MOLECULAR DISSECTION OF SEQUENCE REQUIREMENTS AND INTERACTIONS OF STICKS-AND-STONES

A Thesis in

Integrative Biosciences

by

Kiranmai S Kocherlakota

© 2007 Kiranmai S Kocherlakota

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

December 2007
The thesis of Kiranmai S Kocherlakota was reviewed and approved* by the following:

Wendy Hanna-Rose  
Assistant Professor of Biochemistry, Microbiology and Molecular Biology  
Thesis Advisor  
Chair of Committee

Susan M Abmayr  
Associate Investigator  
Stowers Institute for Medical Research  
Special member

Robert F Paulson  
Associate Professor of Veterinary Sciences

Richard W Ordway  
Associate Professor of Biology

Kyung-An Han  
Associate Professor of Biology

R Scott Hawley  
Investigator  
Stowers Institute for Medical Research  
Special member

Peter J Hudson  
Director  
The Huck Institutes of Life Sciences

*Signatures are on file in the Graduate School
ABSTRACT

In *Drosophila melanogaster*, somatic muscle fibers arise from fusion between two distinct myoblast populations, the founder cells and the fusion competent myoblasts. Sticks-and-Stones (SNS) is a cell adhesion molecule on the surface of fusion competent myoblasts that associates heterotypically with ligands on the surface of founder cells, for their recognition and fusion. Loss-of-function mutants are characterized by an absence of mature myofibers. Intracellular signaling events exist in both myoblast populations to direct actin cytoskeletal changes and recruitment of fusion machinery. Such recruitment is dependent on SNS mediated adhesive contacts in the fusion competent myoblasts.

This study identifies critical functional sequences in the SNS cytodomain by generating deletions and mutations. Using the two-component UAS-GAL4 system, the mutagenized constructs were expressed in the mesoderm of loss-of-function *sns* mutant embryos to test their ability to rescue the myoblast fusion defect. Deletional analysis identified the presence of multiple functional domains within a minimal critical region. SNS is phosphorylated on tyrosine residues and mutagenesis to abolish all phosphorylation affected the ability of the protein to restore wild-type muscle pattern, demonstrating a role for the tyrosine residues for SNS function during myogenesis. Additionally, mutagenesis of proline-rich sequences in the SNS cytodomain revealed a requirement during myoblast fusion. The presence of multiple functional motifs presents the enticing possibility that SNS functions by interaction with multiple intracellular proteins for signaling.
This study also identifies SNS is not essential for initial fusion events to form bi- or tri-nucleated precursor myofibers. This is due to substitution by Hibris (Hbs), the paralog of SNS, as precursor myofibers are absent in double mutants. Endogenous or overexpressed Hbs, however, does not substitute for SNS through additional rounds of fusion for mature myofiber formation. The two proteins associate into hetero-oligomers in cis. Chimeric constructs suggest that SNS and Hbs extracellular and cytodomains function comparably during myoblast fusion but that Hbs is less efficient. This study clarifies the role of Hbs during myogenesis as redundant with SNS for precursor myofiber formation and synergistic during mature myofiber formation.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>viii</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xii</td>
</tr>
</tbody>
</table>

## Chapter 1 The process of Myoblast Fusion .................................................. 1

1.1 Myoblast Fusion .................................................................................. 1
1.2 Mesoderm specification ........................................................................ 2
1.3 The Founder Model ............................................................................. 3
1.4 Two Phase Fusion ................................................................................. 5
1.5 Events leading to myoblast fusion ...................................................... 7
  1.5.1 Role of cell surface molecules ................................................... 7
  1.5.2 Role of intracellular molecules: Signaling during myoblast fusion .... 11
  1.5.3 Cell morphological changes during myoblast fusion .................... 18
1.6 Studies of vertebrate myoblast fusion .............................................. 23
  1.6.1 Key molecules regulating vertebrate myoblast fusion ................... 24
  1.6.2 Cell morphological changes in myoblast fusion .......................... 27
1.7 Present studies ................................................................................... 28

## Chapter 2 Molecular dissection of the SNS cytodomain identifies critical sequences .............................................. 31

2.1 Introduction ......................................................................................... 31
  2.1.1 Function of IgSF proteins in mediating cell adhesion ................. 31
  2.1.2 Role of SNS as a signaling molecule ........................................ 35
  2.1.3 Orthologs of *Drosophila* myogenic IgSF proteins ................... 38
  2.1.4 Critical signaling motifs in cytodomains of IgSF cell adhesion
      molecules .......................................................................................... 40
    2.1.4.1 Role of protein-protein interaction motifs for signaling
            downstream of cell adhesion molecules ................................ ...... 40
    2.1.4.2 Role of phosphorylation in signaling downstream of cell
            adhesion molecules ...................................................................... 42
2.2 Materials and methods ........................................................................ 45
  2.2.1 Fly stocks ..................................................................................... 45
  2.2.2 Antibodies ................................................................................... 45
  2.2.3 Site-directed mutagenesis and constructs .................................. 46
  2.2.4 Immunohistochemistry ................................................................. 48
  2.2.5 Molecular characterization of mutants ....................................... 48
  2.2.6 Quantitation of unfused myoblasts and hemisegments with defects.. 49
  2.2.7 S2 transfection ............................................................................ 50
Chapter 2  Immunoprecipitation and western blotting ................................. 51
2.2.9 Phosphatase treatment ....................................................................... 52
2.3 Results.................................................................................................. 53
  2.3.1 SNS is required for directional migration of fusion competent
myoblasts.................................................................................................. 53
  2.3.2 Development of an assay system based on rescue of sns mutants ...... 54
    2.3.2.1 Rescue using different spatial and temporal expression
    patterns ............................................................................................ 55
    2.3.2.2 Rescue using different epitope tagged forms of SNS .............. 57
    2.3.2.3 Rescue using different levels of expression ......................... 59
  2.3.3 Generation of deletional constructs of SNS ..................................... 61
  2.3.4 Generation of mutational constructs of SNS ................................... 63
  2.3.5 SNS cytodomain is essential for its function ................................. 69
  2.3.6 Multiple functional regions are present in SNS cytodomain ............ 72
  2.3.7 Protein:protein interaction motifs are critical for SNS function ...... 75
  2.3.8 Investigated Serine residues are not essential for SNS function ...... 80
  2.3.9 SNS is phosphorylated on tyrosine residues ................................... 82
  2.3.10 Investigation of a requirement for ligand binding for SNS
    phosphorylation .................................................................................. 86
  2.3.11 Tyrosine residues are critical for SNS function ............................. 88
2.4 Discussion............................................................................................ 93
  2.4.1 SNS directs intracellular events in the fusion competent myoblasts... 93
  2.4.2 SNS cytodomain contains multiple functional regions ................. 94
  2.4.3 SNS requires protein interaction motifs in its cytodomain for
    function ............................................................................................ 96
  2.4.4 SNS requires tyrosine residues in its cytodomain for function ........ 98

Chapter 3  Dissection of physical and genetic interactions of SNS and Hbs .......... 102
  3.1 Introduction........................................................................................ 102
    3.1.1 Specification of distinct populations of myoblasts ...................... 103
    3.1.2 Two phase fusion process ......................................................... 107
    3.1.3 Hibris, a paralog of SNS, is a regulator of myogenesis ............... 110
    3.1.4 Biological properties of Hibris .................................................. 112
  3.2 Materials and methods ..................................................................... 116
    3.2.1 Fly stocks .................................................................................. 116
    3.2.2 Cloning and constructs .............................................................. 116
    3.2.3 Immunohistochemistry .............................................................. 117
    3.2.4 Statistical analysis ..................................................................... 118
    3.2.5 S2 cell culture and transfection ................................................ 119
    3.2.6 Immunoprecipitation and Western blotting ............................... 119
  3.3 Results............................................................................................... 120
    3.3.1 Identity genes are a tool to study two phase myoblast fusion ....... 120
LIST OF FIGURES

Fig. 1.1: Schematic representation of the two phase fusion process. .........................6

Fig. 1.2: Key molecules involved in *Drosophila* myoblast fusion. ......................17

Fig. 1.3: Schematic of the steps of myoblast fusion determined by ultrastructural studies. ..................................................................................................................22

Fig. 2.1: SNS predicted domain architecture and requirement in myoblast fusion. ...32

Fig. 2.2: Schematic for generating mutagenized constructs of SNS......................47

Fig. 2.3: Schematic for generating deletional constructs of SNS. .........................47

Fig. 2.4: SNS is required for directional migration of fusion competent myoblasts in response to attractant Duf/Kirre. .................................................................54

Fig. 2.5: Driving full length cDNA of *sns* in the mesoderm of mutants restores the muscle pattern ..................................................................................................56

Fig. 2.6: Different C-terminal epitope tags did not affect the ability of SNS function in the rescue assay .......................................................................................57

Fig. 2.7: Rescue with full length cDNA does not change at high and low temperatures.......................................................................................................................60

Fig. 2.8: Sequence conservation of SNS cytodomain with orthologous sequences. ..62

Fig. 2.9: Schematic of SNS cytodomain deletions employed in this study. ..........63

Fig. 2.10: Schematic of mutagenized constructs employed in this study. .............68

Fig. 2.11: Truncation of cytodomain following I1163 results in loss of function.....70

Fig. 2.12: The region A1113-H1278 is critical for SNS function. .......................71

Fig. 2.13: Smaller deletions within ΔA1113-H1278 are able to rescue the myoblast fusion defect in *sns* mutants. .................................................................73

Fig. 2.14: Multiple functional domains exist in the SNS cytodomain.................75

Fig. 2.15: Putative SH3 domain binding sites in SNS cytodomain function during myoblast fusion.................................................................77
Fig. 2.16: A requirement for putative SH3 domain binding sites in SNS cytodomain is obvious at lower expression levels.......................................................79

Fig. 2.17: Highly conserved and likely phosphorylated serine residues are not essential.............................................................................................................81

Fig. 2.18: SNS is phosphorylated on tyrosine residues. ........................................83

Fig. 2.19: SNS phosphorylation is specific to tyrosine residues.................................84

Fig. 2.20: Mutagenesis of tyrosine residues in SNS cytodomain causes loss of phosphorylation. ........................................................................................................86

Fig. 2.21: Role of ligand binding for SNS phosphorylation is undetermined. ........88

Fig. 2.22: Tyrosine residues in SNS cytodomain function during myoblast fusion. .......................................................................................................................89

Fig. 2.23: Quantitation of unfused myoblasts in rescued embryos where expression is driven using mef2GAL4 .................................................................90

Fig. 2.24: Quantitation of rescue efficiencies as percent hemisegments with defects in muscle pattern in the lateral transverse muscles in rescued embryos........................................................................................................92

Fig. 3.1: Schematic of two phase fusion process with emphasis on the differences in myofibers...............................................................................................................109

Fig. 3.2: Hbs domain architecture prediction and function during myoblast fusion. .........................................................................................................................112

Fig. 3.3: EVE staining pattern in a wild-type embryo...................................................121

Fig. 3.4: Precursor formation occurs in DA1 in sns mutants........................................123

Fig. 3.5: Comparison of average EVE positive nuclei in DA1 muscle per hemisegment...................................................................................................................124

Fig. 3.6: Progression of DA1 muscle through myoblast fusion in homozygous Df(2R)BB1 mutants........................................................................................................125

Fig. 3.7: Precursor formation occurs in DO1 in sns mutants.........................................126

Fig. 3.8: Precursor formation does not occur in DA1 in Df(1)w67k30 mutants..............127

Fig. 3.9: Precursor formation does not occur in DA1 in mbc[D11.2] mutants. ..........128
Fig. 3.10: Quantitation of differences of requirement for first and second phase of fusion.

Fig. 3.11: Precursor formation does not occur in DA1 in lmd[1] mutants.

Fig. 3.12: IrreC/Rst does not substitute for SNS in precursor formation.

Fig. 3.13: Hbs substitutes for SNS in precursor formation in DA1.

Fig. 3.14: Quantitation to show Hbs substitutes for SNS in the first phase of fusion in DA1.

Fig. 3.15: Hbs substitutes for SNS in precursor formation in DO1.

Fig. 3.16: Quantitation to show Hbs substitutes for SNS in the first phase of fusion in DO1.

Fig. 3.17: Hbs and SNS associate into oligomers.

Fig. 3.18: SNS associates into homo-oligomers.

Fig. 3.19: Hbs is inefficient during later rounds of myoblast fusion.

Fig. 3.20: Hbs:SNS chimera are functional during myoblast fusion.

Fig. 3.21: Hbs is phosphorylated on tyrosine residues.

Fig. A.1: Substitution of Hbs and Syg-2 TM domains for SNS in chimera.

Fig. A.2: Mutagenesis of Glycine residue in the SNS TM domain.
LIST OF TABLES

Table 2.1: Full length cDNA of sns expressed pan-mesodermally rescues mutants to viability.............................................................................................................58

Table 2.2: Several serine residues in the SNS cytodomain are predicted to be candidates for phosphorylation.............................................................................65

Table 2.3: Several threonine residues in the SNS cytodomain are predicted to be candidates for phosphorylation.............................................................................66

Table 2.4: Several tyrosine residues in the SNS cytodomain are predicted to be candidates for phosphorylation.............................................................................67

Table 3.1: Combinatorial code of muscle founder identity genes in specific muscles. ................................................................................................................105

Table B.1: Results of pairwise comparison of number of unfused myoblasts from different transgenic rescue results. .................................................................184

Table B.2: Results of pairwise comparison of percentage of hemisegments with defects in different transgenic rescue results. ..........................................................185

Table B.3: Prediction of candidate sites for phosphorylation among 35 serine residues in the SNS cytodomain. .............................................................................189

Table B.4: Prediction of candidate sites for phosphorylation among 33 threonine residues in the SNS cytodomain. .............................................................................190

Table B.5: Oligonucleotides and restriction enzymes used to generate transgenic constructs. .............................................................................................................191
I dedicate this thesis at the feet of Lord Sai and to my parents.

I begin with sincere acknowledgement to God who has been with me through all big and small events. His Grace has inspired me to finish this thesis and I therefore submit it to Him. My parents’ love and support has gotten me through these years spent apart. They have been understanding, encouraging and supportive in spite of never having experienced anything as intense themselves. They have always given me faith that I can achieve anything.

I thank Dr. Susan Abmayr, my advisor for not only her support and advice but also her patience and perseverance through our years together. Even when I wanted to give up, she gave me the will and opportunity to go on. She has supported my ideas and taught me how to think and execute scientifically.

I am also very thankful to all my committee members for their guidance. Especially, Dr. Wendy Hanna-Rose, who helped me tremendously by offering me a place in her laboratory to finish my work. Also, special thanks to Dr. Scott Hawley, for being understanding and providing me with guidance through tougher times.

I thank everybody at the Stowers Institute for their help and friendship. Particularly, I acknowledge the help of Dr. Stephen Morris, Danny Stark, Cameron Cooper, Sarah Smith and Joel Schwartz in the Imaging facility, Dongxiao Zhu in the Bioinformatics group and Kym Delventhal and Heather Strobietto of the Molecular Biology Core facility and David Duerr and others in the IT group, the fly food facility and media preparation center. I reserve a special mention for Abby Freeman who has helped me deal with personal and professional issues in a sensible manner. Similarly the help of the secretaries at the Huck Institutes and International student advisors at PSU was invaluable.

I thank all the present and past lab members of the Abmayr Lab for the wonderful environment and friendship they offered. Of special mention are Claude Shelton IV, Maggie Chen and Erika Geisbrecht who have been friends more than colleagues. Special thanks to Dr. Jian-min Wu, Jeffrey McDermott, Christy Pucci, David Ash, Sue-Jean
Hong, Kelly Greene, Melissa Mathews and Krista Wimberly for their assistance and collaboration in my project. I also thank Rakhee Banerji whose initial observation of the importance of the cytodomain was the basis for my project.

I am really thankful to all the members of the Hanna-Rose lab, Li, Katie, Tracy and Hongliu, for kindly accepting a fly person in their lab and for making me so comfortable and welcome. They are the best! Li and I have especially grown to be good friends and without this computer guru, I may not have gotten my thesis together.

I am grateful to all the friends and colleagues that have helped me through the difficult times. All my friends and acquaintances through the last six years have helped make this journey wonderful! Without Birjoo, my thesis wouldn’t have printed! I thank Aurelie and Stephen for putting me up each time I visited Kansas City while finishing up my work. Without the constant help, support and love of my best friends Asha and Ian, I would be unable to deal with problems in life. They have always been near when I needed them whatever their personal situations may have been. Mark has been highly instrumental in the completion of my thesis and degree. Without his faith, support, will power and most importantly, ‘firm push’, I may not have made it.
Chapter 1

The process of Myoblast Fusion

1.1 Myoblast Fusion

In complex eukaryotic organisms, the skeletal/somatic or body wall muscles are required for locomotion as well as maintaining body posture. This somatic musculature is comprised of individual muscle fibers formed by the process of myoblast fusion. Each fiber results from fusion between several myoblasts and is hence a multinucleate syncitia with multiple nuclei within a common cytoplasm (reviewed in Abmayr, Balagopalan et al. 2003). Myoblast fusion in vertebrates mostly occurs in embryonic stages in utero and hence the mutants affecting the process result in lethality making identification of mutants and study of the process complicated. The fruitfly, Drosophila melanogaster has been a favored model organism to study the process for several reasons. The embryonic myoblast fusion occurs after egg laying (AEL) and hence embryos can be easily collected and manipulated. The fruitfly life span is relatively short and several mutants and tools are readily available. In addition, unlike in vertebrates where muscle formation takes days, the process is complete within hours in flies. Genetic screens have identified several key molecules involved in the process. This chapter presents the key aspects of the process as understood by various molecular, genetic and ultrastructural studies mainly in Drosophila with a brief mention of studies in vertebrates at the end.
1.2 Mesoderm specification

Following gastrulation, the ventral cells in the embryo give rise to the presumptive mesoderm. The specific mesodermal cell fate depends on the final position of the cell and the intrinsic and extrinsic signals it receives (Baylies and Bate 1996). High levels of a basic-Helix-Loop-Helix (bHLH) transcription factor, Twist (Twi) triggers development into somatic musculature (Baylies and Bate 1996). From these regions of high Twi expression, clusters of cells start expressing the proneural gene lethal of scute (l’sc), another bHLH transcription factor. The cells of this group are equipotent initially and hence termed an equivalence group or cluster and give rise to all the cells of the somatic musculature. Later, by Notch mediated lateral inhibition, L’sc expression is progressively restricted to one cell per equivalence group termed a progenitor cell (Carmena, Bate et al. 1995).

The progenitors move into close contact with the ectoderm and some divide to form two specialized cells called founder cells which attain unique fates and give rise to distinctive muscle fibers (Ruiz-Gomez and Bate 1997; Frasch 1999; Paululat, Breuer et al. 1999). The rest of the myoblasts of the equivalence cluster attain a fusion competent cell fate. By the end of embryogenesis, the embryo is divided into 8 abdominal and 3 thoracic segments by the formation of segmental furrows (Campos-Ortega and Hartenstein 1997). There are 30 distinct founder cells in each abdominal hemisegment from A2-A7 surrounded by large numbers of fusion competent myoblasts. The three thoracic and A1 and A8 segments are slightly different in the muscle pattern from A2-A7 (Bate 1990). Irrespective of these differences between individual fibers, they all share a
common property. Each muscle fiber forms by fusion between one founder cell and multiple fusion competent myoblasts.

1.3 The Founder Model

The founder cell model was originally presented from studies in the grasshopper. Large mesodermal cells create a scaffold on which the pattern of the future body wall muscles is designed. These cells are termed muscle pioneers and when ablated, the resulting muscle fiber is missing in the adult replaced by a loose mass of small mesodermal cells (Ball, Ho et al. 1985). This model has been applied to the development of somatic musculature in *Drosophila*. At the end of mesoderm specification, each abdominal hemisegment has 30 distinct founder cells. Following myoblast fusion, there are 30 uniquely patterned muscle fibers at the sites occupied by the founder cell. Thus, each founder in the fruitfly, similar to each pioneer in the grasshopper seeds the fusion process and prefigures the properties of that muscle fiber (Ball and Goodman 1985; Ball and Goodman 1985; Frasch 1999).

For this purpose, the founder contains the genetic information to specify the unique set of characteristics for each fiber. This genetic information is dependent on expression of a specific set of “identity” genes that make it unique. These are a unique combination of transcription factors specific to different founder or the muscles it specifies (reviewed in Frasch 1999). Direct evidence to the role of these identity genes in specification of unique muscle fibers comes from ectopic expression and genetic loss-of-function experiments (reviewed in Frasch 1999). For example, in *nau* mutants, the subset
of muscle fibers, where NAU is expressed, are missing while none of the other muscles is affected (Balagopalan, Keller et al. 2001).

The fusion competent myoblasts on the other hand appear to be equivalent and do not contain a similar genetic program. From transplantation studies, it appears that the fusion competent myoblasts can fuse with any founder cell (Beer, Technau et al. 1987). The founder recruits neighboring fusion competent myoblasts to fuse with it as required to attain the final size and shape of the muscle (Bate 1990). Upon fusion with specific founder, the latter are reprogrammed and show expression of identity genes associated with that final muscle fiber. This is the central theme of the founder cell model that each founder cell contains the information necessary to pattern a single muscle and it passes this information to “naïve” fusion competent myoblasts upon fusion.

These differences in the properties of the two populations of myoblasts are clear from their behavior in the absence of fusion. In mutants lacking myoblast fusion, the founder cell still attempts to contact the muscle attachment sites and span the territory of the future muscle fiber. It is also innervated correctly consistent with it containing all the information pertaining to the identity of that mature muscle fiber (Prokop, Landgraf et al. 1996). It however becomes long and wispy due to an inability to reach the proper size and shape. The fusion competent myoblasts on the other hand remain rounded and eventually die and are phagocytosed at later stages of embryogenesis (Rushton, Drysdale et al. 1995). Both cell types still express muscle specific markers such as myosin heavy chain (MHC) (Rushton, Drysdale et al. 1995).
1.4 Two Phase Fusion

Each founder cell recruits and fuses with several fusion competent myoblasts, the exact number depending on the final size of the muscle fiber (Bate 1990; Baylies, Bate et al. 1998). These fusion events are not synchronous: not all fusion competent myoblasts fuse simultaneously to the founder. There is an intermediate stage where the founder cells first form bi- or tri-nucleated cells called precursor cells by fusion to one or two fusion competent myoblasts (Fig. 1.1). These initial fusion events deplete the fusion competent myoblasts in the layer immediately internal to them (Beckett and Baylies 2007).

The precursors then extend along the axis of the future muscle fiber and lie over the central nervous system (CNS) without making contact with the epidermis (Bate 1990). They extend projections visualized by dye injections and make contact with sites in the epidermis to form muscle attachments. The precursors are present in the outer layer of mesoderm and the cells in internal layers provide a pool of unfused myoblasts for fusion with these precursors (Bate 1990 and Beckett and Baylies 2007). The two phases of fusion are divided in such a manner that only a limited fusion occurs from 7.5-10.5 hours AEL producing the bi- or tri-nucleated stage and the majority of fusion occurs 10.5-13 hours AEL forming the mature myofibers (Beckett and Baylies 2007).

