p=0.77$; Day 2 $p=0.95$; Day 3 $p=0.34$). *b)* Body mass measurements of Bin1 SH3 and wild type mice. Female transgenic (n=9) and wild type (n=9) and male transgenic (n=9) and wild type (n=8) 10-week-old mice were weighed. Significant differences between male and female mice were observed ($p<0.001$), but no differences between female transgenic and wild type ($p=0.16$) or between male transgenic and wild type ($p=0.57$) were observed.

a


Growth curve in Bin1 SH3 and wild type primary myoblasts
340000
290000
240000
190000
140000
90000
40000
number of cells
0
1
2
3
days
Bin1 SH3 +/+
wild type

b

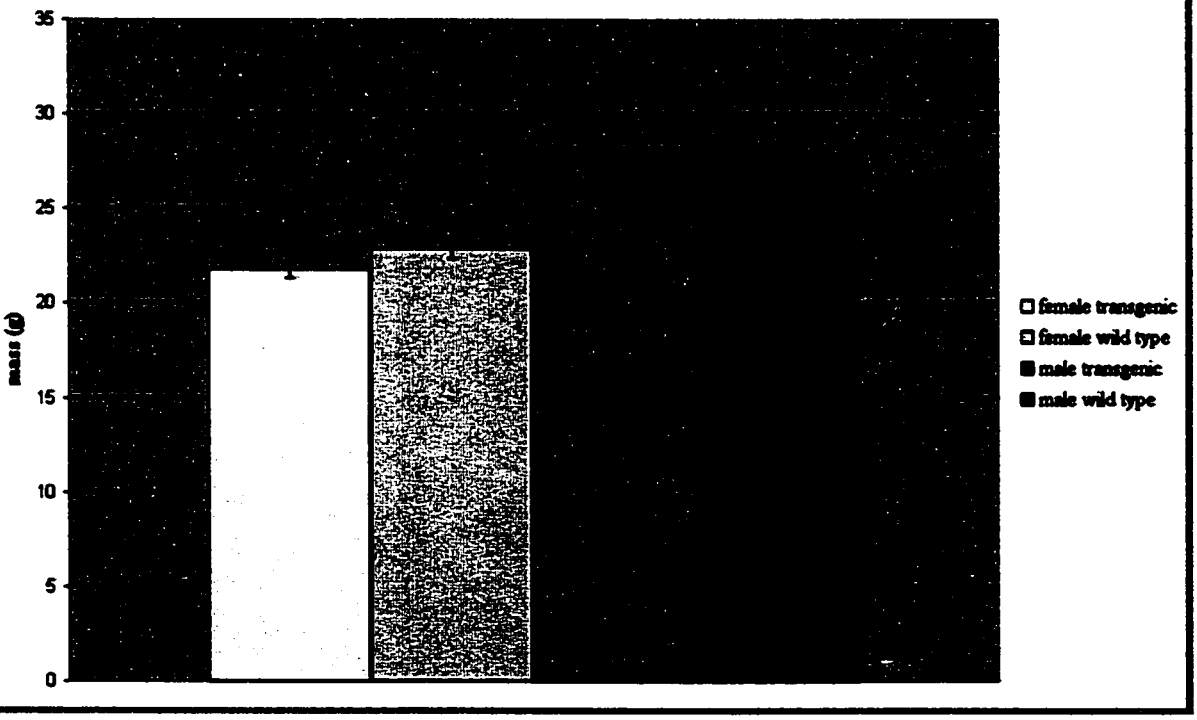
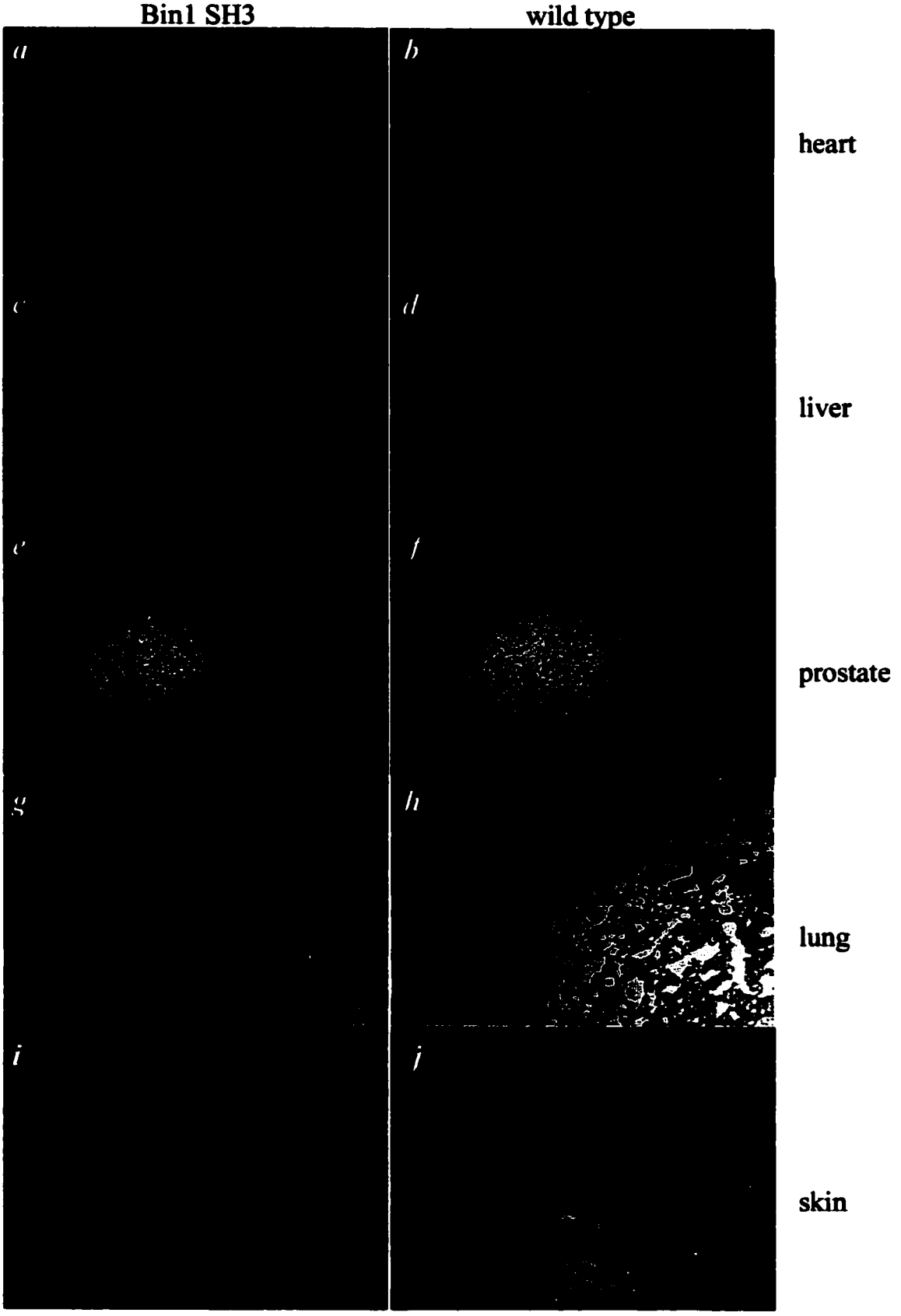