The identity genes allow visualization of the nuclei in specific muscle fibers and quantitation of the numbers of fusion events (Bate 1990). At embryonic developmental stage 16, fusion is completed and muscle size directly correlates with nuclear number. The largest fibers contain as many as 20-25 nuclei, the smallest fibers 3-4 showing that a varied number of cells fuse to form particular muscles (Bate 1990). The individual
founder cells express distinct transcription factors that localize in the nucleus when it is mononucleate. After myoblast fusion, all nuclei of the resulting syncytia express these factors. The distinction into first phase of fusion to make precursors followed by second phase to make mature muscle fibers became possible from the observation of mutants that block these different phases of fusion by marking with such transcription factors to count the number of fusion events in essential myogenic genes.

Fig. 1.1: Schematic representation of the two phase fusion process.

From an equivalent cluster of cells, a founder cell is specified and the remaining cells attain fusion competent cell fate. This founder recruits first one or two fusion competent myoblasts to form a bi- or tri-nucleated precursor myoblast. The precursor extends and recruits further fusion competent myoblasts in multiple rounds to form the final muscle fiber. The total number of fusion events is determined by counting the number of nuclei in the muscle as the nuclei of the fusion competent myoblasts are recruited into the growing myofiber.
1.5 Events leading to myoblast fusion

During myogenesis, several steps occur prior to and during the events of membrane fusion to allow cytoplasmic continuity. These include recognition of, migration toward and adhesion to the founder cells by the fusion competent myoblasts. Recognition is specific and directional and the fusion process is thus asymmetric and founders and fusion competent myoblasts do not fuse with themselves but only with each other. The molecular mechanism for this is the differential expression of specific proteins between the two populations of myoblasts. Following adhesion, the myoblasts align and the two plasma membranes are juxtaposed. In subsequent events, intracellular proteins and structures that are essential for cell migration and membrane fusion are recruited to the plasma membrane, where they likely contribute to reorganization of the cytoskeleton (Abmayr 2005). This reorganization is presumed to be required for extension of filopodial projections leading to cell migration. Other intracellular changes associated with fusion include recruitment of fusion related machinery to sites of cell-cell contact. Membrane breakdown occurs in several places to allow regions of cytoplasmic continuity. The key molecules involved at these various steps are described below.

1.5.1 Role of cell surface molecules

Cell-cell adhesion is critical to the formation and maintenance of tissues and organs in multicellular organisms. Cells adhere to each other via homophilic or heterophilic associations of surface proteins, among which are the cell adhesion molecules of the Immunoglobulin superfamily (IgSF). The first step for the process of
myoblast fusion is the recognition of muscle cells and the juxtaposition of the membranes. Three essential cell adhesion molecules required for this aspect have been identified on the basis of mutant phenotypes characterized by myoblast fusion defects. They belong to the IgSF with a Type I transmembrane topology with the N terminus in the extracellular region and the C-terminus in the cytoplasm connected by a short hydrophobic transmembrane sequence (reviewed in Taylor 2002). These loci are sticks-and-stones (sns) (Bour, Chakravarti et al. 2000), dumbfounded/kin-of-irrec (duf/kirre) (Ruiz-Gomez, Coutts et al. 2000) and irregular chiasm-c/roughest (irrec/rst) (Strunkelnberg, Bonengel et al. 2001). A fourth gene, hibris (hbs) also belonging to this family, was discovered as a non essential regulator of myoblast fusion (Dworak, Charles et al. 2001 and Artero, Castanon et al. 2001).

The mesoderm of sns mutants show complete absence of mature muscle fibers and the corresponding presence of large numbers of unfused myoblasts (Bour, Chakravarti et al. 2000). These myoblasts extend filopodia towards the founder cell and put out random projections without making final contact. SNS is predicted to have 9 Immunoglobulin (Ig) domains and a fibronectin III like (FNIII) domain, a single pass transmembrane (TM) domain and a long cytoplasmic tail. Appearance of sns transcript is coincidental with the timing of myoblast fusion and rapidly drops following fusion. The protein is uniform throughout the mesoderm and is also required for visceral muscle formation (Bour, Chakravarti et al. 2000; San Martin and Bate 2001 and Klapper, Stute et al. 2002). Most interestingly, SNS is expressed exclusively on the surface of fusion competent myoblasts in both somatic and visceral musculature and no expression is
observed in the founder cells (Bour, Chakravarti et al. 2000; San Martin and Bate 2001; Klapper, Stute et al. 2002).

A paralog of SNS was identified independently by two different screens; in a screen for transcripts that were differentially expressed in founder versus fusion competent myoblasts (Artero, Castanon et al. 2001) and through database searches for members of the IgSF in Drosophila (Dworak, Charles et al. 2001). Hbs is 48% identical and 63% similar to SNS and expressed slightly earlier in development. However, it is also restricted to myoblasts by stage 12 and is expressed in a large subset of fusion competent myoblasts (Dworak, Charles et al. 2001; Artero, Castanon et al. 2001). Despite these similarities, hbs mutants are phenotypically very different from sns. Apart from occasional missing muscles in 20% embryos, the musculature is normal (Dworak, Charles et al. 2001 and Dworak and Sink 2002). Overexpression of Hbs however causes a disruption of the musculature indicating a role as a regulator of the fusion process (Artero, Castanon et al. 2001).

Complimentary to SNS and Hbs expression in the fusion competent myoblasts is the expression of another IgSF protein, Duf/Kirre in the founder cell. duf/kirre is very closely associated with irrec/rst which encodes a protein that is 45% similar to Duf/Kirre. A deficiency (Df(1)w67k30) removing both these genes showed complete loss of myoblast fusion (Ruiz-Gomez, Coutts et al. 2000). In these mutants, fusion competent myoblasts extend filopodia but fail to migrate toward and adhere to founder cells. Although the two are functionally redundant in the mesoderm, IrreC/Rst differs from Duf/Kirre by its expression in all founders and a subset of fusion competent myoblasts (Strunkelnberg, Bonengel et al. 2001). The proteins are predicted to have 5 Ig domains, a
single pass transmembrane domain and cytoplasmic tails with putative
autophosphorylation domain.

Duf/Kirre and IrreC/Rst function as cell surface attractants directing migration of
fusion competent myoblasts. When expressed ectopically in the ectoderm of embryos,
either protein can direct migration of fusion competent myoblasts towards such ectopic
sites (Ruiz-Gomez, Coutts et al. 2000; Strunkelnberg, Bonengel et al. 2001). The
differential expression of these cell surface IgSF proteins in the two myoblast populations
provides the mechanistic basis for the directionality of myoblast fusion. During fusion,
SNS is associated into distinct puncta coincident with and dependent on Duf/Kirre or
IrreC/Rst at points of contact between the fusion competent myoblasts and the growing
myofiber (Galletta, Chakravarti et al. 2004).

Other membrane associated and intracellular proteins are required and have been
identified using various methods. The locus singles bar (sing) encodes a multipass
transmembrane protein (Estrada, Maeland et al. 2007). Myoblasts in sing mutants form
clusters, suggesting that early recognition and adhesion of these cells occurs normally.
Sing is highly specific to the mesoderm and is expressed in both myoblasts with a
specific requirement for fusion but not adhesion (Estrada, Maeland et al. 2007). The
second membrane associated protein involved in myogenesis is rolling stone (rost). A P
element inserted in the 5’ untranslated (UTR) region of this gene was identified to cause
defects in myogenesis (Paululat, Goubeaud et al. 1997). Though the analysis of this P
element by itself is complicated due to the discovery of associated second site mutations
in sns, the role for Rost in myoblast fusion was confirmed by inhibition of gene
translation using antisense RNA to reproduce an effect on muscle formation. Rost is
specifically expressed and required in founder cells during myoblast fusion (Paululat, Goubeaud et al. 1997).

Recent studies have identified several intracellular proteins essential for myoblast fusion. Some have been placed in pathways downstream of Duf/Kirre and SNS leading to structural changes in the actin cytoskeleton. These intracellular proteins and described in the next section.

1.5.2 Role of intracellular molecules: Signaling during myoblast fusion

Intercellular recognition is followed by a migration of cells toward each other to facilitate adhesion prior to membrane fusion. Cell movement is mediated by the extrinsic signals for recognition and intracellular regulators of the actin cytoskeleton. Once the cells have established contact, the cytoskeleton of the cell should rearrange itself once again to allow maximum surface contact between the cells and potentially also for membrane breakdown. Fusion should follow at sites of contact between adhered myoblasts to avoid spurious fusion between randomly contacting cells. For this purpose, intracellular proteins must be in place to transmit the adhesive cues from the cell surface interactions to the cytoskeleton.

One of the earliest intracellular proteins identified as essential for the process of myoblast fusion is Myoblast City (MBC). Mutants in \textit{mbc} are characterized by the absence of multinucleate muscle fibers and presence of large numbers of unfused myoblasts (Rushton, Drysdale et al. 1995). The unfused fusion competent myoblasts are attracted to and cluster around the founder cells in \textit{mbc} embryos. Thus, MBC appears to
be required for fusion but not necessarily migration and adhesion. MBC shares extensive homology to a family of guanine nucleotide exchange factors (GEFs) for Rac1, a member of the Rho family of small GTPases. The vertebrate ortholog, Dock180 biochemically interacts with the small adaptor protein Crk (Hasegawa, Kiyokawa et al. 1996) upstream as well as CED-12/ELMO (Gumienny, Brugnera et al. 2001; Zhou, Caron et al. 2001) to function as a Rac-GEF.

The *Drosophila* ortholog of Crk (D-Crk) was identified in a biochemical screen for proteins that interact with MBC and potentially critical to this pathway (Galletta, Niu et al. 1999). Mutants in the *dcrk* locus have not been described although expression of a membrane-targeted myristoylated form (myr-Crk) in the musculature severely perturbs myoblast fusion (Galletta 2004). D-Crk interacts with SNS in S2 cultured cells and potentially regulates downstream events in the fusion competent myoblasts (Kim S 2007). Expression of myr-Crk in the *Drosophila* eye results in a rough-eye phenotype and a screen to identify genetic modifiers of this phenotype identified the locus CG31012 (Galletta 2004). BLAST searches showed extensive homology with CD2-associated protein (CD2AP; Dustin, Olszowy et al. 1998) containing SH3 domains. Examination of deficiencies that disrupt *dcd2ap* revealed occasional missing or duplicated muscles (Galletta 2004). The exact function of this protein during myoblast fusion is unclear.

Consistent with the effects of the regulators of Rac1 activation described above, a direct effect of Rac1 itself on myoblast fusion was observed. Rac1 activation affects actin cytoskeletal rearrangements in migratory eukaryotic cells (Hall 1998). Expression of dominant negative or constitutively active forms of D-Rac1 in the *Drosophila* embryonic mesoderm causes defects in myoblast fusion, presumably by interfering with critical
cytoskeletal events (Luo, Liao et al. 1994). Double mutant loss-of-function alleles of 
drac1 and drac2 have been shown to exhibit significant defects in myoblast fusion (Hakeda-Suzuki, Ng et al. 2002). Consistent with a role for D-Rac1 downstream of MBC, a genetic interaction between these loci has been observed in the Drosophila eye. Loss of one copy of mbc suppresses the overexpression eye phenotype of D-Rac1 (Nolan, Barrett et al. 1998).

The discovery of Antisocial/Rolling pebbles (Ants/Rols) led to the understanding of a biochemical link between MBC with Duf/Kirre. Identified in three independent screens for genes affecting embryonic muscle development (Chen and Olson 2001; Rau, Buttgereit et al. 2001 and Menon and Chia 2001), ants/rols mutants showed a severe myoblast fusion defect. Its expression is specific to the founder cells at a time consistent with myoblast fusion. The predicted Ants/Rols protein contains several domains with the potential to mediate protein-protein interactions (Chen and Olson 2001; Rau, Buttgereit et al. 2001 and Menon and Chia 2001). It interacts biochemically with MBC and Duf/Kirre in cotransfected S2 cells (Chen and Olson 2001). By the latter interaction, it is recruited to points of cell contact. These data suggest a model in which MBC is recruited by Duf/Kirre to discrete points on the membrane via Ants/Rols, leading to localized changes in the cytoskeleton through D-Rac1 activation. Whether D-Crk and/or D-Ced12 have a role in this pathway is currently unclear. A D-Crk-MBC pathway may exist in the fusion competent myoblast downstream of SNS for Rac1 activation.

A second pathway regulating the actin cytoskeleton downstream of Duf/Kirre functions by activation of another small GTPase ADP-ribosylation factor (ARF-6). This activation is the function of an ARF-GEF, Loner. In loner mutant embryos, the fusion-
competent myoblasts appear to recognize and extend filopodia toward the founder cells but do not fuse into syncitia (Chen, Pryce et al. 2003). Interestingly, the subcellular localization of Loner is dependent on Duf/Kirre and/or IrreC/Rst (Chen, Pryce et al. 2003). Since Arf-6 proteins regulate membrane traffic and the actin cytoskeleton, it is possible that Loner directs a separate pathway downstream of cell surface events which also regulate intracellular cytoskeletal events. Consistent with this association between Loner and D-Arf6, expression of a dominant negative form of D-Arf6 in founder cells disrupts the fusion process (Chen, Pryce et al. 2003). Expression of Loner is restricted to founder cells similar to Ants/Rols where it is localized in punctate foci that, in some cases, include the latter (Chen, Pryce et al. 2003). However, Loner localization is not affected in ants/rols mutant embryos and vice versa, suggesting that they function independently even though they can colocalize (Chen, Pryce et al. 2003). Curiously, ARF6 is known to enhance Rac mediated actin cytoskeleton remodeling (Donaldson 2003). Consistent with a link between the seemingly independent pathways, D-Rac1 localization to discrete points in founder cells is lost in loner mutant embryos (Chen, Pryce et al. 2003). These data present an enticing idea that these pathways may converge on similar downstream events. The other possibility is that different cytoskeletal events may function toward filopodia extension and fusion and hence different pathways function downstream of the same cell adhesion molecules to regulate these separate events.

It is clear that the actin cytoskeleton rearrangements are critical to the process of myoblast fusion. Actin-filament-binding proteins control the nucleation, assembly, disassembly and crosslinking of actin filaments, whereas actin-monomer-binding proteins
regulate the size, localization and dynamics of the large pool of unpolymerized actin in
cells (reviewed in (Paavilainen, Bertling et al. 2004). Actin monomer binding proteins are
grouped into six classes, two of which, the Wiskott-Aldrich syndrome protein
(WASP)/WASP family verprolin homologous (WAVE) proteins and verprolin/WASP-
interacting protein (WIP) families have been recently established as essential for
myoblast fusion. Myoblast fusion is severely disrupted in mutants lacking maternal and
zygotic WASP and characterized by clustered groups of unfused myoblasts (Massarwa R
2007; Kim S 2007). The same observations were made independently from the Onel
laboratory using different alleles and deficiencies of the wasp locus (Schafer, Weber et al.
2007). Interestingly, a construct expressing WASP lacking the Arp2/3 binding domain
serves as dominant negative and disrupts myoblast fusion (Schafer, Weber et al. 2007).
Recruitment of the WASP-Arp2/3 system to myoblast contact sites is achieved via D-
WIP and consistent with this function, a small deletion uncovering d-wip caused a severe
myoblast fusion defect (Massarwa R 2007). D-WIP was also simultaneously identified in
a genetic screen for myoblast fusion mutants and called Solitary (Sltr) (Kim S 2007).
Sltr/D-WIP is suggested to be recruited to the sites of fusion by SNS via interactions with
the adaptor protein D-Crk (Kim S 2007). This presents a pathway downstream of SNS in
the fusion competent myoblast regulating the actin cytoskeletal changes.

Kette is a WASP and WAVE regulatory protein shown to sequester WAVE in an
inactive state but activate WASP function at the membrane in the Drosophila nervous
system (Bogdan and Klambt 2003). Mutants in kette are characterized by loss of
myoblast fusion. Removal of one copy of wasp in kette mutants partially suppressed the
kette block of fusion presenting evidence of the anticipated genetic relationship (Schafer,
Weber et al. 2007). The *blown fuse (blow)* locus also interacts genetically with *kette* with loss of one copy of *blow* in a *kette* mutant background resulting in an enhancement of the *kette* phenotype. As a corollary, overexpression of Kette in *blow* mutants caused a partial rescue of the *blow* mutant phenotype (Schroter, Lier et al. 2004). Mutants lacking this cytoplasmic protein are characterized by a lack of somatic muscles and a similar phenotype to *mbc* mutants with clustering of unfused myoblasts around the founder (Doberstein, Fetter et al. 1997).

Other non essential intracellular proteins have been identified as regulators of *Drosophila* myogenesis. Parcas (Pcs) was identified in a genetic screen to identify novel regulators of muscle morphogenesis (Beckett K 2006). A fraction of the mutants lacking maternal and zygotic Pcs show defects in the somatic mesoderm, although the phenotype is not very dramatic (Beckett K 2006). Muscle structural protein Titin, which acts as an elastic scaffold for muscle sarcomeres (Gregorio, Granzier et al. 1999; Trinick and Tskhovrebova 1999), has been identified to function during myoblast fusion. It accumulates on the myoblast surface at points of contact between myoblast and myotube prior to fusion (Zhang and Hsieh 2000; Machado and Andrew 2000) and this recruitment in founder cells is dependent on Ants/Rols (Menon and Chia 2001). It is possible that D-Titin is playing a role in either directly or indirectly organizing the actin cytoskeleton in the two myoblast populations potentially downstream of SNS and Duf/Kirre.

All the above described molecules have been placed in putative pathways based on known interactions and properties of the proteins and this is depicted in the schematic in Fig. 1.3. SNS is required for recruitment of some of these intracellular signaling proteins in the fusion competent myoblasts and SNS transmembrane and cytodomain
sequences are essential for myoblast fusion (Galletta, Chakravarti et al. 2004). Therefore, I hypothesized that the SNS cytodomain contains signaling motifs or sequences that are essential for such interactions and in turn its function during myoblast fusion. I constructed a series of deletions and mutations in the cytodomain to find such critical domains or signaling motifs within it. These results are presented in Chapter II.

---

**Fig. 1.2:** Key molecules involved in *Drosophila* myoblast fusion.

Distribution and interactions of molecules in founder and fusion competent myoblasts is shown. Cell surface molecules Duf/Kirre, IrreC/Rst, SNS and Hbs mediate attraction and adhesion between the myoblasts. Intracellular proteins are shown as associated in pathways leading to actin cytoskeletal rearrangements for structural changes of the cells. Where the links in the pathway are unclear due to lack of direct evidence, dotted lines are used. Intracellular molecules that are not involved in any pathway but known to play roles are shown in the respective myoblast populations to indicate their requirement.
1.5.3 Cell morphological changes during myoblast fusion

The individual steps involved in myoblast fusion have been dissected by genetic analysis combined with studies at the level of the light and electron microscope (EM) of fusing myoblasts in embryos. By such analysis in wild-type embryos, a series of distinct intracellular changes that precede and are tightly associated with fusion have been outlined (Doberstein, Fetter et al. 1997). As mutants with a role in myoblast fusion are identified, they are similarly studied to identify their role at various steps. Interestingly, these studies show that the events in the *Drosophila* embryonic mesoderm during fusion are not unlike those previously found to occur in the fusion of vertebrate myoblasts in culture (Rash and Fambrough 1973). Doberstein found ~40nm vesicles clustered underneath the plasma membranes of neighboring myoblasts. These vesicles though similar to synaptic vesicles in size and shape, can be distinguished from the latter by their electron density. They appear to align with one another on either side of the juxtaposed membranes as if ‘pairing’ to form a structure that has been termed the prefusion complex. Occasionally, single unpaired vesicles have been observed. The actual composition or function of these vesicles remains a mystery. They are considered to be of exocytic origin and bud off from the Golgi in myoblasts (Kim S 2007). They are associated with microtubules and presumably carried to the cell periphery by them (Kim S 2007). Doberstein also observed electron dense patches of ~10nm thick material extending up to ~500nm along the cytoplasmic side of the plasma membranes. These were termed plaques and are rare compared to the prefusion complexes and believed to be a product of
fusion of the paired vesicles with the plasma membranes. Plaques are closely associated with areas of plasma membrane breakdown. Fusion occurs locally by formation of small pores along the length of the apposed membranes. The plasma membranes appear to vesiculate forming membrane sacs enclosing the extracellular space that separates the two myoblasts. Thus the membrane breaks down and free streaming of local cytoplasm is achieved (Fig. 1.3; Doberstein, Fetter et al. 1997).

Although the actual process of membrane breakdown has not been fully understood in fusing myoblasts in *Drosophila*, membrane fusion has been well studied using viral:host cell fusion as a model system. The mechanism is considered to be universally the same for any two fusing plasma membranes. The main steps of viral membrane fusion are membrane juxtaposition, hemifusion, local fusion pore formation, and pore enlargement (Cohen and Melikyan 2004). During membrane fusion, first the contacting monolayer leaflets i.e., the outer layers of the plasma membrane merge maintaining the inner monolayers as such. This stage is called the “hemifusion” stage or the stalk. This stalk bends further to bring the inner monolayers in contact and these finally merge to form a small pore in the membrane called the expanding fusion pore allowing cytoplasmic streaming between the two cells. As suggested by the name, these pores widen to allow the entire intervening plasma membrane to breakdown and make the cells enveloped in a single continuous plasma membrane (reviewed in Cohen and Melikyan 2004; Chernomordik and Kozlov 2005).

Doberstein extended his analysis using ultrastructural studies to studying mutants of *mbc*, *blow*, *rost* and *drac1* that block myoblast fusion. Mutants of *mbc* are characterized by a nearly complete absence of prefusion complexes. Occasionally when a
few do exist, they contain roughly wild-type numbers of paired vesicles, suggesting that the defect lies upstream of the actual assembly of the prefusion complex. On the other hand, in blow mutants, there are normal numbers of prefusion complexes and the progression to plaque formation is blocked. Hence, Blow is required later than MBC. The next step is blocked in mutants of rost where electron dense plaques are observed in higher numbers than in wild-type but membrane breakdown does not occur. In embryos, overexpressing constitutively active drac1, the myoblasts progress to the stage of membrane breakdown although it occurs at very low frequency. Prefusion complexes and plaques are indistinguishable from wild-type embryos. Therefore, it appears these myoblasts are blocked at a late stage of fusion compared to mbc or blow blocking the actual membrane breakdown between the apposed cells. These analyses allow us to envision the proteins function at various steps of the fusion process.

This analysis has been extended to other essential myoblast fusion related genes and helped understand their role in the process further. Sing is required for progression beyond the pre-fusion complex stage of myoblast fusion and fusion pores do not appear in these mutants (Estrada, Maeland et al. 2007). Although this may appear to be similar to blow, there is a difference in the numbers of prefusion complexes. In blow normal numbers of prefusion complexes are observed whereas sing mutants have much higher numbers compared to wild-type or blow. Hence in sing the fusion is blocked at a distinct step than blow. Based on its domain architecture and its loss of function phenotype, Sing is proposed to be required for fusion of electron dense vesicles to the plasma membrane (Estrada, Maeland et al. 2007). In ants/rols mutants, the prefusion complexes and later
steps are missing. Hence this gene function precedes those of \textit{mbc} and \textit{blow} (Rau, Buttgereit et al. 2001).

In \textit{sltr/d-wip} mutants, the electron dense vesicles form but fail to be targeted to the membrane (Kim S 2007). Those that do get targeted are further defective in resolving into plaques and hence remain accumulated in unfused myoblasts and ultimately disappear at later stages (Kim S 2007). Consistent with this, the plasma membranes remain unfused and no cytoplasmic continuity is achieved in these myoblasts. This is in contrast to the results obtained in the Schejter laboratory with analysis of \textit{d-wip} and \textit{wasp} mutants also showing fusion arrest at an advanced stage but with some occasional membrane breakdown (Massarwa R 2007). Multiple discontinuities are apparent in the apposed myoblast membranes suggesting that these proteins are not required for fusion pore formation but for membrane breakdown and removal (Massarwa R 2007). Both groups also did a GFP based diffusion assay and once again obtained conflicting results. The former saw no diffusion of GFP consistent with the absence of membrane breakdown whereas the latter reported diffusion consistent with their EM results (Kim S 2007; Massarwa R 2007). This discrepancy must be resolved to understand the correct role of Sltr/D-WIP during fusion. It is enticing to think that Sltr/D-WIP specifically regulates fusion downstream of SNS.

Consistent with a role for actin regulating proteins in later steps of fusion, mutants of the WASp and WAVE regulator protein Kette also arrest at a late stage. Electron dense plaques are observed in the \textit{kette} mutant myoblasts which are 2-3 times longer than those observed in wild-type embryos (Schroter, Lier et al. 2004). Membrane breakdown is not observed and the prefusion complex remains stably visible until stage 15. Since \textit{blow}
mutants only progress to the prefusion complex stage while the *kette* mutants show plaque formation and there is evidence of genetic interaction between the two loci, Kette is considered downstream of Blow in the process of myoblast fusion (Schroter, Lier et al. 2004). Thus, through the pioneering work of Doberstein and the studies listed above, specific roles for essential genes at the various steps of myoblast fusion have been outlined (Fig. 1.3).

Fig. 1.3: Schematic of the steps of myoblast fusion determined by ultrastructural studies.

Following establishment of sites of contact, electron dense vesicles appear in pairs on either side of the juxtaposed membranes. These resolve into plaques that are considered to be a result of fusion. At these sites, membrane breakdown occurs to allow cytoplasmic continuity between the myoblasts.
To link cell adhesion with subsequent events that ultimately lead to membrane fusion, the subcellular localization of select proteins involved at the cell surface and intracellularly was studied (Kesper, Stute et al. 2007). SNS is observed to be concentrated in a ring shape in the filopodia of the fusion competent myoblasts. Duf/Kirre along with Ants/Rols is concentrated at the opposing sites on the founder cell or the precursor. Intracellular players of the process common to the two cell types such as D-Titin and F-actin are concentrated in this ring in both cell types. Blow which is specific to the fusion competent myoblasts colocalizes with actin. This ring shaped structure has been termed fusion-restricted myogenic adhesive structure (FURMAS; Kesper, Stute et al. 2007). The formation of this structure is dependent on cell:cell contact. It appears to expand with progression of the process reaching up to 5µm in diameter. The authors suggest the adhesive molecules of the FURMAS also limit the width of the fusion pore. The authors also envision the actin reorganization occurs at the pore structure and hence the expansion of the fusion pore may follow the expansion of the FURMAS. Further combination of such immunohistochemistry with the ultrastructural studies can extend this interpretation.

1.6 Studies of vertebrate myoblast fusion

Studies in Drosophila have greatly helped the understanding of myogenesis particularly the process of myoblast fusion. Over the past few decades, the process of myoblast fusion in vertebrates has been understood relying primarily on the strengths of tissue culture model systems. In vertebrates as in Drosophila, skeletal or body wall
muscles arise from segmentally repeated units called somites. Individual muscle fibers are formed by the fusion of mono-nucleated myoblasts to form a syncitial multinucleate myofiber through an ordered sequence of events during myogenesis in vertebrates.

When vertebrate myoblasts are induced to fuse they first become weakly associated in a step that has been defined as recognition (Knudsen and Horwitz 1977). Some cells in aggregates were found to be resistant to disruption and hence considered adhered (Knudsen and Horwitz 1977) and advanced than the recognition step. Following adhesion, the cells elongate and align into linear aggregates prior to fusion. Some cells in culture were resistant even to treatment with fusion inhibitors suggesting that membrane fusion was ongoing (Knudsen and Horwitz 1977). In fact, this has been recorded by visualization of membrane union and vesiculation of the excess plasma membrane at sites of fusion leading to cytoplasmic continuity (Rash and Fambrough 1973). These steps in vertebrate myoblast fusion are similar to the steps in *Drosophila* (reviewed in Bischoff 1978 and Abmayr, Balagopalan et al. 2003). The order and specificity of the fusion process remain largely unknown at a molecular level. There are similarities at the level of individual proteins that participate in the fusion process although the pathways are not entirely conserved.

1.6.1 Key molecules regulating vertebrate myoblast fusion

Similar to *Drosophila*, several cell surface as well as intracellular proteins with roles during myoblast fusion have been identified in vertebrates. Recently, studies in zebrafish have identified the Duf/Kirre ortholog, Kirrel, and outlined its role in myoblast
fusion (Srinivas, Woo et al. 2007). This is further evidence to the conservation of the process through evolution. Orthologs of Duf/Kirre and SNS, Neph1 and Nephrin respectively in higher vertebrates from mouse to man have been identified on the basis of sequence and structure, although functional similarity has not yet been observed. Neph1 and Nephrin are required in the development and maintenance of specialized structures in the kidney for proper filtration of urine (Kestila, Lenkkeri et al. 1998 and Donoviel, Freed et al. 2001).

Treatment of primary chick myoblasts with Neuronal cell adhesion molecule (NCAM) specific antisera causes lack of aggregation (Knudsen, McElwee et al. 1990). NCAM, a member of the IgSF, is present in mononucleate myoblasts in cultured mouse cells and is upregulated as myotubes begin to form (Moore, Thompson et al. 1987). Another membrane protein, Caveolin-3 (Cav-3) which is the principal component of caveolae is required for myoblast fusion (Galbiati, Volonte et al. 1998). This is interesting since caveolae are invaginations in the plasma membrane involved in vesicular trafficking and hence Cav-3 may be regulating similar intracellular vesicles as seen in *Drosophila* myoblast fusion.

Increased intracellular calcium as well as calcium binding calmodulin is required for myoblast fusion (Bar-Sagi and Prives 1983). Calcium and calmodulin can activate signaling pathways involving the nuclear factor of activated T cell (NFAT) family of transcription factors. In fact, proteins that activate the NFATC2 signaling pathway and genes that are regulated by NFATC2 such as IL-4 regulate the process of myoblast fusion and muscle growth (Horsley, Jansen et al. 2003). The NFATC2 and IL-4 pathways further demonstrate that common mechanisms exist between mammalian and *Drosophila*
fusion. IL-4 is expressed in a subset of myoblasts which may form a distinct population similar to founder cells (Horsley and Pavlath 2004). The NFATC2 pathway in the growing muscles regulates fusion of myoblasts with the growing myofiber similar to Drosophila growing myofibers (Horsley and Pavlath 2004; Bate 1990). This has been suggested to form the basis of the directionality of fusion.

As expected from the critical role of actin cytoskeletal rearrangements for fusion in Drosophila, vertebrate myoblast fusion is inhibited by treatment with agents that affect the actin and microtubule networks. However, since these inhibitors also prevent cell motility and proliferation, it is difficult to establish direct effects on membrane fusion from possible interference with migration toward or adhesion between myoblasts and myofibers. Studies in mouse myoblast culture C2C12 cells revealed a function of N-WASP in myoblast migration (Kawamura, Takano et al. 2004). Interestingly, Ants/Rols and Loner-Arf6 pathways leading ultimately to Rac activation may be conserved in vertebrates. There are two mouse orthologs of Ants/Rols termed mants1 and mants2. M-Ants1 is transiently expressed in the mouse mesoderm coincident with the formation of musculature (Chen and Olson 2001). Expression of a dominant-negative form of Arf6 interfered with formation of myotubes in mammalian cultured cells (Chen, Pryce et al. 2003). Recent studies in zebrafish have shown that Rac small GTPase is required for myogenesis (Srinivas, Woo et al. 2007). In contrast to Drosophila however, constitutive Rac activation in zebrafish led to hyperfused giant syncitia potentially indicating functional differences in the two species (Srinivas, Woo et al. 2007).
1.6.2 Cell morphological changes in myoblast fusion

The ultrastructural analysis of myoblast fusion in cultured cells showed both similarities and differences with the observations of Doberstein outlined in Section 1.5.3. The first extensive study of the morphology of fusion was carried out in cultured quail cells (Lipton and Konigsberg 1972). Prior to fusion, they found that myoblasts align in long chains. The earliest fusion site consisted of a small cytoplasmic bridge, about 120 nm in diameter, between apposing cells. Only a single point of cytoplasmic continuity was found between fusing cells leading the authors to conclude that fusion is initiated at a single site which subsequently enlarges for full cytoplasmic connectivity. The authors also noted the prevalence of “fuzzy-coated vesicles” at sites of contact. Similar fusigenic vesicles were observed in myoblasts as well as other cell types and do not appear to be electron dense (Kalderon and Gilula 1979) hence making them potentially different from those observed by Doberstein (Doberstein, Fetter et al. 1997). Ultrastructural analysis of L6 rat myoblasts revealed an electron-opaque material at localized points of cell-cell apposition similar to the electron dense vesicles (Engel, Egar et al. 1986). Further, electron dense patches similar to the plaques of Doberstein were also observed at a later stage of myoblast association (Rash and Fambrough 1973 and Engel, Egar et al. 1986). Thus it appears that the process is conserved at a cellular and molecular level from the fruitfly to higher vertebrates and studies in Drosophila will provide an understanding of the process in general.
1.7 Present studies

In the second chapter, I show that SNS is directly required for the migration of fusion competent myoblasts toward the founder cells in response to the attractant Duf/Kirre. One model for this function is that the SNS cytodomain recruits signaling molecules to the site of adhesion to initiate migration and potentially recruit fusion machinery. Several intracellular proteins have been identified to function within the fusion competent myoblasts. In particular, SNS interacts with D-Crk and recruits Sltr/D-WIP to points of cell contact. The experiments and results described in Chapter II identify motifs and regions within the SNS cytodomain which may be involved in such interactions and downstream signaling. I dissected the cytodomain function by generating deletional and mutational constructs which were then expressed in sns mutants and assayed for ability to rescue.

When \textit{mef2GAL4} (at 25ºC) or \textit{snsGAL4} (at 18ºC) are used to drive the expression of full length, C-terminally tagged \textit{UAS-snsHA} in the mesoderm of \textit{sns[Zf1.4/XB3]} mutants, the wild type muscle pattern is restored. Truncation of 166 amino acids (A1113-H1278) in the cytodomain (\textit{ΔA1113-H1278}) however completely failed to rescue with either GAL4 drivers. Further smaller deletions within this region failed to recapitulate the effect of the larger deletion. However, a combination of sub-deletions, A1113-I1163, Y1233-Y1263 and Y1263-H1278 caused an inability to rescue myoblast fusion which is comparable to \textit{ΔA1113-H1278}. This may be indicative of redundancy within functional domains or an additive effect of multiple functional motifs with subtle effects themselves.
I further demonstrate that the SNS cytodomain undergoes phosphorylation specifically on tyrosine residues. Mutagenizing all 14 cytodomain tyrosines (Y14-F14HA) results in complete loss of phosphorylation. Rescue with Y14-F14HA using mef2GAL4 (at 25°C) showed compromised ability to rescue sns mutant embryos with significantly higher numbers of unfused myoblasts in comparison to rescue with full length cDNA. The muscle pattern itself appeared normal. This inefficiency to rescue became more pronounced when level of expression was lowered using snsGAL4 (at 18°C). Several muscles are missing in the rescued embryos (>70% segments) and a greater increase in unfused myoblasts is observed suggesting that phosphorylated residues are critical for SNS function.

I also identified that SH3 domain binding consensus PXXP motifs are required for SNS function (2xPXXP). Rescue with mef2GAL4 (at 25°C) showed a high number of unfused myoblasts remaining at the end of myoblast fusion although muscle pattern was similar to wild type with all 30 fibers intact. The unfused myoblasts were quantitated and found to be significantly increased. The inefficiency to rescue became more apparent using snsGAL4 (at 18°C) to lower the level of expression. Several muscles were missing in upto 70% of the segments compared to only 12% of defective segments in rescue with UAS-snsHA. Other putative signaling motifs such as serine residues and putative PDZ binding consensus motifs were tested similarly, but found to be not essential using mef2GAL4 and snsGAL4. Thus SNS cytodomain sequences have specific requirements during myoblast fusion.

The third chapter of this thesis presents evidence of physical and genetic interactions between SNS and its paralog, Hbs. SNS is not required for the first phase of
myoblast fusion in the subset of muscles tested; 1-2 fusion competent myoblasts are still able to fuse with the founders to form precursor myotubes. This fusion is not spurious between founders since mutants in transcription factor *lameduck (lmd)*, which lack fusion competent myoblast population do not make precursor myotubes. I hypothesized that the reason for this is a redundancy between SNS and another IgSF protein in the fusion competent myoblast. I tested two candidates, IrreC/Rst and Hbs, expressed on the surface of fusion competent myoblasts and known to bind Duf/Kirre heterotypically. Hbs substitutes for SNS during the first phase of myoblast fusion (Menon, Osman et al. 2005) and double mutants (*sns, hbs*) largely lack precursor myotubes in the muscles under investigation. However, overexpression of *UAS-hbs* in the mesoderm of *sns* mutants does not completely rescue the myoblast fusion defect and hence the substitution does not extend to the second phase. SNS and Hbs form heterodimers in addition to SNS association into homodimers on the surface of myoblasts in embryos. Chimeras of Hbs extracellular domain or cytodomain with SNS are both capable of rescuing *sns* mutants better than *UAS-hbs* but not as well as *UAS-sns*. Thus there is physical and genetic interaction between SNS and Hbs indicating partial redundancy. The functional relevance of the physical interaction for myoblast fusion is yet unclear.
Chapter 2

Molecular dissection of the SNS cytodomain identifies critical sequences

2.1 Introduction

During *Drosophila* myogenesis, active polymerization of Actin occurs coincidentally with myoblast fusion in both myoblast populations. Associations between cell adhesion molecules and intracellular proteins have been identified that regulate these actin rearrangements. Sticks-and-Stones (SNS), present on the surface of fusion competent myoblasts, is an essential cell adhesion molecule for *Drosophila* myogenesis (Bour, Chakravarti et al. 2000). The long cytoplasmic tail of SNS contains several putative protein interaction motifs and signaling sequences. This study identifies the signaling sequences that are critical to protein function. I will begin by introducing SNS and other essential myogenic cell adhesion molecules followed by a summary of known cytoplasmic associations of IgSF cell adhesion molecules in general, as relevant to the SNS sequences under study.

2.1.1 Function of IgSF proteins in mediating cell adhesion

The locus *sns* is essential for myoblast fusion to form mature muscle fibers. It encodes a protein whose domain architecture is predicted by the Simple Modular Architecture Research Tool (SMART, (Schultz, Milpetz et al. 1998) with 9 immunoglobulin (Ig) domains, 1 fibronectin type III (FNIII) like domain, a single pass
transmembrane region and a long cytoplasmic tail consisting of 370 amino acids (Fig. 2.1 A). Mutants are characterized by unfused rounded myoblasts in the mesoderm and a complete lack of syncitial muscle fibers (Fig. 2.1 C; Bour, Chakravarti et al. 2000). Consistent with its function, the \textit{sns} transcript is detected specifically in the somatic and visceral mesoderm prior to the onset of myoblast fusion and antibody staining is maintained as fusion occurs (Bour, Chakravarti et al. 2000). SNS localizes to the plasma membranes exclusively in the fusion competent myoblasts in the mesoderm. In its absence, these myoblasts fail to extend projections and cannot contact founder cells (Bour, Chakravarti et al. 2000).

Fig. 2.1: \textbf{SNS predicted domain architecture and requirement in myoblast fusion.}

A. Schematic of predicted domains for SNS using SMART webtool. B. Wild type embryo. Notice that individual fibers are present in a segmentally repeated pattern. C. An embryo homozygous for \textit{sns} shows loss of mature muscle fibers replaced by unfused rounded myoblasts. All the embryos in this and subsequent figures are at embryonic stage 16 and stained immunohistochemically with a monoclonal antibody to myosin heavy chain (anti-MHC) unless otherwise mentioned. Anterior is to the left and dorsal is to the top in all the images. Scale bars are 10\(\mu\)m.
The locus *dumbfounded/kin-of-irreC* (*duf/kirre*) was identified due to its association with an enhancer trap *rp298-lacZ* which was the first founder cell specific enhancer to be identified (Nose, Isshiki et al. 1998; Ruiz-Gomez, Coutts et al. 2000; San Martin and Bate 2001). Expression of the *duf/kirre* transcript is observed at low levels in the developing mesoderm. The transcript is detectable exclusively in the founder cells prior to fusion and in the growing myofiber during fusion quickly disappearing once mature muscles are formed (Ruiz-Gomez, Coutts et al. 2000).

Another locus, *irrec/rst*, 127 kb upstream of *duf/kirre* encodes a highly related protein. IrreC/Rst is 45% similar to Duf/Kirre and is expressed in founder cells and subsets of fusion competent myoblasts (Strunkelnberg, Bonengel et al. 2001). *duf/kirre* and *irrec/rst* are predicted to encode single pass transmembrane cell adhesion molecules with 5 extracellular Ig-like domains and a cytoplasmic tail (Ruiz-Gomez, Coutts et al. 2000; Strunkelnberg, Bonengel et al. 2001). Duf/Kirre and IrreC/Rst function redundantly during myoblast fusion and a deficiency on the X chromosome removing both genes (*Df(1)w67k30*) results in a complete lack of syncitial muscle fibers and consequently large numbers of unfused myoblasts in the mesoderm (Ruiz-Gomez, Coutts et al. 2000). Fusion competent myoblasts extend projections scanning the neighborhood but fail to establish contacts with founder cells (Ruiz-Gomez, Coutts et al. 2000).

Consistent with their structure predictions, Duf/Kirre, IrreC/RST and SNS all function as cell adhesion molecules and mediate recognition and adhesion of cells. In transiently transfected *Drosophila* Schneider line 2 (S2) cultured cells, SNS, Duf/Kirre and IrreC/Rst are uniformly distributed on the cell surface (Galletta, Chakravarti et al. 2004). Duf/Kirre and IrreC/Rst expressing cells form homotypic cell aggregates. SNS
expressing cells do not associate with themselves under the same conditions but form heterotypic aggregates with cells expressing Duf/Kirre or IrreC/RST (Dworak, Charles et al. 2001; Galletta, Chakravarti et al. 2004). In such aggregated cells, the proteins redistribute in the plasma membrane and get enriched at sites of cell contact. On the surface of fusing myoblasts, SNS colocalizes with Duf/Kirre or IrreC/Rst into discrete punctae (Galletta, Chakravarti et al. 2004). This punctate distribution of SNS is lost in mutants lacking both duf/kirre and irreC/rst. The heterotypic interaction coupled with the differential distribution of SNS and Duf/Kirre in the two myoblast populations provides the basis for cell recognition and adhesion prior to fusion. In the presence of SNS on the surface of fusion competent myoblasts, the Duf/Kirre self interactions are replaced by Duf/Kirre-SNS interactions potentially preventing spurious founder self-fusion events (Menon, Osman et al. 2005).

An interesting insight into the function of these proteins and their interactions came from the observation that Duf/Kirre and IrreC/Rst function as cell surface attractants. Expression of either protein at ectopic sites such as in the embryonic ectoderm is sufficient to target migration of fusion-competent myoblasts towards such ectopic sites (Ruiz-Gomez and Bate 1997 and Strunkelnberg, Bonengel et al. 2001). Clearly, the fusion-competent myoblasts are capable of extending long processes to scan for and recognize founder cells and migrate toward them. The fact that in sns mutants, such filopodial extensions are missing suggests a role for SNS in receiving this attractive signal. A similar mechanism for guidance cues has been observed for Drosophila IgSF members functioning in neuronal development (Bashaw and Goodman 1999; Bashaw, Kidd et al. 2000). Roundabout (Robo) and Frazzled (Fra) receive extracellular cues and
in response direct migration of axons by intracellular signaling events from their cytoplasmic domains. Similarly, cytoplasmic signaling proteins are in place in the fusion competent myoblasts to direct cell migration.

### 2.1.2 Role of SNS as a signaling molecule

The reorganization of cytoskeletal filaments has been shown to affect fundamental processes in cells such as shape change, adhesion, migration and cytokinesis. These changes are mediated by cell surface receptors in response to extracellular cues via conserved signaling motifs in their cytodomains. Such signaling sites are protein-protein interaction motifs or sites for phosphorylation. The former recruit intracellular proteins and cytoskeletal regulatory machinery to the sites of cell surface protein localization. The latter sites function as molecular switches by changing the protein between active and inactive states. When multiple signaling sites are present in proteins, they can function together to direct the same events or regulate different functions downstream.

Coordination of adhesive and cytoskeletal interactions is essential to the process of myoblast fusion. Several actin regulatory genes such as *wasp, wave, kette* and *sltr/d-wip* have been characterized as essential for the process (Kim S 2007; Schroter, Lier et al. 2004; Schafer, Weber et al. 2007; Massarwa R 2007). Further, F-actin rich foci have been tightly associated with points of cell contact in fusing myoblasts. In the fusion competent myoblasts, these foci are dependent on the presence of SNS. One of the important differences in the phenotypes of loss of function mutants of *sns* and *Dff(1)w67k30*
(lacking \textit{duf/kirre} and \textit{irrec/rst}) is that myoblasts in the former remain rounded whereas in the latter, they put out projections trying to find an attractant from the neighborhood. This suggests that in \textit{sns} mutants, cytoskeletal changes required to extend such projections for directional migration are absent.

Specialized protein rich structures termed Fusion-Restricted Myogenic Adhesive Structure (FURMAS; Kesper, Stute et al. 2007) have been implicated in determining the site of fusion and the position and size of the fusion pore (Kesper, Stute et al. 2007). FURMAS are dependent on adhesive contacts between the cells and fail to establish in the absence of the cell adhesion molecules SNS and Duf/Kirre (Kesper, Stute et al. 2007). Hence the recruitment of intracellular molecules required for myoblast fusion follows closely after cell adhesion and is determined by it.

In the founder cell, Duf/Kirre directs activation of two small GTPases, Rac1 and ARF6 (ADP-ribosylation factor) via two separate pathways which are independent but may regulate each other (Section 1.3.3; Chen and Olson 2001; Chen, Pryce et al. 2003). Recruitment of Ants/Rols and MBC downstream of Duf/Kirre signals Rac1 activation to direct cytoskeletal changes. Signaling via Loner, a GEF for ARF6 leads to ARF6 activation and subsequent changes in the actin cytoskeleton regulation of lipid modifications (Chen, Pryce et al. 2003). One enticing possibility is that similar signaling events occur in the fusion competent myoblasts also. Consistent with this, MBC (Balagopalan, Chen et al. 2007) and Rac1 (Luo, Liao et al. 1994) function in both myoblasts and F-actin foci appear on both sides of adjacent fusing myoblast membranes (Kim S 2007).
The pathways in the two myoblasts are not entirely similar and several key molecules are differentially distributed presumably to maintain the directionality of fusion. Ants/Rols and Loner are exclusive to the founder cells (Chen and Olson 2001). Different adaptor proteins may be employed downstream of SNS to signal Rac1 activation. Sltr/D-WIP is a fusion-competent cell-specific protein that potentially links SNS with the actin cytoskeleton (Massarwa R 2007; Kim S 2007). SNS recruits Sltr/D-WIP via interaction through D-Crk in S2 cultured cells (Kim S 2007). This interaction, unless indirect, is mediated via the cytodomain of SNS as both D-Crk and Sltr/D-WIP are intracellular molecules. Thus, a function of SNS in an intracellular signaling pathway emerges.