Body mass in Bin1 SH3 and wild type mice
35
30
25
20
15
10
5
0
mass (g)
female transgenic
female wild type
male transgenic
male wild type

Figure 17. Tissue scan

Bin1 SH3 (left) and wild type (right) tissues from an aged (9 months) high expresser (line 501; refer to Figures 5 and 6) showed no difference in overall cell morphology and organ structure. Similar analysis was performed on lines 502, 487, and 869. Haematoxylin/eosin stained, paraffin-embedded tissues were sectioned at 10 μm. No overt signs of tumour formation were observed (i.e. large masses, tissue discoloration, organ structure shape abnormalities, etc). (*a*) and (*b*) heart; (*c*) and (*d*) liver; (*e*) and (*f*) prostate; (*g*) and (*h*) lung; (*i*) and (*j*) skin. Bar is 0.1 mm.


Bin1 SH3
wild type
a
b
heart
c
d
liver
e
f
prostate
g
h
lung
i
j
skin

CHAPTER 4

4.0 Discussion

This study supports the hypothesis that the SH3 domain of Bin1 has a specific role in skeletal muscle. Endogenous Bin1 is ubiquitously expressed, has roles in cell cycle regulation, and has been shown to inhibit the proto-oncogene c-myc. Therefore, by overexpressing the SH3 domain of Bin1, we were able to assess the function of this particular motif across a variety of tissues. However, skeletal muscle was the only tissue in which a phenotype was evident. Previous experiments support this finding, as conserved features of the Bin1 promoter suggest roles in myogenesis (i.e. MyoD and MEF2 consensus sequences) (Mao et al., 1999). Indeed, Bin1 is upregulated during C2C12 differentiation (Wechsler-Reya et al., 1998), primary myoblast differentiation, and myogenesis in the developing embryo (Megeney et al., unpublished observations). Furthermore, Bin1 exists in stoichiometric excess of c-myc in cells and is present in several isoforms, some of which lack the MBD required for c-myc interaction (Elliott et al., 1999; Wechsler-Reya et al., 1997b; Wechsler-Reya et al., 1998). Together with previous research, our observations suggest a tissue specific role for Bin1 that is regulated by the SH3 domain.