In addition to lack of actin polymerization, myoblasts in sltr/d-wip mutants also lack exocytic vesicle trafficking. This could suggest that SNS functions additionally in recruitment of the electron dense vesicles associated with myoblast fusion extending the role of SNS beyond myoblast recognition and migration. Thus a thorough understanding of functional regions of the SNS cytodomain is required to understand the various aspects of its function. It has been noted previously using a GPI anchored form of SNS, that in the absence of the transmembrane and cytoplasmic sequences, SNS fails to rescue the myoblast fusion defect in sns mutants (Galletta, Chakravarti et al. 2004). Studies on the transmembrane domain are presented as Appendix A in this thesis and this chapter presents a dissection of the cytodomain sequences. To directly identify critical cytodomain sequences of SNS, I generated deletional and mutational constructs and assayed their requirement for a function during myoblast fusion by their ability to rescue the fusion defect in sns mutants. To select the candidate regions/residues to be targeted
for such an analysis, I used sequence conservation and analogy to known signaling sequences in other related IgSF proteins. These are presented in the next sections.

2.1.3 Orthologs of *Drosophila* myogenic IgSF proteins

Blast searches have revealed orthologs of SNS in vertebrates and *C. elegans*. Nephrin and Neph1 have been identified in mammals as the orthologs of SNS and Duf/Kirre respectively. However, these molecules do not have any identified function in myoblast fusion. Mutations cause a defect in the slit diaphragm within kidneys which are the sites of filtration of urine components from blood (Lenkkeri, Mannikko et al. 1999; Donoviel, Freed et al. 2001). Yet, these molecules share many similarities at a molecular level. Nephrin, like SNS has 9 Ig domains and a Fibronectin III like domain in its extracellular region, followed by a transmembrane sequence connecting to a cytoplasmic tail (Kestila, Lenkkeri et al. 1998). Similar to the biologically relevant Duf/Kirre-SNS association, Neph1 and Nephrin interact heterotypically (Gerke, Huber et al. 2003) and can be co-immunoprecipitated from kidney extracts (Barletta, Kovari et al. 2003; Gerke, Huber et al. 2003; Liu, Kaw et al. 2003).

Ectodomain engagement of Nephrin, by clustering with antisera or heterotypic interaction, results in a series of intracellular events. Three independent studies show Nephrin is phosphorylated on conserved tyrosine residues by Src family kinase Fyn (Li, Lemay et al. 2004; Lahdenpera, Kilpelainen et al. 2003; Verma, Wharram et al. 2003). Three of these sites form preferred binding motifs for the Src homology 2 (SH2) domain of adaptor protein Nck. The interaction between Nephrin and Nck at these sites is
enhanced by phosphorylation. This binding in turn facilitates localized actin reorganization in cell culture (Jones, Blasutig et al. 2006). Nephrin also interacts with two other cytoplasmic proteins, the SH3 domain containing CD2-associated protein (CD2AP) and the PDZ domain containing Zona occludens 1 (ZO-1) although a direct requirement for the cytodomain sequences has not been shown (Huber, Hartleben et al. 2003; Huber, Schmidts et al. 2003). These cytoplasmic interactions are crucial for Nephrin function as patients with a truncation of the Nephrin cytodomain exhibit severe proteinuria resulting from lack of proper filtration in the kidney (Lenkkeri, Mannikko et al. 1999).

In *C. elegans*, Synaptic guidepost protein-2 (Syg-2) is the SNS ortholog and is transiently expressed in primary vulval epithelial cells where it serves to guide proper synapse formation (Shen, Fetter et al. 2004). The Duf/Kirre and IrreC/Rst ortholog, Syg-1 is expressed on the HSNL neuron and localizes to the site of future synapses where it serves as a receptor for Syg-2 (Shen and Bergmann 2003; Shen, Fetter et al. 2004). Misexpression of Syg-2 in secondary epithelial cells causes aberrant accumulation of Syg-1 and synaptic markers in HSNL adjacent to the ectopic site of expression (Shen, Fetter et al. 2004) similar to the Duf/Kirre and IrreC/Rst ectopic expression studies in *Drosophila*. Although no functional relevance has been established, Syg-2 is expressed in head and body wall muscles in addition to neurons of the head (Shen, Fetter et al. 2004). So far, no studies have been directed toward understanding a role for the cytoplasmic domains of either protein. As additional information is uncovered, conservation of intracellular events downstream of these related cell adhesion molecules will become apparent.
2.1.4 Critical signaling motifs in cytodomains of IgSF cell adhesion molecules

Several cell adhesion molecules of the IgSF interact with various intracellular adaptors and effectors. Such interactions require specific sequences in the cytodomains and mutagenesis results in loss of function. A single cytodomain may interact with several intracellular proteins from distinct signaling sequences within, for mediating different downstream events as observed with Nephrin, Robo, DSCAM, etc. (Huber, Hartleben et al. 2003; Jones, Blasutig et al. 2006; Bashaw, Kidd et al. 2000; Schmucker, Clemens et al. 2000). SNS also similarly contains two types of protein interaction motifs and putative sites for phosphorylation in its cytodomain. These sequences are introduced below providing roles for similar sequences in other known IgSF proteins with roles in intracellular signaling.

2.1.4.1 Role of protein-protein interaction motifs for signaling downstream of cell adhesion molecules

Src homology 3 (SH3) domains are 50-70 amino acid domains, present in signaling molecules such as adaptors, kinases and GTPase activator proteins (Sudol M, 1998). SH3 domains recognize and bind in most cases a proline-dependent consensus site with a core PXXP motif preceded or followed by a charged residue, with X being any amino acid (Yu, Rosen et al. 1992). The cytodomain of IgSF guidance receptor, Robo, binds three SH3 domain containing proteins Dreadlocks (Dock), Abelson kinase (Abl) and Enabled (Ena). Dock SH3 domains bind Robo through two proline rich regions containing consensus PXXP motifs (Bashaw, Kidd et al. 2000; Fan, Labrador et al. 2003)
and deletion of either region completely abolishes binding. This is different from the redundant binding of Dock via two PXXP motifs in Down syndrome cell adhesion molecule, DSCAM, another IgSF member. Binding affinity to Dock is reduced upon mutating one or the other PXXP motif but completely abolished only when both sites are mutated simultaneously (Schmucker, Clemens et al. 2000).

SNS also has two consensus PXXP motifs that can potentially interact with SH3 domains. Three SH3 domain containing proteins, D-Crk, MBC and CD2AP are identified as regulators of myogenesis and may potentially function downstream of SNS. D-Crk indeed interacts with SNS in S2 cells and recruits Sltr/D-WIP, an actin regulatory protein, to sites of SNS expression (Kim S 2007). MBC functions downstream of Duf/Kirre in the founder cell and is additionally required in the fusion competent myoblasts (Balagopalan, Chen et al. 2007). Embryos overexpressing dCD2AP show a mild myoblast fusion defect (Galletta 2004). Interestingly, Nephrin has been shown to interact with CD2AP in the mouse epithelial M-1 cell line via its third SH3 domain (Palmen, Ahola et al. 2001). Similar interactions may occur with the SNS cytodomain and direct cytoplasmic events critical to myoblast fusion.

PDZ (Post-synaptic density-95/Discs large/Zonula occludens-1) domains are small protein-protein interaction modules that aid in formation of large signaling complexes involving adhesion molecules at the cell surface and cytoplasmic signaling proteins. They have specific consensus binding sites that predominantly reside at the C-terminus of a protein or mimic a free carboxy terminus when present internal to a sequence (Shieh and Zhu 1996; Songyang, Fanning et al. 1997). Nephrin has been suggested to bind ZO-1 through its C-terminus PDZ binding motif (Huber, Schmidts et
al. 2003). Interestingly, this motif is highly conserved between SNS and Nephrin although the rest of the cytodomains have largely diverged. Hence, this C terminal PDZ binding site may be critical for SNS function.

There are also additional internal sequences in the SNS cytodomain which can serve as PDZ domain binding sites. Multiple PDZ binding sites in proteins can be involved in signaling as exemplified by the cytodomain of the sodium-hydrogen exchanger, an unrelated transmembrane protein, where the C terminal PDZ binding motif is the strongest interacting site although internal sites maintain binding in its absence (Weinman, Wang et al. 2003). Hence, the multiple sites in SNS cytodomain may be redundant or differ in their potential for interaction with PDZ domain containing protein(s).

2.1.4.2 Role of phosphorylation in signaling downstream of cell adhesion molecules

Serine phosphorylation

SNS cytodomain consists of 35 serines and 33 threonines, all of which show some degree of conservation among homologs in other species. My attention was attracted to the serines primarily because of the high level of clustering. Serines undergoing phosphorylation in fact have a tendency to cluster and make up -1 and +1 positions of a phosphorylated serine residue (Blom, Gammeltoft et al. 1999). There are 11 conserved serines clustered in the SNS cytodomain of which 7 are predicted to have high probability of phosphorylation. By analogy to cell adhesion molecule, ICAM3 where such a serine rich motif binds intracellular actin remodeling proteins, I envisioned a critical role for this
region in SNS. Mutating one serine at a time in ICAM3 only abolished binding in part but when the whole region is deleted, interactions are greatly reduced (Serrador, Vicente-Manzanares et al. 2002).

Serine phosphorylation of L1CAM is regulated by the L1/NCAM interaction and functions as a switch for signaling downstream (Heiland, Griffith et al. 1998). Phosphorylation of single serine residues in L1CAM cytodomain contributes to neurite outgrowth through different mechanisms with increased growth when some serine residues are modified and decreased by others (Schultheis, Diestel et al. 2007). NCAM itself is phosphorylated on serine/threonine residues by PKC during neuronal differentiation in response to nerve growth factor (Matthias and Horstkorte 2006). It was suggested that this phosphorylation could regulate interaction with components of cytoskeleton although no evidence is provided. In this manner, SNS phosphorylation may also regulate its interactions with the cytoskeleton via modifications on distinct residues in response to binding of ligand.

**Tyrosine phosphorylation**

Tyrosine phosphorylation is a critical post-translational modification that regulates signal transduction pathways. It also affects the stability of many adhesions by cell adhesion molecules and their associated components, with high levels of phosphorylation promoting disassembly. Tyrosine phosphatases can also affect cell-cell adhesions indirectly by regulating the signaling pathways that control the activities of Rho family G proteins (Sallee, Wittchen et al. 2006). Phosphorylated residues can serve as binding sites for different modules in intracellular signaling proteins such as
phosphotyrosine binding (PTB) domains and Src Homology 2 (SH2) domains. Interestingly, Nephrin is phosphorylated on tyrosine residues in its cytodomain by Src family kinases Fyn and Yes (Verma, Wharram et al. 2003; Li, Lemay et al. 2004). This phosphorylation of Nephrin is a critical modification required for downstream signaling in the podocytes and further for the structure of the glomerular slit diaphragm. It regulates Nephrin interaction with the regulatory subunit of PI3K to facilitate Akt signaling (Huber, Hartleben et al. 2003). In an alternate pathway, phosphorylation at specific residues also enhances interaction with Nck which in turn facilitates localized actin reorganization in cell culture (Jones, Blasutig et al. 2006).

Tyrosine phosphorylation can also occur at multiple residues to regulate common or distinct downstream events. In Nephrin, atleast three residues are phosphorylated and serve to enhance downstream signaling by Nck binding (Li, Lemay et al. 2004). In the cytodomain of UNC-5, a transmembrane protein in *C. elegans*, phosphorylation of a single tyrosine is required and largely sufficient for function although other sites are phosphorylated (Killeen, Tong et al. 2002). Phosphorylation can also serve an inhibitory function and result in deactivation of downstream signaling factors as seen in the case of IgSF member, Carcinoembryonic antigen-related cellular adhesion molecule-1 (CEA-CAM1) (Chen, Iijima et al. 2004). The SNS cytodomain contains 14 tyrosine residues of which 6 are predicted to have high probability of phosphorylation. Drawing a comparison to the above examples and others not presented here, SNS cytodomain may also require phosphorylation of critical tyrosine residues to function during myoblast fusion.
2.2 Materials and methods

2.2.1 Fly stocks

All stocks used in this study were maintained on standard cornmeal media at 18°C or 25°C as necessary. The following fly lines were used: mef2GAL4, 24BGAL4, twiGAL4, dllGAL4, sns[Zf1.4] and sns[XB3] have been described elsewhere (Bour, Chakravarti et al. 2000, Ranganayakulu, Elliott et al. 1998). sns[XB3]; mef2GAL4, sns[Zf1.4]; mef2GAL4, sns[Zf1.4]; 24BGAL4 sns[Zf1.4], snsGAL4, sns[Zf1.4], dllGAL4 and sns[XB3], UAS-duf were generated for this study by standard recombination, sns[20-5] was obtained from R. Renkawitz-Pohl (Paululat, Burchard et al. 1995), ethylmethanesulfonate (EMS) induced alleles of sns were generated as described previously (Bour, Chakravarti et al. 2000). UAS-duf was provided by M. Bate (Ruiz-Gomez, Coutts et al. 2000). The transgenes created in this study were generated by using standard methods and mapped to the second chromosome. Balancer chromosomes in all these stocks were either marked with LacZ or GFP to enable identification.

2.2.2 Antibodies

In this study, I used, mouse anti-MHC (1:1000; D Kiehart), rabbit anti-SNS (1:100; Galletta, Chakravarti et al. 2004) and guinea-pig anti-Duf/Kirre (1:500; Galletta, Chakravarti et al. 2004), mouse (Promega) and rabbit (Capella) anti-betagalactosidase (1:1000), mouse anti-phosphotyrosine clone 4G10 (1:1000; Upstate Biotechnology) and horseradish peroxidase conjugated rat anti-HA (1:3000; Roche, Indianapolis, IN).
Secondary antibodies are biotinylated anti-mouse IgG and anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA), horseradish peroxidase conjugated anti-mouse and anti-rabbit (1:5000; Molecular Probes), alexa 488 anti-guinea pig IgG, alexa 660 anti-mouse IgG, alexa 488 anti-rabbit IgG (1:200; Molecular Probes).

### 2.2.3 Site-directed mutagenesis and constructs

All mutagenized constructs of *sns* used in this study were derived from pUAST-*sns* (Galletta, Chakravarti et al. 2004). C-terminal epitope tags were as follows: YPYDVPDYA from influenza hemagglutinin (HA) (SJ Hong), DYKDDDDK (FLAG) and GKIPNPLLGLDST sequence from Simian virus 5 (V5). Tags were engineered by PCR based insertion before the stop codon following the last 4 amino acids (-GHLV) of SNS. Mutations and internal deletions were introduced using PCR amplification with mismatch oligonucleotides (Fig. 2.2 and 2.3) and replacing the relevant region in pUAST-*sns* or pUAST-*sns*HA. The oligonucleotides used for the mutations and deletions generated for this study are provided in Appendix B. C-terminal deletions were generated by single PCR using oligonucleotides introducing a stop codon following the last desired codon (Fig. 2.3). All final constructs were confirmed by sequencing the entire coding region to verify a lack of PCR induced unwanted errors. *snsGAL4* was generated by cloning a 5Kb region from the *sns* genomic region including 2 Kb upstream of the translational start site into pPTGAL vector (Sharma, Cheung et al. 2002). *sns-LacZ* was generated similarly using pH-pelican vector (Barolo, Carver et al. 2000).
Fig. 2.2: Schematic for generating mutagenized constructs of SNS.

Fig. 2.3: Schematic for generating deletional constructs of SNS.

(A) Design of internal deletions and (B) Design of C-terminal deletions.
2.2.4 Immunohistochemistry

Embryos from rescue experiments were collected on agar-apple juice plates at 25°C and aged to 12-18hr after egg-laying at 25°C or 18°C as necessary. After fixing, enzymatic reaction was used to select for mutants by lack of beta-galactosidase (β-gal) activity. Mutants were sorted, verified to be 25% of the population and incubated with anti-myosin heavy chain overnight at 4°C followed by biotinylated secondary antibody for 2 hours at RT. Colorimetric detection was performed using Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA) using diaminobenzidine substrate. For anti-SNS staining, the same protocol was followed with the exception of an additional step of biotinyl tyramide treatment (Vector Laboratories, Burlingame, CA) for enhancement of intensity of signal. Embryos were imaged using Zeiss Axioplan and Axiovision software.

Embryos from the migration assay were aged to 10-15 hr at 18°C and fixed. Double staining was done with anti-MHC and anti-Duf/Kirre with differently labeled fluorescent secondaries. Single Z sections showing both ectodermal and mesodermal layers of the embryos were collected using Zeiss LSM 510 meta microscope and LSM software.

2.2.5 Molecular characterization of mutants

The EMS mutant alleles were balanced using CyO, P[w+mC]=GAL4-twi.G}2.2, P(UAS-2xEGFP)AH2.2. Embryo were collected, dechorionated and sorted to select mutants from the population by lack of green fluorescence protein (GFP) expression on the COPAS PLUS sorting instrument (Union Biometrica). The mutant embryos were
homogenized in Trizol (Invitrogen) to isolate total RNA from which reverse transcription was performed using Superscript III (Invitrogen) with gene-specific oligonucleotides. The products were further amplified using high fidelity polymerase (Roche) and sequenced at the Stowers Institute for Medical Research Molecular Biology Facility. The molecular lesions were confirmed by repeating with independent RNA preparations. These experiments were done by MH Chen.

2.2.6 Quantitation of unfused myoblasts and hemisegments with defects

The unfused myoblasts were counted by using the myoblast specific sns-LacZ expression in the rescue backgrounds. Embryos were stained with indirect immunofluorescence for β-gal using Alexa 488 labelled secondary antibody. Rescued embryos were recognized by lack of β-gal staining in the characteristic wingless (wg) pattern from the balancer chromosome. Rescued embryos were staged by differential interference contrast (DIC) on the Zeiss confocal microscope following the staging system of Campos-Ortega and Hartenstein (1997). A tracheal specific marker was tried to aid in more accurate staining but did not provide the staining pattern expected. This limits the ability to stage accurately. However, from my analysis, the standard deviation was very narrow increasing the reliability of the assay. Three abdominal hemisegments A2, A3 and A4 were imaged in serial 1um Z sections of the ventral musculature using Zeiss LSM software. The images were imported into Imaris (Bitplane) program to quantify fluorescent cells with a diameter of 4um. This limit eliminated counting the faintly stained myotubes. The counted cells were verified visually and an average of counts from
total sample size was calculated and used for comparison with other full length cDNA rescue (n is indicated in the graphs). The comparison was done in pairwise fashion using one-way ANOVA (ANalysis Of VAriance) model followed by Tukey’s HSD (Honestly Significant Differences) test. Since there are 8 cDNA rescues, \( \binom{8}{2} = 28 \) pairwise comparisons were done, and P-values for each of 28 pairwise comparisons are available in Appendix B.

For rescues using sns\textit{GAL4} to drive expression, missing muscles were quantitated to ascertain differences in ability to rescue. 4 abdominal hemisegments A2-A5 were visualized in each embryo. Of the lateral muscles 21-23 (according to Crossley 1978), if any muscle fiber was missing, the hemisegment was counted as defective. A percentage of such defective segments was calculated and comparison drawn by using a simple graphical representation. n values indicate the number of total hemisegments visualized for the analysis for each genotype. Similar to above analyses, \( \binom{14}{2} = 91 \) pairwise comparisons were performed using one-way ANOVA model combined with Tukey’s HSD test. P-values for each of the 91 pairwise comparisons are available in Appendix B.

2.2.7 S2 transfection

S2 cells were grown as described (Cherbas and Cherbas 1998) and plated at 1.6x10^6 cells/ml for transfection. pUAST- constructs were cotransfected with pWAGAL4, a GAL4 expressed from a constitutive Act5C promoter for expression
(Ishimaru, Ueda et al. 2004). pRmHa3 constructs were expressed by adding 700uM copper sulfate. All transient transfections were performed used calcium phosphate (Ashburner 1989).

### 2.2.8 Immunoprecipitation and western bloting

Transgenes to be analyzed were mated to *mef2GAL4* and embryos collected at 25°C for 6 hours and aged at 18°C for 16 hours. The embryos were dechorionated in 50% bleach for 3 minutes and dry weight calculated. They were then homogenized in lysis buffer (2% TritonX-100, 60mM Tris-HCl pH 7.4, 6mM EDTA and 300mM NaCl) at a concentration of 0.1mg/ml in the presence of protease (2mM) and phosphatase inhibitors (1mM). S2 cell lysates were generated by resuspending the cells in lysis buffer and passage through a 25g needle for 10 strokes. The lysates were centrifuged at 20,000xg to remove debris and 1mg total protein for S2 cells and 2-5mg total protein for embryos used for immunoprecipitation.

For immunoprecipitation, lysates were incubated with 20μl anti-HA affinity matrix (Roche, Indianapolis, IN) overnight at 4°C. The immunoprecipitates were washed in lysis buffer three times and eluted by boiling in 40μl Laemmli buffer except where treated with phosphatases (see section below). The eluted proteins were subjected to SDS-PAGE on a 5% acrylamide gel and transferred to a PVDF membrane. Immunoblotting was done using anti-phosphotyrosine (Upstate Biotechnology) and anti-SNS (Galletta, Chakravarti et al. 2004) or anti-HA antibodies (Roche, Indianapolis, IN).
The immunoblots were developed using chemiluminescence by ECL reagents (Amersham GE) and detected by scanning on a Typhoon 9400 (Amersham GE).

2.2.9 Phosphatase treatment

Following immunoprecipitation as described above, the resin was washed in phosphatase buffer three times and incubated with 1μl protein tyrosine phosphatase 1b (3units/μl Upstate Biotechnology) or lambda phosphatase (400 units/μl New England Biolabs) at 30°C for 30 minutes in the presence of Mn2+ containing buffer. The phosphatase was removed by three washes in lysis buffer and treated protein was eluted by boiling in Laemmli buffer and detected on western blots as described above.
2.3 Results

2.3.1 SNS is required for directional migration of fusion competent myoblasts

Duf/Kirre and IrreC/Rst function as attractants directing migration of fusion competent myoblasts to an ectopic site of expression (Ruiz-Gomez, Coutts et al. 2000; Strunkelnberg, Bonengel et al. 2001). Based on the known characteristics of SNS, it is highly likely that it serves as the receptor for this attractive signal and guides the directional migration of fusion competent myoblasts. I tested whether myoblasts of sns mutant embryos show lack of migration towards ectopic Duf/Kirre.

Duf/Kirre was ectopically expressed using dllGAL4 in the ectoderm of wild-type as well as sns mutants. Myoblasts were visualized by immunohistochemical staining with anti-myosin heavy chain (MHC) and Duf/Kirre expression was followed by anti-Duf antisera. In wild-type embryos, myoblasts (red in Fig. 2.4A) were observed to migrate ectopically and found intermingling with cells overexpressing Duf/Kirre (green in Fig. 2.4A) in the ectoderm. However, in the absence of SNS, no myoblasts were observed in the ectoderm at or near the site of Duf/Kirre expression (Fig. 2.4B). Lots of unfused myoblasts were still present in the underlying mesoderm. Hence, Duf/Kirre directed ectopic migration requires SNS on the surface of fusion competent myoblasts. This is consistent with the myoblast fusion phenotype of loss of function mutants of sns where
myoblasts fail to extend projections and make contact with the elongated founder cells (Bour, Chakravarti et al. 2000).