4.1 Myogenin expression in Bin1 SH3 transgenic mice

In support of a role for the Bin1 SH3 domain in myogenesis, Bin1 SH3 founder embryos (Figure 7) and homozygous embryos from line 487 (Figure 8) displayed a severe reduction in myogenin message. Evidence from myogenin null mice suggests that myogenin is indispensable for transcript (or messenger RNA) (Hasty et al., 1993; Nabeshima et al., 1993; Venuti et al., 1995). In particular, myogenin is essential for the development of secondary myofibres (Venuti et al., 1995). To assess the phenotypic

impact of decreased myogenin expression in Bin1 SH3 mice, we analysed fibre size in p10-p15 mice. Bin1 SH3 mice had much larger myofibre cross sectional area, diameter, and perimeter, confirming an effect of myogenin expression on secondary myofibre development. Interestingly, the differences in these variables decreased as the mice aged. For example, at p10, Bin1 SH3 cross sectional area in the soleus muscle was ~2 fold greater than wild type, and this decreased to a 25% difference by p15 (refer to Table 3).

This trend toward normalization of fibre size with age was consistent in each variable and time point measured and may be explained by examining myogenin expression. Myogenin is downregulated postnatally during muscle maturation, i.e. 15-fold after the third week of development (Eftimie et al., 1991). Therefore, other muscle specific genes may be able to compensate in the Bin1 SH3 mice as the importance of myogenin decreases with age. For example, MRF4 expression remains elevated throughout adult muscle maturation and is responsible for maintaining the differentiated state (Megeney and Rudnicki, 1995; Perry and Rudnicki, 2000).

Interestingly, the tibialis anterior muscle was less affected by myogenin disruption than the soleus muscle (i.e. – fibre size differences between Bin1 SH3 and wild type in the tibialis anterior muscle were not as large as in the soleus muscle). The greater decrement in soleus versus tibialis anterior may be a result of the fibre type composition differences between these two muscles. Indeed, the soleus and tibialis anterior muscles were selected for their variable level of slow twitch versus fast twitch fibre type composition, respectively. Soleus muscle is composed primarily of slow twitch (type I) fibres and is

used in activities that require low muscle tone for long periods of time (e.g. – standing) (Hauschka, 1994). Conversely, tibialis anterior is composed primarily of fast twitch (type IIa and type IIb) fibres and is a muscle used in activities that require high muscle tone for shorter periods of time (e.g. – running) (Hauschka, 1994). However, staining for slow and fast isoforms of MHC revealed that myogenin does not affect the development of specific muscle fibre types (Venuti et al., 1995). Nevertheless, it is possible that the Bin1 SH3 domain may affect fibre type development in addition to affecting secondary myogenesis through myogenin disruption. Further experiments in Bin1 SH3 mice, such as studying expression of slow and fast isoforms of MHC, will be important in assessing a role for this domain in fibre type development.

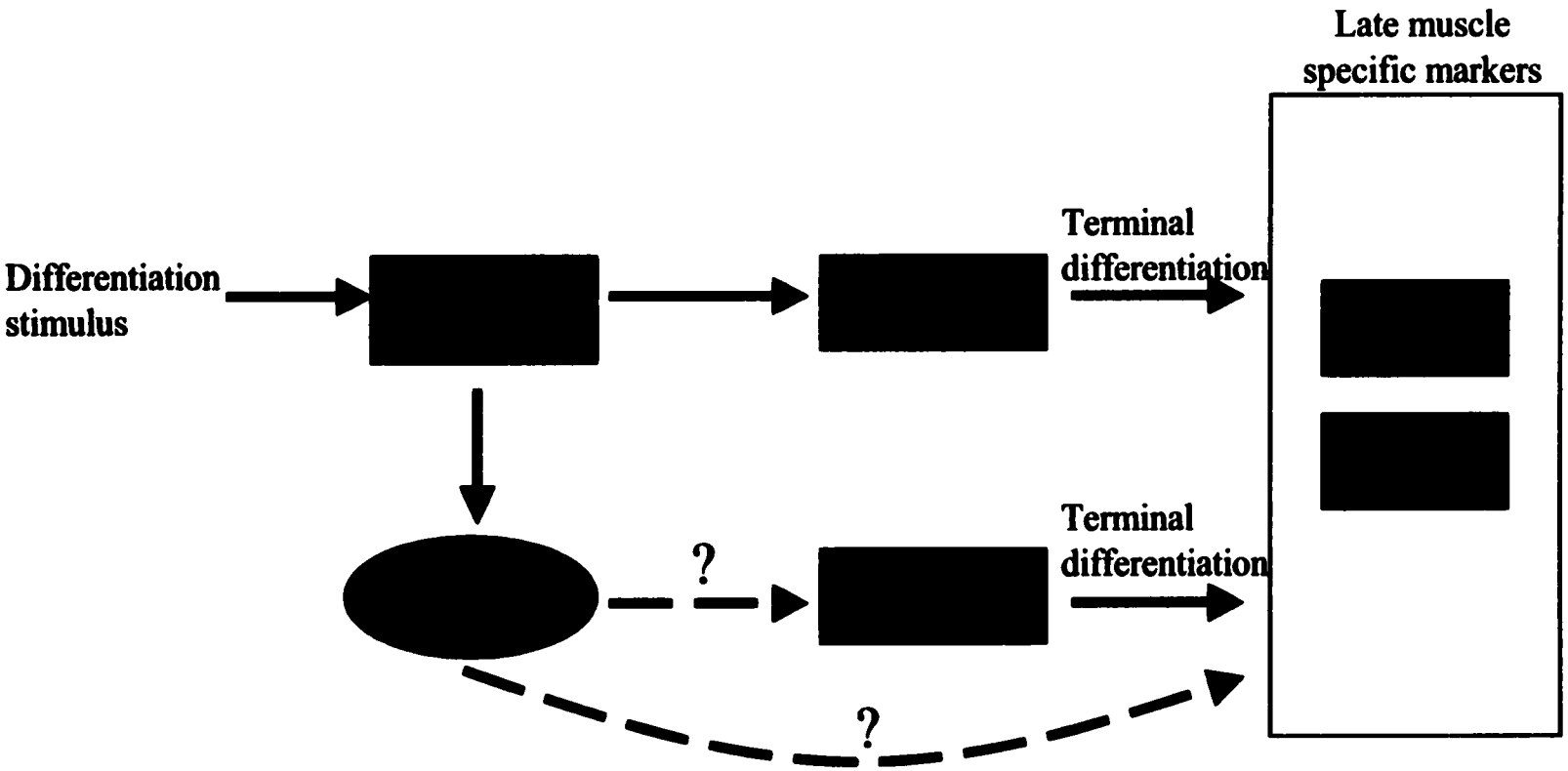
4.2 Disrupted MRF expression in Bin1 SH3 myoblasts