---

Fig. 2.4: **SNS is required for directional migration of fusion competent myoblasts in response to attractant Duf/Kirre.**

Expression of Duf/Kirre (Green) in the ectoderm of wild-type (A; *UAS-duf/dllGAL4*) and *sns* mutants (B; *sns[Zf.14], dllGAL4/sns[XB3], UAS-duf*). Myoblasts (red) migrate toward the ectopic site in A (arrows) but not in B. m and e indicate mesodermal and ectodermal layers respectively.

---

### 2.3.2 Development of an assay system based on rescue of *sns* mutants

The goal of this study is to identify critical functional sequences/motifs within the SNS cytodomain by generating deletions and mutations. To test the in vivo function of mutant constructs, the two component UAS-GAL4 system (Brand and Perrimon 1993) is used to express these constructs in the mesoderm of *sns* mutants and assaying for the ability to restore wild-type muscle pattern. Since there are several different GAL4 drivers to choose from, I first wished to establish a good rescue using full length cDNA to ensure ability to characterize the mutant constructs in comparison. To be able to follow
expression and perform biochemical analyses, I placed convenient epitope tags. I first tested different epitope tags to determine one with the least hindrance to SNS function. Lastly, I tested different levels of expression to find ideal conditions where a difference between full length \textit{sns} and the mutagenized sequences can be established.

2.3.2.1 Rescue using different spatial and temporal expression patterns

Different GAL4 lines in \textit{Drosophila} have different patterns of expression depending on the gene associated with the specific enhancer traps (Brand and Perrimon 1993). Since SNS expression is controlled in a spatial and temporal manner in embryos, the mutant transgene expression should closely mimic it to obtain the best rescue conditions. Recently, a fusion competent myoblast specific GAL4 driver has been generated in our laboratory by fusing 5KB of \textit{sns} genomic region to GAL4 sequence (JP McDermott). Expression of full length cDNA of \textit{sns} using this \textit{snsGAL4} rescued the myoblast fusion defect although occasional missing muscles were observed in dorsal, ventral and lateral views (Fig. 2.5D). Some muscles that do form are thin compared to those in wild-type embryos. This may be due to the absence of some regulatory sequences and the GAL4 does not recapitulate endogenous \textit{sns} expression pattern completely.

I also employed three pan-mesodermal GAL4 drivers with differences in temporal expression (\textit{twiGAL4}, \textit{mef2GAL4} and \textit{24BGAL4}). Traditionally, our laboratory used \textit{twiGAL4} which has an early onset of expression before specification of the mesoderm and is downregulated once the mesoderm is patterned (R Banerji). Using \textit{twiGAL4}, the
mutant phenotype was largely rescued and all muscle fibers were made (Fig. 2.5A).

However, the muscles became thin and stretched out in later stages and failed to achieve proper attachments (Fig. 2.5A). The two other pan-mesodermal GAL4 drivers rescued the myoblast fusion defect better than *twiGAL4*. Using *mef2GAL4* (Fig. 2.5B) and *24BGAL4* (Fig. 2.5C; Galletta, Chakravarti et al. 2004), all muscle fibers appear to be similar to wild-type in shape and size and not many unfused myoblasts remain at the end of fusion at embryonic stage 16. Further, these drivers rescue the *sns* mutant embryos to adulthood (Table 2.1).

---

Fig. 2.5: Driving full length cDNA of *sns* in the mesoderm of mutants restores the muscle pattern.

(A) *twiGAL4; sns[XB3]/sns[Zf1.4]; UAS-sns*  (B) *sns[Zf.14], UAS-sns/sns[XB3];mef2GAL4/+*  (C) *sns[Zf.14], UAS-snsHA/sns[Zf.4];24BGAL4/+* and (D) *sns[Zf.14],snsGAL4/sns[Zf1.4], UAS-snsHA*. Some defects in the muscle pattern are observed when *snsGAL4* is used. Pan-mesodermal expression rescues well although *twiGAL4* rescued poorly with thin muscles and occasional defects at later stages.
2.3.2.2 Rescue using different epitope tagged forms of SNS

For the study of modifications of the protein as well as protein:protein interactions, it is important to be able to purify the protein and analyze it biochemically. To do this with all of the mutant and deletional constructs of SNS, I placed convenient epitope tags. However, epitope tags have been demonstrated to sometimes interfere with proper folding of the protein or form a physical hindrance for interaction with regular binding partners. Hence, I tested different epitope tags at the N and C terminus of SNS. Epitope tags were placed at the C terminus to enable me to ascertain full translation of the protein. Three short epitope tags, HA (9 amino acids, SJ Hong), V5 tag (14 amino acids) and FLAG tag (8 amino acids) were tested (see Materials and Methods). All three did not interfere dramatically with the function of SNS as evidenced by robust rescue of myoblast fusion defects in sns mutants using *mef2GAL4* to drive expression (Fig. 2.6). I further tested rescue to adulthood and found SNS-HA rescues appreciably although not as well as untagged form of SNS (Table 2.1). V5 and FLAG tagged forms also rescued to

Fig. 2.6: Different C-terminal epitope tags did not affect the ability of SNS function in the rescue assay.

Epitope tags HA (A), V5 (B) and FLAG (C) do not interfere with the ability of full length *sns* cDNA to rescue the myoblast fusion defect in mutants. (A) *sns[Zf.14], UAS-snsHA/sns[XB3];mef2GAL4/+* (B) *sns[Zf.14], UAS-snsV5/sns[XB3];mef2GAL4/+* and (C) *sns[Zf.14], UAS-snsFLAG/sns[XB3];mef2GAL4/+.*
viability although the percentages were lower. In addition, when expressed in an
otherwise wild-type embryo using *mef2GAL4*, no dominant effects were observed with
*UAS-snsHA* (data not shown). Therefore, I used the HA tag for the mutant constructs in
this study.

Table 2.1: **Full length cDNA of sns expressed pan-mesodermally rescues mutants to
viability.** The genotypes are indicated on the left. The expected percentage of straight
winged adults for each genetic combination listed is 33%. The value represented on the
right is the actual percentage of straight winged adults from the total adults obtained.
*UAS-sns* rescues as expected with either *24BGAL4* or *mef2GAL4*. Tagged forms are
slightly less efficient. The least interference to rescue to viability is seen with HA tag.

<table>
<thead>
<tr>
<th>Stocks</th>
<th>% Straight winged adults</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sns[Zf1.4]; 24BGAL4 X sns[XB3]; UAS-sns</em></td>
<td></td>
</tr>
<tr>
<td>CyO 24BGAL4  CyO UAS-sns</td>
<td>34%</td>
</tr>
<tr>
<td><em>sns[XB3]; mef2GAL4 X sns[Zf1.4]; UAS-sns</em></td>
<td></td>
</tr>
<tr>
<td>CyO mef2GAL4  CyO UAS-sns</td>
<td>34%</td>
</tr>
<tr>
<td><em>sns[XB3]; mef2GAL4 X sns[Zf1.4],UAS-snsHA</em></td>
<td></td>
</tr>
<tr>
<td>CyO mef2GAL4  CyO</td>
<td>23%</td>
</tr>
<tr>
<td><em>sns[XB3]; mef2GAL4 X sns[Zf1.4],UAS-snsFLAG</em></td>
<td></td>
</tr>
<tr>
<td>CyO mef2GAL4  CyO</td>
<td>14%</td>
</tr>
<tr>
<td><em>sns[XB3]; mef2GAL4 X sns[Zf1.4],UAS-snsV5</em></td>
<td></td>
</tr>
<tr>
<td>CyO mef2GAL4  CyO</td>
<td>18%</td>
</tr>
</tbody>
</table>
2.3.2.3 Rescue using different levels of expression

GAL4 expression is sensitive to changes in temperature. I employed temperature shifts to modulate the level of expression of transgenes to test the effect on ability to rescue the myoblast fusion defect in sns mutants. High levels of expression can result in dominant effects of proteins, complicating analysis of sequence requirements by rescue abilities. On the other hand, low levels of expression may cause inadequate rescue due to lack of robust interactions. There are two ways to temper level of expression in Drosophila-by changing temperature of incubation during development or by increasing number of transgenes.

I reasoned that with mutagenized constructs, some compromised function may go unnoticed if higher expression level compensates for the lack of rescue. I was interested in looking for subtle defects that may be missed when high levels of protein are expressed. Thus, I established an assay where I can temper the level of expression to reveal such compromised function if necessary by lowering temperature of incubation. Two GAL4 drivers, the pan-mesodermal mef2GAL4 and the fusion competent myoblast specific snsGAL4 were tested at 25°C and 18°C where GAL4 induction is lowered. Using mef2GAL4 at 25°C or 18°C, there was no difference in the efficiency of rescue (Fig. 2.7 A&C). Using snsGAL4 at 25°C or 18°C did reveal some missing muscles and increased numbers of unfused myoblasts but the rescue capability did not change at the two temperatures. By western analysis, it was confirmed that snsGAL4 driven expression was reduced compared to mef2GAL4 driven expression at 18°C (data not shown). There is the possibility that changes in temperature can affect the rate of progression of
developmental processes resulting in an effect on the ability to rescue. Using *snsGAL4* at 18°C however, did not change the ability of *UAS-snsHA* to rescue the myoblast fusion defect. Hence, tempering the level of expression did not change the capability of full length cDNA to function during myogenesis. Since the defects are mild, an enhancement will be quantifiable and hence, this assay can be used to test other mutagenized constructs for efficiency of rescue capabilities in comparison to full length *sns* cDNA.

---

**Fig. 2.7:** Rescue with full length cDNA does not change at high and low temperatures.

Rescue using *mef2GAL4* at 25°C (A) and 18°C (C) *sns[Zf.14],UAS-snsHA/sns[XB3]*; *mef2GAL4* and rescue using *snsGAL4* at 25°C (B) and 18°C (D) *sns[Zf.14],UAS-snsHA/sns[Zf1.4],snsGAL4*. No defects were observed using *mef2GAL4* at either temperature. Rescue embryos in C and D have minor defects but the muscle pattern is normal and does not change between the two temperatures.
2.3.3 Generation of deletional constructs of SNS

SNS full length cDNA is translated into a protein with 1482 amino acids with a predicted molecular weight of 162KDa. The actual migration of SNS protein purified from the mesoderm is ~180KDa, potentially due to post-translational modifications. SNS sequence has two Glutamine (Q) rich regions that have been reported at different lengths resulting in changes in the amino acid positions of SNS from the published sequence (Bour, Chakravarti et al. 2000). Hence, I have represented my deletion constructs to show both the residue position and the amino acid.

Domain architecture prediction by the SMART webtool predicts SNS cytodomain to start at amino acid R1108 (Schultz, Milpetz et al. 1998). The first few amino acids are hydrophilic making up the stop transfer signal to prevent this region passing through the plasma membrane. Hence, I consider amino acid A1113 as the start of the cytodomain and V1482 as the last. All deletional and mutational constructs in this study are targeted at sequences within these amino acids.
Amino acids A1113-H1278 form a large block of highly conserved sequences (Fig. 2.8). Two large deletions were generated in the SNS cytodomain, one deleting from A1113-H1278 and the other deleting the remaining region from L1279-V1482 to determine which region was critical (in collaboration with Dr. R Banerji). I also generated 4 smaller deletions within A1113-H1278. These deletions were later combined to make constructs to test redundancy or additive effect of multiple functional groups in the original set of deletions and to identify the minimal critical region within the cytodomain. These deletional constructs are shown in the schematic below.
2.3.4 Generation of mutational constructs of SNS

The SNS cytodomain contains 2 consensus PXXP motifs that can form sites for interaction with SH3 domains in intracellular proteins. The first of these sites from P1239-P1242 sits in a short proline rich region (1235-1243). The second site is farther downstream from P1373-P1376 and followed by a basic histidine. I mutated 6 prolines in the first stretch individually (UAS-1xPXXP), and in combination with the 2 prolines of the second PXXP (UAS-2xPXXP) to alanine residues.

The C-terminal 4 amino acids G1479-V1482 form a highly conserved putative PDZ binding site. This site is deleted in the ΔL1279-V1482 construct. In addition to this, there are 4 other internal PDZ binding sites in SNS that can bind PDZ domains. Hence,
with the assistance of Dr. Jian-Min Wu, first two of the highly conserved sites and then all five PDZ binding sites were mutagenized using amino acid substitutions as shown below in the schematic (UAS-2xPDZ and UAS-5xPDZ-see figure below for residue positions).

There are a total of 35 serine, 33 threonine and 14 tyrosine residues in the SNS cytodomain. The best candidate sites for phosphorylation were selected using 3 prediction programs, Netphos2.0 (Blom, Gammeltoft et al. 1999), Disphos 1.3 (Iakoucheva, Radivojac et al. 2004) and PONDR (Romero et al. 2001). These results are shown in tables 2.2, 2.3 and 2.4 along with candidate kinases or binding partners for the relevant sites. For serine and threonine, only the predicted sites are shown in the interest of space. Prediction results for all 35 serine and 33 threonine residues are provided in Appendix B. For tyrosines, all 14 residues and their prediction results are shown.

Eight serine residues scored very high (>0.9 probability; Table 2.2) using the widely used Netphos2.0 program and were first mutated to alanines (UAS-snsS8-A8-see figure below for residue positions). There were also 6 other serine residues with a probability score higher than the threshold used by the programs (>0.5). Conservation of residues was also considered with the view that critical phosphorylation sites are probably conserved through evolution. Also, due to the high clustering of conserved serines in the beginning of the cytodomain, some second site phosphorylation can occur in the absence of the preferred site. Hence, with the assistance of JP McDermott, I mutated a total of 17 residues (of 19 in A1113-H1278) based on prediction scores and high level of conservation (UAS-S17-A17HA-see figure below for residue positions).
Threonine mutagenesis has not been performed in this study although the scores from the prediction programs did reveal some candidate sites (Table 2.3).

Table 2.2: Several serine residues in the SNS cytodomain are predicted to be candidates for phosphorylation.

Of the 35, only the predicted candidate sites are shown here.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Netphos</th>
<th>Pondr</th>
<th>Disphos</th>
<th>Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1131</td>
<td>0.996</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1132</td>
<td>0.690</td>
<td>✓</td>
<td>0.601</td>
<td>PKC/CKII</td>
</tr>
<tr>
<td>S1143</td>
<td>0.779</td>
<td>✓</td>
<td></td>
<td>CKII</td>
</tr>
<tr>
<td>S1144</td>
<td>0.796</td>
<td>✓</td>
<td>0.705</td>
<td>CKII/PKC/Cdc2</td>
</tr>
<tr>
<td>S1146</td>
<td>0.918</td>
<td>✓</td>
<td>0.804</td>
<td>CKII/Cdc2</td>
</tr>
<tr>
<td>S1151</td>
<td>0.991</td>
<td>✓</td>
<td></td>
<td>CKII/ATM</td>
</tr>
<tr>
<td>S1157</td>
<td></td>
<td>✓</td>
<td>0.830</td>
<td>CKII/DNAPK/ATM</td>
</tr>
<tr>
<td>S1158</td>
<td>0.983</td>
<td>✓</td>
<td>0.595</td>
<td>PKA</td>
</tr>
<tr>
<td>S1172</td>
<td>0.954</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1179</td>
<td></td>
<td>✓</td>
<td>0.690</td>
<td></td>
</tr>
<tr>
<td>S1215</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1229</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1236</td>
<td>0.684</td>
<td></td>
<td>0.557</td>
<td>DNAPK</td>
</tr>
<tr>
<td>S1238</td>
<td>0.678</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1246</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1252</td>
<td>0.976</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1257</td>
<td>0.678</td>
<td></td>
<td>0.557</td>
<td></td>
</tr>
<tr>
<td>S1337</td>
<td>0.807</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1459</td>
<td>0.678</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3: Several threonine residues in the SNS cytodomain are predicted to be candidates for phosphorylation.

Of the 33, only the predicted candidate sites are shown here.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Netphos</th>
<th>Pondr</th>
<th>Disphos</th>
<th>Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1114</td>
<td>0.631</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1120</td>
<td></td>
<td>✓</td>
<td>0.577</td>
<td>CKI</td>
</tr>
<tr>
<td>T1134</td>
<td></td>
<td></td>
<td></td>
<td>PKC</td>
</tr>
<tr>
<td>T1171</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1181</td>
<td></td>
<td></td>
<td></td>
<td>DNAPK</td>
</tr>
<tr>
<td>T1219</td>
<td></td>
<td>✓</td>
<td></td>
<td>CKII</td>
</tr>
<tr>
<td>T1287</td>
<td></td>
<td></td>
<td></td>
<td>PKC</td>
</tr>
<tr>
<td>T1292</td>
<td></td>
<td></td>
<td></td>
<td>PKC</td>
</tr>
<tr>
<td>T1308</td>
<td>0.784</td>
<td>✓</td>
<td></td>
<td>PKC</td>
</tr>
<tr>
<td>T1331</td>
<td></td>
<td></td>
<td></td>
<td>CKI/PKC</td>
</tr>
<tr>
<td>T1352</td>
<td></td>
<td></td>
<td></td>
<td>CKI</td>
</tr>
<tr>
<td>T1358</td>
<td></td>
<td></td>
<td></td>
<td>CKI</td>
</tr>
<tr>
<td>T1365</td>
<td></td>
<td>✓</td>
<td></td>
<td>PKC</td>
</tr>
<tr>
<td>T1369</td>
<td></td>
<td>✓</td>
<td></td>
<td>PKC</td>
</tr>
<tr>
<td>T1444</td>
<td></td>
<td></td>
<td></td>
<td>PKC</td>
</tr>
<tr>
<td>T1452</td>
<td></td>
<td></td>
<td></td>
<td>PKC</td>
</tr>
<tr>
<td>T1472</td>
<td></td>
<td>✓</td>
<td></td>
<td>CKII</td>
</tr>
</tbody>
</table>

Of the 14 tyrosine residues, six scored high probabilities for being phosphorylated by the most widely accepted prediction program Netphos 2.0 (Table 2.4). These were first mutated to phenylalanine which is structurally similar to tyrosine but does not have the functional –OH group (UAS-Y6-F6HA-see figure below for residue positions).

Considering other prediction programs and sequence conservation, 11 tyrosine residues were identified as potential sites for phosphorylation and mutated to phenylalanine residues (UAS-Y11-F11HA-see figure below for residue positions). Lastly, I mutated all 14 tyrosine residues in one single construct to eliminate any likelihood for redundancy or substitution (UAS-Y14-F14HA-see figure below for residue positions).
Table 2.4: Several tyrosine residues in the SNS cytodomain are predicted to be candidates for phosphorylation.

All the cytodomain tyrosines and their scores are presented here.

<table>
<thead>
<tr>
<th>Residue position</th>
<th>Netphos2.0 Score</th>
<th>Pondr prediction</th>
<th>Diphos1.3 Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1137</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1143</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Y1152</td>
<td>0.971</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Y1162</td>
<td>0.984</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Y1174</td>
<td></td>
<td>✓</td>
<td>0.508</td>
</tr>
<tr>
<td>Y1186</td>
<td>0.600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1196</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1233</td>
<td>0.907</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Y1247</td>
<td>0.966</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Y1248</td>
<td></td>
<td>✓</td>
<td>0.636</td>
</tr>
<tr>
<td>Y1255</td>
<td>0.950</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1258</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1263</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1474</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2.10: Schematic of mutagenized constructs employed in this study.

The name is provided on the left and the original residue and its position are shown on top.
2.3.5 SNS cytodomain is essential for its function

Previously, it was shown that a truncation lacking the transmembrane and cytodomains of SNS is unable to rescue myoblast fusion defect in sns mutants demonstrating a role for these sequences in SNS function during myoblast fusion (Galletta, Chakravarti et al. 2004). The first direct evidence to a requirement of the cytodomain itself came from an EMS allele of sns resulting in a truncated form lacking most of the cytodomain (sns[20-5]). The mutation is a splice donor site change in intron 18 resulting in a read through into the intronic sequence ultimately reaching a stop codon after 46 amino acids. The resultant protein is a truncation with intact SNS sequence from M1-I1163 which includes A1113-I1163 residues of the cytodomain. This allele is still expressed and protein can be detected in the myoblasts using an antibody to the N-terminal region of SNS (Fig. 2.11 A&B). The mutants however are defective for myoblast fusion and appear similar to other null alleles of sns (Fig. 2.11 C).

To confirm the loss of function phenotype is due to the truncation, I constructed a C-terminal deletion by placing an HA epitope tag and a stop codon following amino acid I1163 (UAS-sns20-5HA). This construct does not contain the intron readthrough sequences of sns[20-5]. Western blot analysis showed expression of this protein at the expected size in crude membrane preparations (data not shown). Targeting expression to the mesoderm using mef2GAL4 in sns mutants completely failed to rescue the myoblast
fusion defect (Fig. 2.11D). The rescued embryos look similar to the sns[20-5] allelic mutants confirming a requirement for the sequences in the cytodomain following I1163.

Fig. 2.11: Truncation of cytodomain following I1163 results in loss of function.

Schematic showing the truncated construct UAS[20-5]. (A) sns[20-5]/sns[20-5] and (B) sns[20-5]/CyO stained with anti-SNS showing comparable expression in the mutant and heterozygote. (C) sns[20-5]/sns[20-5] stained for MHC to show loss-of-function phenotype. (D) sns[Zf1.4], UAS-[20-5HA]/sns[XB3]; mef2GAL4/+. The construct is unable to rescue sns mutants.
To determine the critical region within the cytodomain, initially two deletions were generated; one deleting the C-terminal 204 amino acids (ΔL1279-V1482, R Banerji) and another deleting 166 amino acids following the stop transfer sequence proximal to the transmembrane domain (ΔA1113-H1278HA). These transgenes were expressed using mef2GAL4 to test the ability to rescue sns mutants. UAS-ΔA1113-H1278HA was entirely unable to rescue the myoblast fusion defect (Fig. 2.12A). This inability was not due to lack of stable expression or targeting to the membrane as these were comparable to wild-type (data not shown). In contrast, ΔL1279-V1482 was able to rescue the myoblast fusion defect in the rescue assay. All 30 myofibers were able to form in the absence of this region. Some muscles are occasionally wispy but fusion does occur to a large extent in these mutants (Fig. 2.12B). These results show that the region from A1113-H1278 contains absolutely essential sequences for SNS function.