Disruptions in MRF expression are also evident during myotube formation in Bin1 SH3 primary myoblasts. In addition, when antisense Bin1 is expressed in C2C12 cells, they are also unable to differentiate (Wechsler-Reya et al., 1998). Wild type differentiating C2C12 cells express myogenin within 24 hours of beginning the differentiation process. Myogenin is required for the expression of cyclin dependent kinase inhibitor, p21, and for myoblasts to permanently exit the cell cycle. Myoblasts must accomplish these initial steps before they begin expressing myofibrillar (MHC) and enzymatic proteins (MCK), and before they fuse into multinucleate myotubes (Perry and Rudnicki, 2000). In agreement with an early upregulation of Bin1 message, differentiating Bin1 SH3 primary myoblasts demonstrate a 12-hour delay in myogenin upregulation. Bin1 SH3 myoblasts are also unable to fuse properly, and demonstrate decreased MHC expression (Figure 12,

13, Table 3). The delayed and overall decrease in myogenin and MEF2C expression suggests that these proteins are inhibited in their ability to further the differentiation process. In contrast, the normal expression of MyoD in Bin1 SH3 and wild type differentiating primary myoblasts suggests that the initial differentiation cues are intact (Figure 13a). Indeed, our lab has observed reductions in Bin1 expression concurrent to the delayed muscle differentiation in both MyoD and Myf5 null embryos, suggesting that Bin1 expression is subject to regulation by the primary MRF proteins (Megeney et al, unpublished observations). Together, these observations suggest a model whereby Bin1 functions downstream of MyoD and upstream of myogenin in the differentiation program (Figure 18).

Figure 18. Skeletal muscle differentiation: Context of Bin1

Schematic depicting a model for Bin1 in skeletal muscle differentiation. Differentiation stimuli are known to activate skeletal muscle specific transcription (solid horizontal arrows) through the bHLH proteins (*red* boxes). In addition, MyoD can activate Bin1 (*blue* oval) in response to differentiation stimuli (solid vertical arrow). Our observations suggest that Bin1 exerts its effects post-induction of differentiation at the level of myogenin expression (dashed horizontal arrow), although the mechanism for this effect is unknown. Furthermore, Bin1 may affect other differentiation factors (*purple* boxes) through an unknown mechanism (dashed curved arrow).


Late muscle
specific markers
Terminal
differentiation
Differentiation
stimulus
Terminal
differentiation
?
?

4.3 Bin1 SH3 and primary myoblast differentiation

Given that Bin1 SH3 myoblasts are unable to form myotubes, what could be causing the differences in fusion index between Bin1 SH3 and wild type myoblasts? First, the overexpression of the Bin1 SH3 domain may have an indirect effect on myotube formation. For example, myogenin, MEF2C, and MHC expression is disrupted in differentiating Bin1 SH3 myoblasts (Figure 12a, b, c) and these proteins are all required for the coordination and completion of the differentiation program. Indeed, inhibition of MyoD, myogenin, and Myf5 can specifically prevent myotube fusion in C2C12 cells (Dedieu et al., 2002), suggesting that these MRFs may activate another subset of muscle specific genes important for myoblast fusion. Therefore, the inability of Bin1 SH3 myoblasts to fuse into myotubes could be secondary to a disruption in the activity/expression of the MRFs.