Fig. 2.12: The region A1113-H1278 is critical for SNS function.
(A) sns[Zf1.4],UAS [Δ1113-1278HA]/sns[XB3]; mef2GAL4/+ and (B) sns[Zf1.4],UAS-[Δ1279-1482]/sns[XB3]; mef2GAL4/+. The former is unable to rescue the myoblast fusion defect whereas the latter restores muscle pattern.
2.3.6 Multiple functional regions are present in SNS cytodomain

To establish a minimal critical region within A1113-H1278, I designed 4 smaller deletions within this region (ΔA1113-I1163HA, ΔD1164-V1232HA, ΔY1233-Y1263HA and ΔY1263-H1278HA). For simplicity, these will simply be referred to as ΔA, ΔB, ΔC and ΔD respectively (Fig. 2.13). Surprisingly, of these 4 smaller deletions, none recapitulated the dramatic lack of rescue seen with ΔA1113-H1278HA. Of note, in the absence of Y1233-Y1263 (ΔC), the protein is compromised and several unfused myoblasts still remained at late stages after fusion was completed although all muscle fibers are formed. Quantitation showed these unfused myoblasts were significantly more numerous than rescue using UAS-snsHA (Graph for unfused myoblasts-Fig. 2.23). Unfused myoblasts were also observed with the other deletions (ΔA, ΔB and ΔD) but have not been quantitated and appear to be comparable to UAS-snsHA. Further, rescue using ΔC but not ΔA expressed using snsGAL4 at 18°C to lower the expression level showed obvious defects such as missing muscles. Quantitation of percentage of defective hemisegments showed this difference in ability to rescue was significant (Fig. 2.24). Such defects with ΔC are however not as severe as those observed with ΔA1113-H1278 indicating there are other functional sequences within (Fig. 2.24).
There are two possible explanations to why the smaller deletions were functional while ΔA1113-H1278HA was completely unable to rescue the myoblast fusion defect. One is that there are redundant functional groups in the cytodomain that need to be simultaneously mutated to show an obvious effect. The second possibility is that multiple functional groups exist in the cytodomain that individually have subtle effects and contribute additively to the function of the protein. When these are all removed, the

Fig. 2.13: Smaller deletions within ΔA1113-H1278 are able to rescue the myoblast fusion defect in sns mutants. (A) sns[Zf1.4], UAS [Δ1113-1163HA]/sns[XB3]; mef2GAL4/+, (B) sns[Zf1.4], UAS [Δ1164-1232HA]/sns[XB3]; mef2GAL4/+, (C) sns[Zf1.4], UAS [Δ1233-1263HA]/sns[XB3]; mef2GAL4/+, (D) sns[Zf1.4], UAS[Δ1263-1278HA]/sns[XB3]; mef2GAL4/+.

All 4 constructs restore the muscle pattern.
protein is appreciably compromised in function. Thus deletions were made in combinations and tested by the rescue assay. Note that the results with these constructs only allow us to identify a smaller functionally critical region than A1113-H1278 and do not help in distinguishing between the two above outlined possibilities.

ΔA1113-V1232HA combines the deletions ΔA and ΔB (Δ1113-1163HA+Δ1164-1232HA) and can restore muscle pattern although the muscle pattern is not entirely wild type with occasional missing muscles (Fig. 2.14A). The transgene UAS-ΔD1164-H1278HA combines individual deletions, ΔB, ΔC and ΔD (Δ1164-1232HA+Δ1233-1263HA+Δ1263-1278HA). This construct was also able to partially rescue the myoblast fusion defect in sns mutants (Fig. 2.14B). Although fusion occurs in the mutants and the muscle fibers are restored, many muscles are entirely missing or defective compared to wild-type. Increased numbers of unfused myoblasts in comparison to rescue with UAS-snsHA are also observed although these were not quantitated. Thus this deletion affects protein function without completely abolishing it.

A striking difference in rescue was obtained with ΔA1113-I1163+ΔY1233-H1278HA which combines the three deletions ΔA, ΔC and ΔD (Δ1113-1163HA+Δ1233-1263HA+Δ1263-1278HA). This deletion resulted in a loss of ability to rescue comparable to that with ΔA1113-H1278HA (Fig. 2.14C). Two independent transgenes gave comparable results indicating the level of expression is not responsible for this inability to rescue. Further, the protein expression was confirmed by western blot analysis (data not shown). Percentage of defective segments calculated from rescued embryos using this deletion and ΔA1113-H1278HA expressed using snsGAL4 at 18°C, showed a comparable lack of ability to rescue (Fig. 2.24).
The fact that the combination is more severe than the internal deletions, lends weight to the idea that multiple redundant or additive functional domains exist in this region. The redundancy could be between various serine, threonine or tyrosine residues that are evenly spread in this region. Alternatively, the lack of rescue may be due to the additive losses of these and/or the protein interaction motifs. Hence, I mutagenized putative signaling sequences to identify the critical residues within SNS cytodomain.

2.3.7 Protein:protein interaction motifs are critical for SNS function

SNS cytodomain contains consensus sequences for binding both SH3 and PDZ domain containing proteins. There are five consensus putative PDZ domain binding sites. All of these sites are conserved among the insect genomes. But the most striking site is at

---

**Fig. 2.14: Multiple functional domains exist in the SNS cytodomain.**

In order from left to right are combinations of ΔA+ΔB; ΔB+ΔC+ΔD; ΔA+ΔC+ΔD. (A) sns[Zf1.4], UAS [Δ1113-1232HA]/sns[XB3]; mef2GAL4/+, (B) sns[Zf1.4], UAS [Δ1114-1278HA]/sns[XB3]; mef2GAL4/+, (C) sns[Zf1.4], UAS [Δ1113-1163+Δ1233-1278HA]/sns[XB3]; mef2GAL4/+. Only the combination ΔA+ΔC+ΔD results in a complete lack of ability to rescue similar to that of Δ1113-1278.
the very C-terminus of the protein. The last 4 amino acids which make a hydrophobic pocket that can serve as a putative PDZ domain binding site is conserved throughout evolution even in the highly diverged mammalian orthologs of SNS (Fig. 2.8). However, ΔL1279-V1482 deletes this site but is able to rescue the myoblast fusion defect to a considerable extent (Fig. 2.12B). I reasoned that this may be indicative of functional redundancy between this and other internal putative PDZ binding sites. Mutagenizing a second internal highly conserved putative PDZ domain binding motif along with the C-terminal four residues did not affect the ability of the protein to function during myoblast fusion (Fig. 2.15A). To address further redundancy, all 5 putative PDZ binding sites were mutagenized simultaneously using conservative amino acid changes. Expression of this transgene was driven in embryonic musculature using both mef2GAL4 at 25°C and snsGAL4 at 18°C. In both these rescue conditions, 5xPDZ rescued the myoblast fusion defect of sns mutant embryos similar to full length sns cDNA (Fig. 2.15C and Fig. 2.16A-C). This shows that these sites are by themselves not absolutely required for SNS function. Even lowered expression of the protein with snsGAL4 at 18°C did not reveal any lowered ability to function during myoblast fusion. Hence, the mutations do not affect protein function appreciably.
Fig. 2.15: Putative SH3 domain binding sites in SNS cytodomain function during myoblast fusion.

(A) sns[Zf1.4], UAS [2xPDZ]/sns[XB3]; 24BGAL4/+; (B) sns[Zf1.4], UAS[1xPXXP]/sns[XB3]; twiGAL4/+; (C) sns[Zf1.4], UAS [5xPDZ]/sns[XB3]; mef2GAL4/+; (D) sns[Zf1.4], UAS[2xPXXP]/sns[XB3]; mef2GAL4/+ . Several unfused myoblasts are observed in the rescue with 2xPXXP but not with 5xPDZ. For quantitation see Fig. 2.23.
SNS cytodomain contains two conserved proline rich consensus PXXP putative SH3 binding motifs. Six proline residues in the first site were mutated to alanines (UAS-1xPXXP) and restored in the mesoderm of sns mutants using twiGAL4. The resulting rescued embryos showed comparable rescue of the myoblast fusion defect to full length sns cDNA control (Fig. 2.15B). Many unfused myoblasts were observed in the rescued embryos, but note that this rescue was performed using twiGAL4 and should be repeated using mef2GAL4 or snsGAL4 to be able to ascertain any compromised function.

To knock out both PXXP consensus sites and overcome any potential redundancy, a construct was generated mutating all 6 proline residues of the first proline and the two proline residues of the second proline rich regions to alanine residues (UAS-2xPXXP). The resultant transgene was expressed in the mesoderm of sns mutants with mef2GAL4 at 25°C. The rescued embryos showed the presence of all 30 intact muscle fibers in each hemisegment (Fig. 2.15D). However, large numbers of unfused myoblasts were observed in the focal planes beneath the musculature. Quantitation of unfused myoblasts at stage 16 when fusion should be completed showed an increase that was significant in comparison to UAS-snsHA (Fig. 2.23).

This suggested lowered efficiency of protein function for directing myoblast fusion. The effect was further enhanced by lowering the expression level using snsGAL4 at 18°C, whereby, the inefficiency of the transgene to restore muscle pattern became more apparent (Fig. 2.16D-F). Several muscles were missing and a quantitation revealed defects in ~70% of the hemisegments in comparison to rescues using UAS-snsHA (12% defective hemisegments) or UAS-5xPDZ (20% defective hemisegments; Fig. 2.24). Some
muscles had additional defects which appeared to be due to lack of proper attachments although this may be a result of incomplete fusion and needs to be investigated further. It is clear however that the consensus PXXP motifs in the SNS cytodomain are required for proper muscle morphogenesis, but this requirement can be compensated by increased expression of the protein.

Fig. 2.16: A requirement for putative SH3 domain binding sites in SNS cytodomain is obvious at lower expression levels.

Rescue embryos of sns[XB3], UAS [5xPDZ]/sns[Zf1.4]; snsGAL4/+ (A-C) and sns[Zf1.4], UAS [2xPXXP]/sns[Zf1.4]; snsGAL4/+ (D-F). Notice the muscle defects are especially obvious in D-F whereas the pattern is largely normal in A-C.
2.3.8 Investigated Serine residues are not essential for SNS function

SNS cytodomain has 35 serine and 33 threonine residues, of which potential signaling sites were selected using prediction programs (see section 2.3.4). We were unable to establish whether SNS was phosphorylated on serine or threonine residues using the available commercial antibodies (data not shown). The eight serine residues predicted to be highly likely to be phosphorylated by Netphos2.0 were first mutagenized to alanine residues (*UAS-S8-A8*). This transgene was capable of rescuing the myoblast fusion defect in *sns* mutants comparable to *UAS-snsHA* (Fig. 2.17A&B).

Combining the prediction results from Netphos2.0 server with two other prediction programs and sequence conservation, 17 highly conserved and likely to be phosphorylated serine residues were chosen and mutagenized to alanine residues simultaneously (*UAS-snsS17-A17HA*). When this mutant construct was targeted to the mesoderm of *sns* mutant embryos using either *mef2GAL4* at 25°C or *snsGAL4* at 18°C, wild-type muscle pattern was restored (Fig. 2.17C). With *mef2GAL4*, all 30 muscle fibers were present in each hemisegment and there was no difference in the numbers of unfused myoblasts compared to SNS-HA (Fig. 2.23).

Occasional defects were observed when *UAS-S17-A17HA* was expressed using *snsGAL4* at 18°C but these were not significantly different from those observed using *UAS-snsHA*. From two independent transgenes of S17-A17HA, there were 20% and 12% hemisegments with muscle defects respectively, compared to 12% hemisegments in *UAS-snsHA* rescued embryos under the same conditions (Fig. 2.24). From this data, I concluded that these serine residues are not absolutely essential for SNS function in
myogenesis. However, there are many unmutated serine residues still present in the cytodomain. Promiscuous phosphorylation or substitution for function by these sites cannot be eliminated and may result in rescue despite a requirement of the mutagenized sites.

Fig. 2.17: Highly conserved and likely phosphorylated serine residues are not essential.

(A) sns[Zf1.4],UAS-snsHA/sns[XB3]; mef2GAL4/+ (B) sns[Zf1.4],UAS [S8-A8]/sns[XB3]; mef2GAL4/+ (C) sns[Zf1.4],UAS [S17-A17HA]/sns[XB3]; mef2GAL4/+ D-F) sns[Zf1.4],UAS [S17-A17HA]/sns[Zf1.4]; snsGAL4/+ . Mutagenized serine residues do not affect the ability of the protein to restore muscle pattern in sns mutants.
2.3.9 SNS is phosphorylated on tyrosine residues

Prediction programs identify several of the tyrosine residues in SNS cytodomain as potential sites for phosphorylation. I employed biochemical methods to establish SNS phosphorylation using non-sequence-specific anti-phosphotyrosine antibody. SNS expressing construct was transiently transfected in S2 cells and immunoprecipitated with SNS antisera in the presence of sodium orthovanadate, a phosphatase inhibitor. Western blotting with anti-SNS and anti-phosphotyrosine antibodies identified single bands at the expected molecular size for SNS (Fig. 2.18A Lane 4).

This result was confirmed from embryonic musculature to ensure this is not a cell line specific phenomenon. C-terminally HA tagged SNS was expressed in the embryonic mesoderm using mef2GAL4. Immunoprecipitation using anti-HA coupled resin in the presence of orthovanadate followed by western blotting showed an immunoreactive band with anti-phosphotyrosine at the same size as on the anti-SNS blot (Fig. 2.18B Lane 1). Double labeling with anti-SNS and anti-phosphotyrosine antibodies, confirmed the bands are the same (data not shown).
To confirm the immunoreactivity is specific to phosphorylated residues, the immunoprecipitation was done in the absence of orthovanadate, the tyrosine specific-phosphatase inhibitor. This resulted in a loss of the phospho-tyrosine signal (Fig. 2.19 Lane1). Further, the immunoprecipitated protein was treated with lambda phosphatase (λ-PPase) and protein tyrosine phosphatase 1b (PTP-1b) separately. Under these treatments also, the phospho-tyrosine reactive band was completely lost indicating the observed phosphorylation is specific to tyrosine residues in SNS (Fig. 2.19 Lane 3 and 4).

Fig. 2.18: SNS is phosphorylated on tyrosine residues.
Immunoprecipitation of SNS from S2 cell lysates-comparison of untransfected and pRmHa3-sns transfected S2 cells (A) and embryonic mesoderm-comparison of mef2GAL4 and mef2GAL4/UAS-snsHA (B) shows a phosphotyrosine immunoreactive band (Lane 1).
To identify the specific phosphorylated residues in SNS, the tyrosine mutant constructs outlined in Fig. 2.10 were used. Mutagenizing the 6 candidate sites (Y6-F6HA) which scored very high probability using Netphos 2.0 server only reduced the phospho-tyrosine specific signal by 40% (Fig. 2.20 Lane 2). Since phosphorylation is not
completely abolished, these are either not the only sites to be phosphorylated or other sites are capable of phosphorylation in the absence of these preferred sites. A reduction of 80% in phospho-tyrosine signal intensity was observed by mutagenesis of 11 tyrosines (Y11-F11HA) (Fig. 2.20 Lane 3). Residual phosphorylation was still observed in ΔA1113-H1278HA, which removes 13 of the total 14 tyrosine residues in the SNS cytodomain, although this was not quantitated (Fig. 2.20 Lane5). Mutagenizing all 14 cytodomain tyrosines (Y14-F14HA) finally showed complete absence of phosphorylation (Fig. 2.20 Lane 4). This modification further supported our hypothesis of SNS function as a signaling molecule in addition to the cytodomain requirement.
2.3.10 Investigation of a requirement for ligand binding for SNS phosphorylation

In transiently transfected S2 cells, SNS is phosphorylated. This phosphorylation is not dependent on the presence of Duf/Kirre or IrreC/Rst expressing cells. Further, the intensity of the phospho-tyrosine signal did not change appreciably in the presence of Duf/Kirre expressing cells (Fig. 2.21A Lanes 2 & 3). This result has not however been
quantitated with proper controls for expression of SNS and extent of Duf/Kirre association.

To directly test a requirement for ligand binding for tyrosine phosphorylation in the embryonic mesoderm, I generated a construct lacking all 9 Ig domains of SNS. Since the heterotypic interaction with ligands is mediated by the extracellular domain (Galletta, Chakravarti et al. 2004), I reasoned that this construct should be incapable of ligand binding although it is targeted to the membrane in crude membrane preparations from lysed embryos (data not shown). However, immunoprecipitated protein only showed very weak immunoreactivity with phospho-tyrosine antibody as compared to full length protein (Fig. 2.21B Lanes 5&6). This trace phosphorylation suggests phosphorylation is not dependent on ligand binding. This suggests that ligand binding might enhance phosphorylation. However, since this construct has a large deletion, the structure may be affected in a way as to interfere with the phosphorylation. Hence, the two approaches both have their limitations. Since the results do not entirely agree, it is difficult to conclude at this point whether ligand binding regulates phosphorylation. Further, different residues may be phosphorylated in the presence of absence of ligand and either of the assays do not address this possibility.
2.3.11 Tyrosine residues are critical for SNS function

To study the functional requirement for tyrosine phosphorylation, I tested the different tyrosine mutant constructs for ability to rescue sns mutants. mef2GAL4 directed expression of UAS-Y6-F6HA, where the 6 highly predicted candidates for phosphorylation are mutated, restored wild-type muscle pattern (Fig. 2.22A). This result combined with the knowledge that phosphorylation is only reduced by 40% in this mutant, suggested that other phosphorylated residues may substitute or be sufficient to rescue. Targeted expression of UAS-Y11-F11HA using mef2GAL4 restored wild-type

Fig. 2.21: Role of ligand binding for SNS phosphorylation is undetermined.
(A) SNS is phosphorylated in S2 cells in the absence of association with Duf/Kirre expressing cells. (B) In the embryonic mesoderm, expression of a construct lacking all 9 Ig domains of SNS, shows very little immunoreactivity with phosphotyrosine antibody.
muscle pattern (Fig. 2.22 B). Since, this construct also does not lose all phosphorylation, I considered other unpredicted sites were capable of functioning in the absence of the mutagenized sites. In light of this, I focused on UAS-Y14-F14HA, which removes all phosphorylation.

When UAS-Y14-F14HA was expressed in the mesoderm using mef2GAL4, considerable rescue of the myoblast fusion defect in sns mutants was observed (Fig. 2.22 C). All 30 muscles were still formed similar to rescue using UAS-snsHA. However, the
muscles are wispy in comparison and there are several unfused myoblasts present even at late stages when fusion should be completed. Two independent transgenes were used and the number of unfused myoblasts quantitated and found to be significantly higher than the rescue with *UAS-snsHA* (Fig. 2.23). This increase is not due to a dominant effect as overexpression of this transgene in otherwise wild-type embryos did not reproduce this. The muscles were all normal and I did not observe an increased number of unfused myoblasts (data not shown).

![Fig. 2.23: Quantitation of unfused myoblasts in rescued embryos where expression is driven using mef2GAL4.](image)

The transgene names and sample size (n) are as indicated. Two transgenes were tested to confirm the result. Increased numbers of unfused myoblasts are observed in rescue using *UAS-Y14-F14HA, UAS-2xPXXP* and *UAS-Δ1233-1263HA* in comparison to *UAS-snsHA* and *UAS-S17-A17HA*. ** indicate the difference is statistically significant; P-values (Appendix B) were calculated by one-way ANOVA model combined with Tukey’s HSD tests. Error bars correspond to the standard errors of means of each group.
The unfused myoblasts indicated that fusion is not following through to completion and the protein function may be compromised although not severely. The reason for the normal muscle morphology may be due to higher level of expression from the strong \textit{mef2} promoter compensating for such compromised function. Hence, expression was driven using \textit{snsGAL4} at 18°C. Two independent transgenes of Y14-F14HA with \textit{snsGAL4} at 18°C were inefficient in rescuing \textit{sns} mutant myoblast fusion defect compared to full length SNS-HA (Fig. 2.22 D-F). Several missing muscles and much higher numbers of unfused myoblasts were observed although fusion was not completely abolished. To quantify this difference, the percentage of hemisegments with missing or defective muscles was calculated and found to be significantly higher in \textit{UAS-Y14-F14HA} (70%) compared to \textit{UAS-snsHA} (12%) (Fig. 2.24). Hence, the phosphorylation of the tyrosine residues in the cytodomain is significant to SNS function but can be compensated by increased expression of the mutagenized transgene. At this point it is unclear which tyrosine residues are essential as \textit{UAS-Y6-F6HA} or \textit{UAS-Y14-F14HA} have not been tested similarly by quantitation of unfused myoblasts or defects arising from tempering level of expression.
Fig. 2.24: Quantitation of rescue efficiencies as percent hemisegments with defects in muscle pattern in the lateral transverse muscles in rescued embryos.

Transgenes and sample size (n) are as indicated. Two transgenes were tested for some samples to confirm the result and the results have been presented together. Transgene expression was driven using snsGAL4 at 18ºC. n value indicates the number of total hemisegments analyzed. To determine whether each pairwise comparison was statistically significant at 0.05 level, all pairwise P-values (Appendix B) were calculated by one-way ANOVA model combined with Tukey’s HSD tests. ** indicate the difference is statistically significant.
2.4 Discussion

2.4.1 SNS directs intracellular events in the fusion competent myoblasts

Each somatic myofiber in *Drosophila* embryos is formed by fusion between two myoblast populations, the founder cells and fusion competent myoblasts. Differentially expressed IgSF proteins, SNS, Duf/Kirre and IrreC/Rst on the surfaces of the two myoblasts mediate recognition and adhesion (Bour, Chakravarti et al. 2000; Ruiz-Gomez, Coutts et al. 2000 and Strunkelnberg, Bonengel et al. 2001) and colocalize to the adhesive points (Galletta, Chakravarti et al. 2004). Following adhesion, intracellular events such as formation of F-actin rich foci and recruitment of fusion machinery occur beneath the apposed membranes (Kim S 2007; Doberstein, Fetter et al. 1997). Intracellular pathways directing such events depend on the cell adhesion molecules to ensure membrane breakdown specifically occurs at points of cell contact.

Duf/Kirre and IrreC/Rst function as attractants capable of directing myoblast migration to an ectopic site of expression (Ruiz-Gomez and Bate 1997; Strunkelnberg, Bonengel et al. 2001). SNS is required on the fusion competent myoblasts for such directional migration. An enticing possibility is that the SNS cytodomain contains sequences that direct intracellular events specifically necessary for this directional migration.

Consistent with this, a GPI anchored SNS, lacking the transmembrane and cytodomain sequences, which mediates heterotypic aggregation with Duf/Kirre cells did not rescue the myoblast fusion defect and myoblasts failed to make contact with founders in such rescued embryos (Galletta, Chakravarti et al. 2004). Thus, I undertook a thorough
study of the cytodomain to identify such functional sequences with the assumption that they will be critical for function and mutagenesis will reveal a functional requirement.

Interestingly, intracellular pathways leading to actin rearrangements, which may be required for cell motility, have been noted in both myoblast populations. Some components of this actin regulatory pathway, MBC and Rac1 also function in other migratory cell types (Kinchen, Cabello et al. 2005). In the founder cell, two such pathways exist downstream of Duf/Kirre leading to activation of small GTPases Rac1 and ARF6 (Chen and Olson 2001; Chen, Pryce et al. 2003). In the fusion competent myoblast, Sltr/D-WIP functions downstream of SNS and directs F-actin foci formation (Kim S 2007; Massarwa R 2007). It is suggested that this actin polymerization is required for the transport of electron dense vesicles which are associated with the fusion machinery (Kim S 2007). This is an interesting observation and links SNS to not just actin rearrangements for cell migration but potentially to recruitment of electron dense vesicles for cell fusion. Several other intracellular proteins are in place in fusion competent myoblasts and play essential roles during myoblast fusion. These raise the potential for multiple signaling sequences within the SNS cytodomain to signal independent processes downstream for myoblast migration and fusion.

2.4.2 SNS cytodomain contains multiple functional regions

I tested the requirement of the cytodomain by generating several deletional constructs. Deleting the region A1113-H1278 resulted in a complete loss of ability to function during myoblast fusion in sns mutants suggesting this region contains functional
sequences critical for SNS. ΔA1113-H1278 does not appear to affect expression, membrane localization or assembly into oligomers (data not shown) showing a clear functional requirement towards migration and/or fusion.

Further dissection of the A1113-H1278 region showed the presence of multiple functional domains. Individual deletions ΔA1113-I1163, ΔY1233-Y1263 and ΔY1263-H1278 restored the muscle pattern in sns mutants while the combination ΔA1113-I1163+Y1233-H1278 is entirely unable to rescue. Further, the latter deletion appears to recapitulate the dramatic lack of rescue with ΔA1113-H1278 and hence must contain most if not all of the functional sequences of this region.