Alternatively, a disruption in Bin1 SH3 expression could directly affect myoblast fusion, possibly through an interaction with cytoskeletal proteins. Indeed, the BAR family RVS proteins interact with a number of components of the actin cytoskeleton (Bon et al., 2000). For example, RVS167, the yeast homologue of Bin1, interacts with actin specifically through its SH3 domain (Amberg et al., 1995). Furthermore, Bin1 is expressed in the T-tubules of murine skeletal muscle (Butler et al., 1997), which contain several cell adhesion and associated cytoskeletal proteins (Tuvia et al., 1999). Recently, an amphiphysin null mutant in drosophila has demonstrated disruptions in the excitation-contraction mechanism, concurrent to T-tubule formation and actin expression, both of which rely on proper cytoskeletal arrangement and function (Razzaq et al., 2001). For

these reasons, the subcellular changes in Bin1 localization throughout differentiation should be carefully characterized and compared to cytoskeletal protein expression patterns, as Bin1 localization during distinct stages of differentiation may be informative.

It is also possible that the Bin1 SH3 domain may not affect cytoskeletal rearrangement during myoblast fusion and differentiation, but may interact with other factors through an as yet uncharacterized pathway. This argument is supported by the observation that targeted mutations in RVS161 result in separable actin versus cell fusion defects. In addition, actin and endocytosis defects appear with the same mutation (Brizzio et al., 1998). Subsequently, the link between RVS161 and cell fusion was supported by the localization of RVS161 to the cell fusion zone in prezygotes and its interaction with Fus2p (Brizzio et al., 1998). Fus2p is a protein associated either with a vesicular or cytoskeletal structure that localizes to the region of cell fusion and plays a specific role in this process during zygote formation in yeast (Elion et al., 1995). In addition, both RVS161 and Fus2p appear to act in the same pathway and the stability of the Fus2p protein depends on the presence of RVS161 (Brizzio et al., 1998). Thus, it is possible that Bin1 may interact with a Fus2p homologue to promote myoblast fusion and myotube formation. To summarize, our studies demonstrate that the Bin1 SH3 domain affects early differentiation cues through MRF expression, which in turn facilitate proper myotube formation. However, more direct roles for this domain during the later stages of differentiation, such as myoblast fusion, rearrangement of the cytoskeleton, and establishment of the excitation-contraction mechanism (T-tubules) may also be important.

4.4 In vitro versus in vivo phenotype discrepancy

Interestingly, the overexpression of the Bin1 SH3 domain elicits a more severe phenotype *in vitro* than *in vivo*. Intuitively, an inability to differentiate and properly form myotubes is significantly more disruptive than an increase in fibre diameter post-natally. Competing theories may explain this disparity. Most obviously, the *in vivo* system is a highly coordinated network that we can only begin to imitate *in vitro*. A complex network of communication and protein regulation exists *in vivo* that is better able to compensate for the aberrations in Bin1 function than in an *in vitro*, single cell system. For example, innervation of primary myofibres has been demonstrated as an important requirement for proper muscle development (Ashby et al., 1993; Duxson and Sheard, 1995; Wilson and Harris, 1993).

Alternatively, the phenotype discrepancy may occur from the different origins of *in vitro* myoblasts compared to the population examined *in vivo*. Myoblasts used in cell culture are derived from adult satellite cells that originate during a third wave of differentiation from the somite in late embryogenesis (Perry and Rudnicki, 2000). They are generally quiescent, but are activated and fuse to existing myofibres during muscle regeneration after physical stresses, such as injury or exercise (Seale and Rudnicki, 2000). Considering the continued high expression of Bin1 in adult skeletal muscle, it is possible that a more severe *in vivo* phenotype could be elicited when testing muscle regeneration (e.g. – crush experiments, exercise stress, etc.). Indeed, this is the case with MyoD null mice that demonstrate severe impairment of muscle regeneration after injury (Megeney and Rudnicki, 1995).

4.5 Bin1 complex formation occurs early in differentiation

SH3 domains are protein interaction domains that encourage complex assembly (Mayer, 2001). Therefore, Bin1 function is likely dependent on formation of a multi-protein complex. To further characterize Bin1 during differentiation, we analyzed full-length Bin1 complex assembly during myoblast differentiation in both wild type and Bin1 SH3 primary myoblasts. The formation of a Bin1 protein complex between 12 and 24 hours of differentiation in wild type myoblasts suggests that Bin1 is important during the early stages of differentiation. These observations are supported by other data from our lab that demonstrate that Bin1 message is upregulated within 24 hours of differentiation (Megeney et al., unpublished observations). In contrast, Bin1 complex formation was perturbed and appeared unstable in Bin1 SH3 myoblasts, suggesting that the Bin1 SH3 domain is functioning in a dominant-negative manner. To date, the proteins present in the Bin1 complex are unknown. However, the differentiation specific formation of the Bin1 complex suggests that assembly may require other proteins that regulate skeletal muscle differentiation, such as signalling proteins. For example, P38 has been shown to positively affect MRF expression/activation (Bergstrom et al., 2002).

4.6 Bin1 SH3 primary myoblasts are not delayed in differentiation

Importantly, the inability of Bin1 SH3 primary myoblasts to properly differentiate *in vitro* is not simply representative of a differentiation delay. Neither MEF2C nor myogenin reach equivalent expression levels compared to wild type myoblasts, and myogenin expression tapers off by 4 days of differentiation (Figure 13 b, c). In addition,

full length Bin1 complex formation is not delayed in forming, but rather, is assembled prematurely and quickly breaks down (Figure 13d). Finally, extended time in differentiation serum does not result in myotube formation in Bin1 SH3 myotubes (up to seven days).

4.7 Tissue specific function of Bin1

This study argues that the Bin1 SH3 domain has a specific role in skeletal muscle. In contrast, full-length Bin1 has pro-apoptotic roles in Bin1 null malignant cells (Sakamuro et al., 1996) and decreases proliferation when introduced into C2C12 myoblasts (Wechsler-Reya et al., 1998). The presence of a NF-κB consensus sequence in the Bin1 promoter also suggests a role for Bin1 in apoptosis (Mao et al., 1999). Taking these previous observations into account, it is reasonable to attribute a pro-apoptotic role to the Bin1 SH3 domain. Therefore, Bin1 SH3 myoblasts may be expected to demonstrate an increase in proliferation and, possibly, a decrease in apoptosis. However, these events were not observed when proliferation was measured in cultured myoblasts over three days (Figure 16a), and when apoptosis was measured during growth and differentiation using the Annexin-V assay (Figure 15). Unexpectedly, apoptosis appeared slightly elevated in the Bin1 SH3 differentiating myoblasts. The reason for this is unknown, but may be secondary to the decreased differentiation in the transgenic myoblasts and would need to be studied further.

Similar to Bin1 SH3 myoblasts, a role for the SH3 domain *in vivo* was not observed in apoptosis during embryogenesis (Figure 14). A role for the Bin1 SH3 domain was

equally not apparent during growth in adult body mass measurements (Figure 16b). Bin1 expression is absent or deregulated in breast, prostate, lung and liver carcinomas (Ge et al., 2000a; Ge et al., 2000b; Negorev et al., 1996; Sakamuro et al., 1996). Therefore, the effect of the Bin1 SH3 domain on tumour formation in a variety of tissues was examined histologically. The absence of aberrant morphology in Bin1 SH3 transgenic mice (greater than 9 months old) in prostate, lung, liver and other tissues further suggests that the Bin1 SH3 domain has no specific role in cell cycle regulation or apoptosis (Figure 17).

4.8 Bin1 SH3 function in the brain

Bin1 is also highly expressed in the brain and the Bin1 SH3 domain has been shown to be a potent and specific inhibitor of endocytosis in neural cell culture. Therefore, an *in vivo* model for Bin1 SH3 overexpression would offer a useful tool to corroborate these *in vitro* observations. Unfortunately, Bin1 SH3 transgene expression in the brain was infrequent and only weakly expressed in line 501 (Figure 5c). In addition, a few founder embryos had smaller heads, but whether this was from an effect on endocytosis, or from another event was not pursued. It is possible that lines with high levels of Bin1 SH3 expression in the brain are not viable. Certainly, *in vivo* investigations of the Bin1 SH3 domain transgene in the brain are worth pursuing.

4.9 Bin1 accomplishes tissue specificity through a modular SH3 domain

Increasingly, research on proteins traditionally viewed as cell cycle regulators has uncovered multiple roles, or identities, for some of these proteins. Importantly, these

roles are distinct and separable from their roles in cell cycle regulation. For example, the ability of Rb to gate S phase entry was considered its most important function. More recently, its role in differentiation has been highlighted as an additional function. For example, mutant Rb that does not bind E2F can still stimulate differentiation of skeletal muscle (Zhang, 1999). The presence of Rb has been proven necessary for transcriptional activation of differentiation specific genes in skeletal muscle, the ocular lens, keratinocytes, and adipocytes (Zhang, 1999). Indeed, mice with weak Rb expression and Rb-/- mice rescued to term by E2F1 inactivation display defects in myogenesis (Zacksenhaus et al., 1996; Tsai et al., 1998, respectively). Rb deficiencies affect the later stages of differentiation and this ability to affect differentiation may be explained by the requirement for Rb in MyoD-mediated activation of the MEF2C transactivation domain (Novitch et al., 1999).

Similar to Rb, Bin1 is involved in a variety of cellular functions. The results from this study suggest an appealing, and biologically simple concept. In this model, distinct modular protein domains regulate the ability of a protein to participate in numerous and separable cellular functions. Indeed a definitive role for the neural-specific domain (exons 12A-D) and the SH3 domain of Bin1 has been described in the brain in endocytosis. The MBD and BAR domains are important for c-myc interaction and Bin1 suppression of transformation. Extrapolation of these observations leads us to believe that a role for Bin1 in muscle is also based on a modular domain concept. Indeed, our study defines a role for the Bin1 SH3 domain in coordinating the early stages of muscle differentiation *in vitro* and in secondary myotube formation *in vivo*. Further

characterization of this domain in muscle and the overexpression of other Bin1 domains individually and in combination will continue to provide insight into the role of the modular domains of Bin1.