Two possibilities can explain the effect of the combined deletion. One is that these functional sequences are redundant and hence only show an effect when all such sites are removed. The second possibility is that these individual domains are required for function but play subtle roles which have an additive effect by combinatorial loss in the larger deletion. The latter idea is supported by the fact that proline and tyrosine residues in the cytodomain (see below) are both required for SNS function but mutagenesis of either only results in compromised function and not total loss of it. Thus, multiple functional sequences may have subtle additive effects. Since this region is coated with all of the potential protein interaction sites and candidates for phosphorylation, it is further necessary to use a mutagenesis approach to identify specific functional sequences.
2.4.3 SNS requires protein interaction motifs in its cytodomain for function

By analogy to known pathways downstream of Duf/Kirre, SNS may also initiate multiple signaling events/pathways to regulate myoblast migration and/or fusion. The closely related IgSF proteins, Nephrin and Robo, among others, physically interact with multiple intracellular proteins from various sequences in their cytodomains. In particular two proline rich consensus putative SH3 domain binding motifs in the cytodomain of Robo are required for interaction with the SH3 domain of adaptor protein Dock for directional migration of neurons in response to extracellular signals (Fan, Labrador et al. 2003).

SNS contains two PXXP motifs which when mutated affect the ability to function during myoblast fusion (UAS-2xPXXP). It is thus enticing to think the SNS cytodomain is involved in interactions with intracellular SH3 domain containing proteins to direct myoblast fusion. From the data presented here, it is unclear which of the two PXXP motifs is the critical binding site or whether there is functional redundancy between the two. But some inferences can be made by the rescue result using ΔY1233-Y1263. This deletion removes the entire proline rich region housing the first PXXP motif in the SNS cytodomain. Rescue results with this construct show normal muscle morphology but several unfused myoblasts remain at stages when myoblast fusion should be complete and quantitation showed these were comparable to UAS-2xPXXP. Defects resulting from lowered expression of this deletion were also comparable to UAS-2xPXXP. Further, rescue using 1xPXXP expression directed by twiGAL4 results in normal muscle pattern but also with large numbers of unfused myoblasts which have not been quantitated and
compared. These data suggest the first proline rich region from P1235-P1243 is the functionally relevant binding site, although it needs to be directly tested by proper quantitation. This will indeed be interesting since this region is highly conserved compared to the second PXXP site.

Three SH3 domain containing proteins function during Drosophila myogenesis: MBC, D-Crk and CD2AP (Balagopalan, Chen et al. 2007; Galletta, Niu et al. 1999; Galletta 2004). SNS interacts with cytoplasmic adaptor D-Crk in Drosophila S2 cells although this interaction may involve the SH2 and/or SH3 domains of the latter (Kim S 2007). MBC requires its SH3 domain for function during myoblast fusion (Balagopalan, Chen et al. 2007). Loss of cd2ap results in disruption of somatic muscle fibers (Galletta 2004). Nephrin interacts with CD2AP to activate Akt signaling downstream in mouse kidney cells (Huber, Hartleben et al. 2003). Nephrin binds the SH3 domain in CD2AP and both Nephrin and CD2AP associate with the actin cytoskeleton (Yuan, Takeuchi et al. 2002). Nephrin has a conserved putative SH3 domain binding PXXP motif in its cytodomain, although a direct requirement for this motif in CD2AP binding has not been identified. SNS also contains two additional non-conventional proline rich sites not mutated in this study that are similar to identified CIN85 binding sites. Since CIN85 is the ortholog of CD2AP, it is likely that SNS interaction is mediated from these sites independently, in addition to the PXXP sites providing an explanation for the lowered efficiency rather than an absolute effect of mutagenesis of the latter.

SNS also contains consensus sequences for PDZ domain binding. IrreC/Rst binds the PDZ domain containing adaptor protein X11L/Dmint1 from its C terminus, an interaction conserved with its ortholog, Neph1 which binds PDZ domain containing ZO-1
SNS also contains an absolutely conserved C-terminal consensus putative PDZ binding sequence conserved in Nephrin. Nephrin is also suggested to interact with ZO-1 similar to Neph1. ZO-1 is present at the leading edge of cells suggesting a role in migration and adhesion (Huber, Schmidts et al. 2003). Hence we investigated if this sequence was critical for SNS function. Deletion of C-terminal 204 amino acids removing the highly conserved putative PDZ binding site or mutagenizing all candidate PDZ binding sites did not affect the ability to rescue myoblast fusion in mutants. Thus, these PDZ binding sites are not by themselves essential for SNS function during myoblast fusion. It will be interesting to investigate a role for these sequences in a function for SNS later in development such as in muscle attachment during larval stages or in other tissues.

2.4.4 SNS requires tyrosine residues in its cytodomain for function

Tyrosine phosphorylation is a key regulatory modification in cell surface receptors. Nephrin is phosphorylated on tyrosine residues by Src family kinase Fyn (Li, Lemay et al. 2004). This phosphorylation allows signaling to two downstream pathways: one signaling PI3K for Akt activation and the other directing actin rearrangements via Nck. Both the pathways are critical for Nephrin function in the assembly of the slit diaphragm which is the filtering apparatus in kidneys (Huber, Hartleben et al. 2003; Jones, Blasutig et al. 2006). I identified phosphorylation of SNS on tyrosine residues in its cytodomain.
UAS-Y14-F14HA showed a compromised function of the protein in the rescue assay which is exacerbated when level of expression is tempered. This may indicate a potential interaction which becomes weaker by mutagenesis but not completely lost. High level of expression can then compensate by increasing the numbers of such interactions and making up for the weak binding. This is indeed the case with Nephrin where Nck binding is enhanced by tyrosine phosphorylation but is not dependent on it (Jones, Blasutig et al. 2006). Specific phosphorylated residues in Nephrin cytodomain interact with the SH2 domain in Nck (Jones, Blasutig et al. 2006). Specific tyrosine residues in the guidance receptor PVR (PDGF and VEGF related receptor) are required for function in a cooperative manner (Bianco, Poukkula et al. 2007). Such specific requirements can be tested by various different combinations of tyrosine mutants of SNS. This may also identify whether the highly likely candidate sites for phosphorylation are indeed the critical regulators of SNS function.

SNS interacts with the SH2-SH3 domain containing cytoplasmic adaptor protein D-Crk in S2 cells (Kim S 2007). It is enticing to think this interaction is dependent on phospho-tyrosine residues and this possibility can be tested by checking for a lack of interaction with the mutagenized construct. Specific residues required for interaction can be determined by using the different mutated constructs. Although loss-of-function mutants are not available to ascertain a direct role of D-Crk during myogenesis, overexpression of a myristoylated form causes disruption of muscle pattern in wild-type embryos (Galletta, Niu et al. 1999). If D-Crk is indeed functioning upstream of MBC and D-Ced12 for Rac1 activation as in migrating vertebrate cells, then it is possible that sequestering D-Crk with a myristoylation tag results in overactivation of the pathway and
has an activated Rac1-like phenotype. SNS recruitment of D-Crk may signal Rac1 activation downstream and phosphorylation can serve as a regulatory switch to control this function.

Additionally, the interaction between SNS and D-Crk has been shown to direct the recruitment of Sltr/D-WIP (Kim S 2007). Sltr/DWIP has multiple functional implications such as regulation of F-actin foci at sites of cell contact and channeling of fusion related electron dense vesicles (Kim S 2007). These pathways make an exciting scenario whereby SNS cytodomain mediates migration and fusion related downstream events. These actin rearrangements may have different implications than the Rac1 activation pathway or the two pathways may function together. Genetic interactions between these different proteins and placement into pathways on the basis of physical interactions may shed further light.

The inefficiency to function during fusion for some of my transgenes was not appreciable when expressed at high levels in the rescue assay. They only became more appreciable when the level of expression was reduced. This is an important observation and suggests that the protein function is only compromised by mutagenesis of these sites and not completely abolished. It shows that these residues do not by themselves have an absolute requirement for protein function. Higher level of expression is able to overcome the compromised function due to mutagenesis. Hence, in performing such analyses, while it may be better to have high expression to be able to obtain the best rescue, sometimes, it may be better to reduce the levels to look for more subtle effects. A good assay system is critical for identifying such subtle requirements.
I conclude with the remark that this study has shed light on two functionally critical sequences in the SNS cytodomain. As expected from the complex functions of SNS, there are multiple sequence requirements. There appears to be the potential for redundancy between functional sites as well as an additive effect of multiple functional regions to determine various aspects of SNS function. The constructs generated in this study can further be used to determine direct interactions with intracellular proteins as well as requirements for recruitment of F-actin foci, FURMAS or fusion related machinery in fusing myoblasts in vivo.
Chapter 3

Dissection of physical and genetic interactions of SNS and Hbs

3.1 Introduction

The larval somatic musculature of Drosophila is established during embryonic development by a complex set of events. Each larval muscle is prefigured by a single founder cell, which seeds muscle formation by fusing with surrounding naïve fusion-competent myoblasts. The number of fusion events is variable (3-25), depending on the specific muscle (Bate 1993). The resulting mature muscle fibers are arranged in a repeated segmental array of 30 muscle fibers per hemisegment. Each muscle is distinguished by a distinctive set of properties including size, shape, orientation, innervation and epidermal attachment. These differences in the muscle fiber are accounted for by the specification of their precursors by a unique combination of regulatory molecules. A number of such genes have been identified that are expressed in distinct subsets of muscle fibers forming a combinatorial code. Mutants in these genes result in absence of specific muscle fibers, and conversely ectopic expression results in fate changes.

When fusion is blocked, the founder cells still make mono-nucleated mini-muscles that occupy the position of the myofiber and are correctly innervated and properly oriented showing all the genetic information of the myofiber is present in these cells. These muscles, however, are very thin and extended due to the absence of
contribution to shape and size by fusion competent myoblasts. The other population, fusion-competent myoblasts remain undifferentiated, although they can express general muscle-markers showing their committed fate as myoblasts (Rushton, Drysdale et al. 1995). Differentially expressed cell adhesion molecules in both the founder and fusion competent myoblasts mediate recognition of the cells prior to fusion. These are proteins of the Immunoglobulin superfamily (IgSF) and include Sticks-and-Stones (SNS), Dumbfounded/Kin-of-IrreC (Duf/Kirre) and Irregular Chiasm-C/Roughest (IrreC/Rst) (Bour, Chakravarti et al. 2000; Ruiz-Gomez, Coutts et al. 2000; Strunkelnberg, Bonengel et al. 2001) which have been discussed in detail in chapters I and II. A fourth member of this family is Hibris (Hbs), which is highly similar though not essential for myogenesis (Artero, Castanon et al. 2001; Dworak, Charles et al. 2001). In the introduction to this chapter, I will briefly review how a muscle acquires its particular identity and the sequential fusion events for its formation. I will then describe Hibris and its role in myogenesis outlining its similarities and differences with its paralog SNS.

3.1.1 Specification of distinct populations of myoblasts

In the Drosophila embryo, the mesoderm arises from the ventral region and subdivides into domains that will give rise to the various mesodermal tissues such as the somatic and visceral muscles, the fat body and the dorsal vessel. Initially, the cells in this region have relatively unrestricted developmental potentials (Beer, Technau et al. 1987). Mesodermal segmentation manifests by the generation of alternating domains of high and low expression of a transcription factor, Twist (Twi), along the antero-posterior axis; the
somatic muscles arising from within the region of high Twi expression (Borkowski, Brown et al. 1995; Baylies and Bate 1996). Once definitive positions are established by cell migration, the adjacent ectoderm induces specific mesodermal derivatives (Baker and Schubiger 1995). A large subset of somatic muscles depends on Wingless (Wg), an ectodermally derived Wnt family member (reviewed in Frasch 1999). In response to complex ectodermal signaling and responsive receptor tyrosine kinase activities, mesodermal cells switch on the proneural bHLH transcription factor encoding gene, *lethal of scute* (*l’sc*) (Carmena, Murugasu-Oei et al. 1998).

The mesoderm is subsequently divided into territories with small clusters of L’Sc expressing equipotent cells from which one cell is selected to become a muscle progenitor by lateral inhibition governed by Notch and its ligand Delta (Carmena, Bate et al. 1995). The fates of several muscle progenitors have been well studied. Progenitor cells divide asymmetrically to produce a pair of sibling cells with distinct fates, at least one of which becomes a muscle founder cell which seeds the formation of individual muscle fibers. The remaining cells in the L’Sc cluster become fusion competent myoblasts and have distinct properties from the founder cell. This is an essential step in the formation of somatic muscles, controlling the specification and segregation of these two distinct populations of myoblasts that will later fuse and form muscle fibers.

While the specification of founder cells is well studied, little is known about how the information and processes within a founder cell coordinate and determine specific muscle traits. An interesting observation providing the molecular basis for this next step is the specific combinations of signaling inputs that a given founder cell receives resulting in a unique set of molecular determinants called the founder “identity” genes.
These are aptly named as they are expressed in distinct patterns in different founder cells and appear to underlie the determination of its unique set of characteristics or “identity”.

These encode transcription factors and include *nautilus* (*nau*) (Keller, Grill et al. 1998), *S59* (Dohrmann, Azpiazu et al. 1990; Knirr, Azpiazu et al. 1999), *kruppel* (*kr*) (Ruiz-Gomez and Bate 1997), *even-skipped* (*eve*) (Frasch, Hoey et al. 1987), *apterous* (*ap*) (Bourgouin, Lundgren et al. 1992), *vestigial* (*vg*) (Zider, Paumard-Rigal et al. 1998; Williams, Bell et al. 1991), *ladybird* (Jagla, Bellard et al. 1998), *collier* (Crozatier and Vincent 1999) and *muscle segment homeobox* (*msh*) (Lord, Lin et al. 1995). These nine identity genes are expressed in different or sometimes overlapping patterns in different founder cells (Table 3.1). Loss and gain-of-function experiments on seven of these identity genes show a direct role in determining final muscle characteristics (Frasch 1999).

### Table 3.1: Combinatorial code of muscle founder identity genes in specific muscles.

<table>
<thead>
<tr>
<th>marker</th>
<th>muscles</th>
<th>other tissues</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Even-skipped</td>
<td>1</td>
<td>pericardial cell and CNS</td>
<td>Landgraf, etc. 1999; Frasch, etc. 1987</td>
</tr>
<tr>
<td>Knüppel</td>
<td>1 4 6 7 9 13 16 22 24 27 30</td>
<td>peripheral nerve</td>
<td>Rushton, etc. 1995</td>
</tr>
<tr>
<td>Vestigial</td>
<td>1 2 3 4 6 7 12 13</td>
<td></td>
<td>Keller, etc. 1998</td>
</tr>
<tr>
<td>Nautilus</td>
<td>3 4 5 9 10 11 16 26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S59/Slouch</td>
<td>5 11 18 26 28 29 29</td>
<td></td>
<td>Rushton, etc. 1995; Keller, etc. 1998</td>
</tr>
<tr>
<td>Apterous</td>
<td>21 22 23 24 27 29</td>
<td>dorsal neuroblast and longitudinal glialblasts</td>
<td></td>
</tr>
<tr>
<td>Msh</td>
<td>9 10 20</td>
<td></td>
<td>Nose, etc. 1998</td>
</tr>
<tr>
<td>Collier</td>
<td>3</td>
<td></td>
<td>Crozatier, etc. 1999</td>
</tr>
</tbody>
</table>

*marker* myosin heavy chain to mark somatic and visceral muscles
Three progenitors among the dorsal mesodermal cells divide to give rise to 3 different founders. The founder of the pericardial cells expresses the pair-rule gene, eve, and gives rise to two pericardial cells (Frasch, Hoey et al. 1987). The founder cell for dorsal muscle, dorsal acute 1 (DA1, muscle 1) expresses eve and zinc finger transcription factor encoding, kr (Ruiz-Gomez and Bate 1997) and the founder for dorsal muscle, dorsal oblique 1 (DO1, muscle 9) expresses kr, msh and nau (Frasch 1999). I will refer to the latter two as the DA1 and DO1 from here onwards. Consistent with their function as transcription factors, antibody staining shows tight localization of these markers to the nucleus in the founder cell initially. As fusion proceeds, the nuclei of the fusion competent myoblasts get incorporated into the growing muscle fiber and also start expressing these same markers in their nuclei. In this manner, as the muscle grows, the number of fusion events can be determined by counting the number of nuclei incorporated into the muscle by using these specific markers. This is also true for the other identity genes mentioned above and can be used to visualize distinct subsets of fibers. Table 3.1 shows the combination of muscle markers and the unique subset of muscle fibers marked by each. Note from here that EVE facilitates visualization of the single muscle DA1 while Kr marks DA1 and DO1 in the dorsal region along with five lateral and four ventral muscles.

As described above, each founder cell is unique in its properties. In contrast to this, the fusion competent myoblasts have been considered to be equivalent regardless of their position in the mesoderm and acquire the specific muscle fate upon fusion with a specific founder cell. A member of the Gli superfamily of transcription factors, Lameduck/Myoblasts incompetent/Gleeful (Lmd/Minc/Gfl) is essential for the
specification of all fusion competent myoblasts (Furlong, Andersen et al. 2001; Duan, Skeath et al. 2001; Ruiz-Gomez, Coutts et al. 2002). In mutants, sns transcript levels are drastically reduced throughout the embryonic mesoderm suggesting shortage of fusion competent myoblasts. The founder cells however remain normal and form mini-muscles (Duan, Skeath et al. 2001; Ruiz-Gomez, Coutts et al. 2002). A role for secreted protein Jelly Belly (Jeb) and the receptor tyrosine kinase Milliways/Anaplastic lymphoma kinase (Mili/Alk) in the differentiation of fusion competent myoblasts is also evident. In the absence of these proteins, the fusion competent myoblasts express lmd/minc/gfl but lack expression of sns suggesting that initial determination takes place, but the subsequent differentiation is blocked (Stute, Schimmelpfeng et al. 2004). Once the two myoblast populations are specified, they are attracted to each other and fuse to form multinucleate muscle fibers.

3.1.2 Two phase fusion process

In Drosophila, the establishment of muscle founder cells is a key step in determining the pattern of the larval somatic musculature required for locomotion of the larva (Bate 1993). Each seeds the formation of individual distinct muscle fibers, and to achieve the final size and shape of each such fiber, the founder cell will recruit cells from a neighboring pool of fusion competent myoblasts to fuse with it.

Fusion is not synchronous so that all fusion is not completed simultaneously. Not only do distinct founder cells fuse at different times during development, but also recruit fusing partners sequentially rather than simultaneously. They first fuse with one or two
fusion competent myoblasts to form an intermediate stage of bi- or tri-nucleated cells
called precursor cells in the first phase of myoblast fusion (Fig. 3.1). For the first phase, the founder cell employs fusion competent myoblasts from the layer directly beneath it in the mesoderm (Beckett and Baylies 2007). Like the muscle pioneers in grasshopper, precursor myotubes extend growth cones over the epidermis and contact attachment sites in it (Bate 1990). However, the former are a distinctive class of single cells, which in many cases enlarge and extend growth cones to attachment sites before fusion begins, but in the latter fusion occurs before and throughout the phase of growth and attachment (Bate 1990).

The precursors are also present in the outer layer of the mesoderm like the founder cell (Bate 1990; Beckett and Baylies 2007). In a second phase of fusion, the precursors recruit more fusion competent myoblasts from further internal mesodermal layers along their length for fusion to form the fully mature muscle fiber (Fig. 3.1; Beckett and Baylies 2007). The final muscle fiber has multiple nuclei incorporated from the fused myoblasts and may have 3-25 nuclei depending on its final size and the number of fusion events (Fig. 3.1). Recent experiments in mammalian cell culture also have shown that myoblast fusion takes place in two different rounds: first the nascent myotubes form, and then, additional myoblasts fuse to the nascent myotube (Horsley and Pavlath 2004).
These distinct phases for the formation of mature muscle fibers could require
distinct factors to regulate the differences. Mutations in different fusion genes do affect
the formation of the initial precursors or mature muscle fibers indicating distinct
requirements for the two phases (Rau, Buttgereit et al. 2001; Schroter, Lier et al. 2004;
Menon, Osman et al. 2005; Estrada, Maeland et al. 2007). The muscle identity genes
serve as excellent markers to determine these differences. By counting the number of
nuclei within a muscle, the number of fusion events undergone by that myotube can be

Fig. 3.1: Schematic of two phase fusion process with emphasis on the differences in
myofibers.

In the first phase of myoblast fusion, the mono-nucleated founder cell fuses with one to
two fusion competent myoblasts to form a di- or tri-nucleated precursor myofiber. This
precursor extends and fuses with more fusion competent myoblasts to form the fully
mature myofiber in the second phase of fusion. Each founder cell is unique and gives rise
to a myofibers with unique characteristics.
determined. In mutants where fusion is completely blocked such that first and second phases of fusion are both lost, only a single nucleus is present in each muscle fiber. By contrast mutants in those factors required only for progression past the precursor stage, precursor myotubes are observed with two to three nuclei although the second phase does not occur and mature muscle fibers are entirely lacking.

3.1.3 Hibris, a paralog of SNS, is a regulator of myogenesis

The locus hibris (hbs) encodes a cell adhesion molecule of the IgSF which is a paralog of SNS. Hbs was identified in two independent screens; one designed for identification of transcripts that were differentially expressed in founder cells versus fusion-competent cells (Artero, Castanon et al. 2001) and the second designed to find members of the IgSF in Drosophila by database searches (Dworak, Charles et al. 2001). Embryonic expression starts earlier than SNS but becomes restricted to fusion competent myoblasts by embryonic stage 12 although it is not present throughout the population (Artero, Castanon et al. 2001; Dworak, Charles et al. 2001). Co-staining with SNS and Hbs antibodies showed that Hbs was present in a subset of SNS expressing myoblasts. There is a subset of fusion competent myoblasts that only show SNS expression and lack Hbs (Artero, Castanon et al. 2001). When present together in the myoblasts, Hbs and SNS colocalize to discrete points on the cell surface (Artero, Castanon et al. 2001). Hbs is present at other sites of SNS expression such as in the eye imaginal discs (Kocherlakota and Abmayr unpublished; Dworak, Charles et al. 2001).
Hbs is predicted to have the same domain architecture as SNS with 9 Ig-like domains, a FN-III domain, a transmembrane spanning region and a cytoplasmic region (Fig. 3.2A; SMART webtool; Schultz, Milpetz et al. 1998). Though the domain architecture prediction of SNS and Hbs is identical, the cytodomain of Hbs is much smaller than SNS. Hbs is 48% identical and 63% similar to SNS. Like the latter, the cytodomain of Hbs contains potential protein-protein interaction motifs and phosphorylation sites that are conserved (Artero, Castanon et al. 2001). Prediction programs for identifying highly likely sites for phosphorylation identify several residues which are conserved in SNS cytodomain. Several common putative functional sites exist in the cytodomains of the two such as candidate sites for phosphorylation by Casein Kinase II (CKII) and Protein Kinase C (PKC) (Artero, Castanon et al. 2001) and proline rich consensus sites for Src Homology 3 domain (SH3) containing proteins. Some of these motifs are indeed critical for SNS function and potentially serve as interfaces for intracellular adaptor proteins (Chapter II). Hence SNS and Hbs cytodomains may function in common intracellular pathways in the fusion competent myoblasts.