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Appendix

Appendix A: Murine Bin1 (SH3p9) nucleic acid sequence

Underlined portion denotes SH3 domain (1262-1480) and represents the sequence cloned into the pcAGGS vector for injection (GenBank Accession Number U60884.1).

1 cctcactcgc tctccccgcg cacgctccgt ctccgtcagt cccctgagct gttctagtgc
61 gcggcgtgga gccagggctc aggctggtgg agcggccggg gctggaggct gggagtgcgg
121 cgcgcacggc ctccccgcgc cattatccgc gctcgcttcg ggcgaggccg gcgccaggat
181 ggcagagatg gggagcaagg gggtgacggc ggggaagatc gccagcaacg tacagaagaa
241 gctgacccga gcgcaggaga aggtcctgca gaaactgggg aaggcggacg agacgaagga
301 cgagcagttt gagcagtgtg tccagaactt caataagcag ctgacagagg gtacccggct
361 gcagaaggat cttcggacct atctggcttc tgttaaagcg atgcacgaag cctccaagaa
421 gctgagtgag tgtcttcagg aggtgtacga gcccgagtgg cctggcaggg atgaagcaaa
481 caagattgca gagaacaatg acctactctg gatggactac caccagaagc tggtggacca
541 ggctctgctg accatggaca cctacctagg ccagttccct gatatcaagt cgcgcattgc
601 caagcggggg cggaagctgg tggactatga cagtgcccgg caccactatg agtctcttca
661 aaccgccaaa aagaaggatg aagccaaaat tgccaaggca gaagaggagc tcatcaaagc
721 ccagaaggtg ttcgaggaga tgaacgtgga tctgcaggag gagctgccat ccctgtggaa
781 cagccgtgta ggtttctatg tcaacacgtt ccagagcatc gcgggtctgg aggaaaactt
841 ccataaagag atgagtaagc tcaatcagaa cctcaatgat gtcctggtca gcctagagaa
901 gcagcacggg agcaacacct tcacagtcaa ggcccaaccc agtgacaatg cccctgagaa
961 agggaacaag agcccgtcac ctcctccaga tggctcccct gctgctaccc ctgagatcag
1021 agtgaaccat gagccagagc cggccagtgg ggcctcaccc ggggctacca tccccaagtc
1081 cccatctcag ccagcagagg cctccgaggt ggtgggtgga gcccaggagc caggggagac
1141 agcagccagt gaagcaacct ccagctctct tccggctgtg gtggtggaga ccttctccgc
1201 aactgtgaat ggggcggtgg agggcagcgc tgggactgga cgcttggacc tgccccccggg
1261 attcatgttc aaggttcaag cccagcatga ttacacggcc actgacactg atgagctgca
1321 actcaaagct ggcgatgtgg tgttggtgat tcctttccag aacccagagg agcaggatga
1381 aggctggctc atgggtgtga aggagagcga ctggaatcag cacaaggaac tggagaaatg
1441 ccgcggcgtc ttcccggaga attttacaga gcgggtacag tgacggagga gccttccgga
1501 gtgtgaagaa cctttccccc aaagatgtgt g

Appendix B: Mouse Colony

Line 869

869 male DOB 29/11/99

M	tg	F	tg		Numbers
5	4	4	3	Litter 1 crossed with wt female DOB 7/2/00	322-329,376
3	2	5	4	LItter 2 crossed with wt female DOB 7/2/00	330-337
4	3	4	4	Litter 3 crossed with wt female DOB 15/2/00	338-345
4	3	5	4	Litter 4 crossed with wt female DOB 8/3/00	414-422
5	4	3	2	Litter 5 crossed with wt female DOB 31/3/00	444-451

F1 Heterozygous crosses

M	tg	+/-	+/+	F	tg	+/-	+/+	DOB	Numbers
5	5	2	3	4	3	1	1	31-05-00	495-499,491-494
3	3	1	1	3	3	1		14-06-00	521-526
2	1			4	4	1		20-06-00	531,532,527-530
2	2	1	1	4	3		2	06-11-00	675,676,671-674
4	3	3	0	0	0			19-12-00	744-747
1				2				07-02-01	1050-1052

F1 heterozygous x wt

M	tg	F	tg	DOB	numbers
6	3	4	2	24-09-00	634-639,622-625

F2 heterozygous x wt

M	tg	F	tg	DOB	numbers
3	0	5	0	27-12-00	776-783
3	0	1	0	11-01-01	814-816,813
5	3	6	3	21-02-01	882-892

F2 homozygous x wt

M	tg	F	tg	DOB	numbers
1	1	0	0	07-02-01	865
4	4	3	3	04-03-01	904-907,901-903

F2 homozygous x homozygous

M	tg	+/-	+/+	F	tg	+/-	+/+	DOB	Numbers
1	0?			2	2			25-02-01	881,879,880
4	3	1	2	1	1	1		14-03-01	915-918,914
2	2	1	1	6	5	3	2	19-03-01	919-923,926-927
1	1	1	0	1	1	0	1	19-03-01	924,925
4	4			3	3			17-06-01	1185-1191,1182-1184
2	2			3	2			09-07-01	1192-1193,1189-1191
4	4			4	4			01-08-01	1194-1201

Line 501

501 female DOB 21/6/00

M	tg	f	tg		Numbers
6	2	4	2	Litter 1 cross with wt male DOB 6/12/00	784-789,790-793
8	8	3	3	Litter 2 cross with wt male DOB 29/1/01	840-847, 837-839

F1 heterozygous x wt

M	tg	F	tg	DOB	numbers
3		4		19-04-01	1043-1049
5	0	4	3	15-06-01	1206-1210,1202-1205
2	0	5	1	27-06-01	1211-1217
3	3	2	2	20-07-01	1220-1222,1218,1219

Line 482

482 male DOB 21/6/00

M	tg	F	tg		Numbers
4	1	3	0	Litter 1 crossed with wt female DOB 21/8/00	592-595,589-591
7	0	3	0	Litter 2 crossed with wt female DOB 1/11/00	677-686
5	0	5	0	Litter 3 crossed with wt female DOB 2/1/01	808-812,803-807
4	0	5	1	LItter 4 crossed with wt female DOB 13/1/01	829-832,824-828
5	5	3	0	Litter 5 crossed with wt female DOB 12/3/01	893-900
3	0	6	0	Litter 6 crossed with wt female DOB 2/4/1	953-961

F1 heterozygous x wt

M	tg	F	tg	DOB	numbers	
2	0	7	5	04-04-01	951-952,944-950	950 tg was small

Line 487

487 female DOB 21/6/00

M	tg	F	tg		Numbers
6	2	2	2	Litter 1 crossed with wt male DOB 21/9/00	614-621
5	1	6	3	LItter 2 crossed with wt male DOB 17/10/00	656-660,650-655

F1 heterozygous crosses

M	tg	+/-	+/+	F	tg	+/-	+/+	DOB	Numbers
2	1			4	4			26-11-00	704-709
0	0			2	2			29-11-00	710-711
3	1	1	0	4	2	0	2	24-03-01	933-935,929-932

F1 tg cross

M	tg	F	tg	DOB	Numbers
3	1	4	1	12-01-01	817-823
2	1	6	5	02-02-01	857-864

Line 485 - not expressing

485 male DOB 21/6/00

M	tg	F	tg		Numbers
3	1	4	3	Litter 1 crossed with wt female DOB 25/8/00	596-603
3	2	5	3	Litter 2 crossed with wt female DOB 25/8/00	604-611
5	4	3	2	Litter 3 crossed with wt female DOB 25/9/00	629-633,626-628

F1 heterozygous crosses

M	tg	+/-	+/+	F	tg	+/-	+/+	DOB	Numbers
4	4			4	1			08-11-00	687-694

F1 heterozygous x wt

M	tg	F	tg	DOB	numbers	
6		1		14-04-01	1027-1033	Not genotyped
2		7		16-04-01	1034-1042	Not genotyped

Line 502

502 female DOB 21/6/00

M	tg	F	tg		numbers
4	0	6	3	Litter 1 crossed with wt male DOB 17/12/00	712-721

F1 heterozygous x wt

M	tg	F	tg	DOB	numbers
7		2		16-04-01	1018-1026

Line 508

508 male DOB 21/6/00

M	tg	F	tg		numbers
1	0	0	0	Litter 1 crossed with wt female DOB 11/10/00	649
4	1	5	2	Litter 2 crossed with wt female DOB 1/11/00	700-703,695-699
3	1	6	2	Litter 3 crossed with wt female DOB 26/1/1	848-856

Line 510 - no transgenics born

510 female DOB 21/6/00

M	tg	F	tg		numbers
4	0	3	0	Litter 1 crossed with wt male DOB 26/12/00	794-800
1	0	2	0	Litter 2 crossed with wt male DOB 22/01/01	835,833,834
2	0	1	0	Litter 4 crossed with wt male DOB 20/6/1	1224-1226
0	0	1	0	LItter 3 crossed with wt male DOB 25/6/1	1223

Line 512 - one transgenic born

512 female DOB 21/6/00

M	tg	F	tg	
1	1	0	0	Litter 1 crossed with wt male DOB 21/01/01

F1 heterozygous x wt

M	tg	F	tg	DOB	numbers
5	2	3	1	28-03-01	939-943,936-938
7	3	5	0	14-04-01	967-973,962-966

Line 514

514 male DOB 21/6/00

M	tg	F	tg		numbers
5	1	5	0	Litter 1 crossed with wt female	722-732
7	4	4	2	LItter 2 crossed with wt female DOB 2/12/00	737-743,733-736