Despite the high similarity between the sequences of Hbs and SNS, loss-of-function alleles suggested that *hbs* plays a very different role in myoblast fusion from *sns*. It is not embryonic lethal and *hbs* mutant embryos only exhibit a modest increase in the number of unfused myoblasts, and occasional missing or smaller muscles (Fig. 3.2C; Artero, Castanon et al. 2001, Dworak, Charles et al. 2001). Moreover, expression of Hbs in the developing mesoderm causes muscle loss and an increased number of unfused myoblasts. This effect is mediated by the cytodomain of Hbs, since expression of a truncated form lacking the entire extracellular domain but not a truncation of the
cytodomain mimics the overexpression phenotype (Artero, Castanon et al. 2001). In contrast, the *sns* loss-of-function phenotype is dramatic with complete absence of mature muscle fibers and overexpression of *sns* does not result in any muscle abnormalities (Bour, Chakravarti et al. 2000; Kocherlakota and Abmayr unpublished).

3.1.4 Biological properties of Hibris

Hbs and SNS are very similar in structure, sequence, expression and even ligand binding. However, the loss of function phenotypes of the two in somatic musculature are very different and they do not always appear to function in concert. There is only one orthologous sequence in vertebrates which encodes Nephrin, an essential mediator of cell

![Diagram](image)

Fig. 3.2: **Hbs domain architecture prediction and function during myoblast fusion.** (A) Schematic of Hbs predicted domain architecture using SMART webtool (B) and *hbs*2593/*hbs*459 mutant embryos. Dorsal is to the top and anterior is to the left of the stage 16, myosin-heavy-chain stained embryos.
adhesion at the slit diaphragm which constitutes the filtration apparatus in kidneys (Lenkkeri, Mannikko et al. 1999). No function has been established for Nephrin in myogenesis in vertebrates. The Malpighian tubules form the simple renal tubules of invertebrates and are composed of two cell types, principal and stellate. Hbs is expressed in the stellate cells of the malpighian tubules and controls the normal incorporation of stellate cells and the later physiological activity of the mature tubules (Artero, Castanon et al. 2001; Denholm, Sudarsan et al. 2003). Other myoblast fusion intracellular pathway proteins such as Ants/Rols, MBC, Rac1 also have a role in malpighian tubule morphogenesis and can be regulated by signaling from Hbs (Putz, Kesper et al. 2005). Hbs is unique in the mesectoderm and the CNS midline cells.

Like SNS, Hbs also interacts with the cell adhesion molecules Duf/Kirre and IrreC/Rst. In S2 cells, Hbs expressing cells fail to associate homotypically similar to the SNS expressing cells. Further, they do not associate in heterotypic aggregates with SNS expressing cells. They however, do form heterotypic aggregates with Duf/Kirre expressing cells. Hbs can further co-immunoprecipitate IrreC/Rst from heterotypic aggregates of transfected S2 cells. IrreC/Rst is not similarly co-immunoprecipitated in aggregates with SNS expressing cells (Bao and Cagan 2005). This interaction has been presented as a mode of action of Hbs and IrreC/Rst during eye development. Both hbs and irrec/rst mutants hence have similar rough-eye phenotypes where the alignment of individual units making up the Drosophila eye is lost. Misexpression of hbs but not sns changes IrreC/Rst localization in the cells of the eyes.

An antagonistic relationship between Hbs and SNS has been previously outlined by studying genetic interactions during myogenesis. The mild myoblast fusion defect of
hbs mutant embryos is dominantly suppressed by loss of one copy of sns. Conversely, sns mutations enhance the phenotype seen by overexpression of Hbs in the mesoderm (Artero, Castanon et al. 2001). These genetic interactions are also manifested in the visceral and cardiac mesoderm. The authors propose that SNS and Hbs could compete for the same extracellular ligand and hence antagonize one another. This is conceivable since Hbs expressing S2 cells indeed form heterotypic aggregates with Duf/Kirre expressing cells similar to SNS expressing cells. If this model were correct, the extracellular domain of Hbs mediating such interaction should contribute to the dominant effect and not the cytoplasmic domain. However, the latter was found to be the mediator of the dominant overexpression phenotype and hence, this possibility does not explain the genetic interaction. Another possibility presented by the authors is that Hbs and SNS may interact in cis to form a “negative” receptor. This co-receptor may respond differently to ligand than the preferred SNS receptor. This is likely as the two proteins colocalize into puncta on the surface of myoblasts. Lastly, the authors propose Hbs and SNS may converge on an intracellular downstream target that plays a role in regulating fusion. This possibility is also acceptable as the cytoldomain mediates the negative interaction and thus may be doing so by titrating one or more intracellular proteins from their preferred interaction at the membrane with SNS at adhesive contacts.

Since Duf/Kirre and IrreC/Rst belong to the same family of proteins as Hbs and SNS and implicated in myoblast fusion, a potential functional relationship with hbs may exist. Removing one copy of duf/kirre and irrec/rst in loss or gain-of-function backgrounds of hbs did not alter the phenotype of the latter. Hence, no genetic interaction was observed between hbs and duf/kirre and/or irrec/rst similar to that observed between
hbs and sns (Artero, Castanon et al. 2001). Thus the genetic interaction with SNS is specific and probably a result of high functional, structural and sequence similarity.

In this chapter, I present evidence for the two phases of myoblast fusion by identification of mutants blocking the process at the two different phases. SNS, which is required for mature myofiber formation is not required for precursor formation. This is due to substitution for the first phase by Hbs. Thus, the data presented here reveals a redundant relationship between SNS and Hbs at least for the first phase of fusion. SNS associates with itself in homo-oligomers and with Hbs in hetero-oligomers. Hbs is capable of functioning similarly to SNS as domain swaps of extracellular or intracellular domains of the two proteins still result in functional forms capable of rescuing myoblast fusion in sns mutants. The Hbs cytodomain undergoes tyrosine phosphorylation which is a modification essential for SNS cytodomain also and this provides further evidence to a function in common intracellular processes.
3.2 Materials and methods

3.2.1 Fly stocks

All the following mutants have been reported previously: \textit{sns[XB3]}, \textit{sns[Zf1.4]}, \textit{Df(2R)BB1} (Bour, Chakravarti et al. 2000), \textit{mbc[D11.2]} (Erickson, Galletta et al. 1997), \textit{lmd[1]} (Duan, Skeath et al. 2001), \textit{Df(1)w67k30} (Ruiz-Gomez, Coutts et al. 2000), \textit{irrec[UB883]} or \textit{irrec1} (Strunkelnberg, Bonengel et al. 2001), \textit{hbs[459]} and \textit{hbs[2593]}, \textit{UAS-hbs} (Artero, Castanon et al. 2001), \textit{mef2GAL4} has been used to drive expression of transgenes in the mesoderm (Ranganayakulu, Elliott et al. 1998). \textit{sns[s660]} was a gift from Elizabeth Chen (isolated in the screen described in Chen and Olson 2001). The molecular lesion was sequenced to be a single base change at nucleotide position 1547 changing amino acid Y333 to a stop codon, by MH Chen in our laboratory. \textit{UAS-snsHA}, and \textit{UAS-snsV5} are described in section 2.2.

3.2.2 Cloning and constructs

Standard procedures were used to construct the SNS-Hbs chimera. For SESTHC-HA (with SNS extracellular and transmembrane fused to Hbs cytodomain), the SNS TM and Hbs cytodomains were amplified using oligonucleotides that combine the two regions by a short region of overlap. This whole region was placed into \textit{pUAST-sns} using AflIII and XbaI. HESTSC-HA (with Hbs extracellular fused to SNS transmembrane and cytodomain) was made by amplification of SNS transmembrane and cytodomains with the 5’ end matching the last few nucleotides of the Hbs extracellular domain and the 3’ end with an HA tag and an XbaI restriction site for convenient cloning. This segment was
replaced into *pUAST-hbs* using BglII and XbaI. An HA tag was engineered in each of the 3’ oligonucleotides before the stop codon and the XbaI restriction sites. The entire length of the cloned cDNAs was sequenced.

### 3.2.3 Immunohistochemistry

Anti-EVE antibody (1:1000) was a generous gift from Dr. Manfred Frasch, Anti-Pericardin was purchased from Developmental Society Hybridoma Bank (1:10; DSHB) and Anti-Kr (1:300) antibody (originally reported in Kosman, Small et al. 1998) was obtained on request from the East Asian Distribution Center for Segmentation Antibodies at National Institute of Genetics, Division of Developmental Genetics (Mishima, JAPAN). Anti-beta-gal antibody (1:1000; Promega), anti-MHC monoclonal antibody (1:500; Dr. D Kiehart), Alexa 488, Alexa 546 and Alexa 660 labelled anti rabbit, anti-guinea pig and anti-mouse (1:200; Molecular Probes), biotinylated anti mouse and anti rabbit IgG (1:200; Vector Laboratories, Burlingame, CA), horseradish peroxidase conjugated anti-mouse (1:5000; Molecular Probes), anti-HA (Roche, Indianapolis, IN), anti-V5 (Invitrogen) and anti-FLAG (Sigma) were also used for this study. Embryos were collected and staged at 25°C according to standard procedures. After fixing, enzymatic reaction was used to select for mutants by lack of beta-galactosidase activity. Primary antisera were first preadsorbed on 0-2 hour WT embryos and incubated with samples overnight at 4°C followed by secondary for 2 hours at room temperature. Colorimetric detection was performed using Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA) using diaminobenzidine substrate.
3.2.4 Statistical analysis

Beta gal negative mutant embryos were stained with anti EVE and anti Kr followed by biotinylated or fluorescently tagged secondary antibodies. Images were collected on Zeiss Axioplan2 for colorimetrically stained and Zeiss LSM 510 Meta confocal microscope for fluorescently stained embryos at 40X (for EVE) or 100X magnification (for Kr). The nuclei were manually counted from individual hemisegments and the data analyzed by two different methods to confirm the significance of the difference in genotypes. One method was the simple average of data represented in graphical form as average ± standard deviation across the population (n=sample size, the number of hemisegments analyzed). The Student’s t test was used to determine significant differences between groups. In an alternate method, the percentages of hemisegments with multiple (>1) nuclei was calculated from total hemisegments analyzed (n). The following statistical analysis was done to ascertain the data is significant at a 95% confidence interval (Milton, 1992):

\[ L_1 = p - z\sqrt{p(1-p)/n} \] \[ L_2 = p + z\sqrt{p(1-p)/n} \]

where \( z = 1.96 \) for a 95% confidence interval, \( L_1 \) is the lower bound and \( L_2 \) is the upper bound, \( p \) is the proportion of segments with multiple nuclei, and \( n \) is the sample size. The error bars in the graphical representation are the limits obtained from this analysis.
3.2.5 S2 cell culture and transfection

S2 cells were grown as described (Cherbas and Cherbas 1998). Transient transfections were done using calcium phosphate (Ashburner 1989). Plasmids pUAST-snsV5, pUAST-snsHA, pUAST-snsFLAG and pUAST-hbs were cotransfected with pWAGAL4 (Ishimaru, Ueda et al. 2004), which expressed GAL4 in S2 cells from the Act5C promoter. Transfected cells were recovered by washing in PBS and allowed to grow for at least 24 hours to allow expression and association of the relevant proteins before proceeding with immunoprecipitation.

3.2.6 Immunoprecipitation and Western blotting

For lysis of S2 cells, transfected cells were resuspended in 250 µl lysis buffer (2% Triton X-100, 50 mM Tris–HCl pH 7.4, 300mM NaCl, 2 mM PMSF) and passed through a 25 g needle 10 times. For lysis of whole embryos, appropriately aged embryos were homogenized at a final concentration of 0.1mg embryos/ml in lysis buffer. Once lysed, the steps of immunoprecipitation are common for S2 cells and embryos: 2-4mg of total protein was mixed with 20µl relevant antibody coupled resin (Anti-V5-agarose (Invitrogen, Carlsbad, CA), anti-HAclone3F10-affinity matrix (Roche Applied Science, Indianapolis, IN) or anti-FLAG-M1-agarose (Sigma) as required) overnight at 4ºC. The beads were washed with lysis buffer and protein eluted by boiling in 40µl Laemmlli buffer. Co-immunoprecipitated proteins or phospho-tyrosine signals were detected in Western blots using ECL (Amersham, Piscataway, NJ) by scanning on a Typhoon 9400 (Amersham GE).
3.3 Results

3.3.1 Identity genes are a tool to study two phase myoblast fusion

To fully understand the requirements of essential genes during myoblast fusion, it is important to determine the specific phase at which fusion is blocked in mutants. EVE expression can be used to identify requirements of specific genes for the first versus second phase of myoblast fusion since it allows visualization of the nuclei in a single muscle fiber without interference from neighboring nuclei. Before starting this analysis with mutants, I wished to determine the pattern of EVE in wild type at various stages for comparison.

At the onset of myogenesis, EVE is present in two pericardial cells and a closely situated founder cell of DA1 per hemisegment (Fig. 3.3A: red circles and green box respectively). As development proceeds, more nuclei show EVE expression indicating that fusion is occurring and fusion competent myoblast nuclei are being added to the myofiber (Fig. 3.3 C). Initially, the nuclei are clustered but slowly spread out as the muscle elongates and stretches to span the territory of the future myofiber (Fig. 3.3 D; Rau, Buttgereit et al. 2001). An average of 11 nuclei are present in the DA1 muscle of wild type by the time fusion is completed although counting becomes difficult at later stages due to clumping and faint expression (Menon, Osman et al. 2005). At the end of myogenesis, EVE expression begins to go down and the nuclei become lighter. Thus, if fusion is proceeding normally, precursor formation occurs by stage 14 and mature muscle formation is complete by stage 16. My attention was first drawn towards differences in requirements for the first and second round of fusion by comparison of *sns* mutants with
embryos lacking *duf/kirre* and *irrec/rst* where I noticed multiple nuclei in the myofibers of the former while the latter myofibers were mono-nucleated.

---

Fig. 3.3: **EVE staining pattern in a wild-type embryo.**

(A) Onset of myogenesis, 2 EVE positive nuclei of the pericardial cells (red circles) and one nucleus of the founder of the DA1 muscle (green box) are seen. Through rounds of fusion, the nuclei increase in number (A-F). By stage 16, fusion is complete and the fiber is stretched and the multiple nuclei are situated at the ends of the fiber (green box in F). EVE staining starts to go down at the end of myogenesis.

---

### 3.3.2 SNS is not required for the first phase of myoblast fusion in DA1 and DO1

In *sns* mutants, adhesion of the fusion competent myoblasts to founder cells should be lacking and hence a complete block of fusion is expected. Using myosin-heavy chain antibody to mark myoblasts and myotubes, I observed that although mature muscles were never observed in these mutants, the stretched mini-muscles at the end of myogenesis were not mono-nucleated indicating some fusion could occur in the absence of *sns* (Fig. 3.4A) This was especially obvious in the dorsal region where only four total
muscles are present in each hemisegment and in the absence of fusion, only 4 nuclei (one per unfused founder cell) should be present. However, in each hemisegment, I observed multiple nuclei within the same myofiber indicating fusion was occurring to some extent (Fig. 3.4A).

I followed DA1 muscle using EVE in sns[Zf1.4] mutants. This allele was characterized to have a nonsense mutation at amino acid R356. At stage 13 by the onset of myoblast fusion, there are two pericardial cells and one founder nucleus per hemisegment. Stage 15 onwards, more nuclei appeared to express EVE in the region of the dorsal muscle. Fusion did not progress past two or three times as seen by 3-4 nuclei in the mutants (Fig. 3.4B). The staining continued to remain strong in the pericardial and the founder cells.
Fig. 3.4: **Precursor formation occurs in DA1 in sns mutants.**

(A) homozygous *sns[s660]* embryo stained for myosin-heavy chain. Arrows mark the dorsal myofibers with multiple nuclei within a single membrane. Homozygous mutant embryos of *sns[Zf1.4]* and (B), *sns[s660]* (C) stained for EVE. Homozygous mutant embryo of *sns[XB3]* (D) stained for EVE and pericardin, a pericardial cell marker. All *sns* mutants show multiple EVE positive nuclei in DA1.

One possibility for this result is that the *sns[Zf1.4]* allele is an incomplete stop which allows read-through during translation to make small amounts of full length protein. This can account for partial fusion but lack of fully mature muscles and explain how in the seeming absence of SNS and hence adhesion of myoblasts, fusion is still able to proceed. To test this possibility, I used two other alleles of *sns, sns[s660]* with a nonsense mutation at Y333 and *sns[XB3]*, a splice site change leading to read-through of intron 11 changing the protein sequence (Fig. 3.4 C and D). I also tested a deficiency (*Df(2R)BB1*) that removes the gene entirely. EVE expression in all these mutants shows
that small amounts of fusion can occur in the absence of SNS and is represented graphically in Fig. 3.5.

![Graph showing average EVE positive founder nuclei per hemisegment](chart)

**Fig. 3.5: Comparison of average EVE positive nuclei in DA1 muscle per hemisegment.**

Comparison of different *sns* alleles and a deficiency removing the entire genomic region of *sns*. All show multiple nuclei in DA1 indicating a lack of requirement for SNS in the first phase of myoblast fusion.

An analysis of the formation of the EVE positive precursor myofiber in *Df(2R)BB1* is shown in Fig. 3.6. At early stages, the muscle founder is identified as mono-nucleated (Fig. 3.6A: green box and red circles respectively) but by stage 15, 2-3 nuclei are seen at the place where the dorsal muscle should form (Fig. 3.6D). Occasional mono-nucleated DA1 founder cells were observed (Fig. 3.6F). However, 80% hemisegments in *Df(2R)BB1* mutants contained multiple nuclei. Taken together, these
results show that SNS is not required for the first phase of fusion to form precursors in the DA1 muscle.

Fig. 3.6: Progression of DA1 muscle through myoblast fusion in homozygous Df(2R)BB1 mutants.

EVE staining shows two pericardial and one founder nuclei for DA1 at early stages (red circles and green box in A). At later stages, fusion progresses to precursor myotube stage but not further (B-F). At the end of fusion, DA1 is di- or tri-nucleated (green box in F).

To test if this event may be a general mechanism that occurs during muscle formation or is specific to the DA1 muscle, I visualized an additional muscle DO1, which is conveniently marked by Kr staining. Kr is expressed in the founders of both DA1 and DO1, hence I needed to stain for EVE and Kr together to be able to distinguish and count the nuclei of the latter muscle specifically. By completion of myoblast fusion, when multiple EVE nuclei are seen in DA1, multiple nuclei are also observed in all hemisegments in DO1 (Fig. 3.7). Thus, fusion is able to occur in the absence of SNS in two distinct muscle fibers.
3.3.3 Genes required for first phase of myoblast fusion in DA1

As mentioned earlier, other IgSF proteins are required for muscle development and it is of interest to see at which phase they are required. In mutants lacking *duf/kirre* and *irrec/rst (Df(1)w67k30)*, there is a single founder expressing EVE at the onset of...
myogenesis (Fig. 3.8A). In contrast to sns mutants, fusion does not proceed in this case and the founder cells continue to be mono-nucleated (Fig. 3.8F).

Previously, in mbc mutants, it was established that the founder remains mono-nucleated in loss of function mutants (Rushton, Drysdale et al. 1995; Erickson, Galletta et al. 1997). I analyzed mbc[D11.2] mutants using EVE staining. A single founder nucleus was observed in each hemisegment (Fig. 3.9A) which remained mono-nucleated throughout myogenesis (Fig. 3.9F; Schroter, Lier et al. 2004). As the founder cell elongates, it becomes thin and the founder nuclei move away from the pericardial cells. Thus, mbc expression is required for fusion past the founder cell stage.

**Fig. 3.8: Precursor formation does not occur in DA1 in Df(1)w67k30 mutants.**

EVE staining in embryos carrying the deficiency Df(1)w67k30 lacking both duf/kirre and irrec/rst expression. The founder cell for DA1 starts off (green box in A) and remains (green box in F) mono-nucleated during the stages when myoblast fusion is occurring in wild-type embryos.
The average number of EVE positive DA1 nuclei in the above mutants and embryos carrying the deficiency $Df(2R)BB1$ lacking $sns$ expression were compared (Fig. 3.10A). Occasionally, mono-nucleated founders were seen in $sns$ and di-nucleated precursors observed in $mbc$ mutants. In $sns$ mutants, 80% of the hemisegments as opposed to 2% and 10% in mutants lacking $duf/kirre$ and $irrec/rst$ and $mbc$ mutants respectively contained multiple EVE positive nuclei within a precursor myotube (Fig. 3.10B). Thus, a requirement for $mbc$ and $duf/kirre$ and $irrec/rst$ is clear for the first phase of myoblast fusion, while $sns$ is only required for the second phase.

**Fig. 3.9:** Precursor formation does not occur in DA1 in $mbc[D11.2]$ mutants.

EVE staining in homozygous mutants shows the founder cell for DA1 starts off (green box in A) and remains (green box in F) mono-nucleated during the stages when myoblast fusion is occurring in wild-type embryos.
Fig. 3.10: Quantitation of differences of requirement for first and second phase of fusion.

Comparison of A) EVE positive nuclei in DA1 per hemisegment and B) percentage of hemisegments with multiple EVE positive nuclei. Both methods show that SNS is required only for second phase of myoblast fusion while MBC and Duf/Kirre and IrreC/Rst are essential for the first phase. * indicates statistical significance.
3.3.4 First phase of myoblast fusion requires fusion competent myoblasts

One possible explanation for the observation that myoblasts in sns mutants can get past the mono-nucleated founder cell stage in certain muscles can be that this fusion is spurious and occurs between two neighboring founder cells. In sns mutants, the association of Duf/Kirre into discrete puncta on the surface of neighboring founder cells is observed (Menon, Osman et al. 2005). This association is lost in the presence of SNS, presumably due to the preferred interaction between Duf/Kirre and SNS compared to Duf/Kirre with itself. To address this possibility of spurious founder fusion events resulting in precursor formation in sns mutants, I looked at the number of EVE nuclei in loss-of-function mutants of lmd/minc/gfl (Duan, Skeath et al. 2001; Furlong, Andersen et al. 2001; Ruiz-Gomez, Coutts et al. 2002). Anti-SNS staining in these mutants shows lack of fusion competent myoblast specification, and thus the majority of the fusion competent myoblast population is lacking in the mesoderm (Duan, Skeath et al. 2001; Ruiz-Gomez, Coutts et al. 2002).

At embryonic stage 13 in lmd/minc/gfl mutants, when myoblast specification is completed, there are two EVE positive pericardial cells and one founder for the DA1 (Fig. 3.11A). This shows that founder specification is unaffected in these mutants as has been reported previously. Occasionally, a founder nucleus could not be determined in the DA1 muscle. This may be due to faint staining or displacement to such an extent as to make the distinction between pericardial and founder nuclei difficult. At later stages of development when fusion is progressing in wild type and sns mutants, the founder cell remains mono-nucleated in lmd/minc/gfl mutants (Fig. 3.11D-F). The founder does not
undergo any fusion and no precursor formation is observed in a total of 87 hemisegments under analysis. Average nuclei per hemisegment in the DA1 muscle were counted and found to be 0.9±0.27. These data show that precursor formation is between founder cell and fusion competent myoblasts and not due to spurious founder-founder fusion events. I hypothesized that in the absence of SNS, another IgSF protein is able to substitute and hence allow precursor formation. I discuss these results in the next sections.

3.3.5 IrreC/Rst does not substitute for SNS during the first phase of myoblast fusion

IrreC/Rst, although redundant with Duf/Kirre, differs from the latter in being expressed on the surface of fusion competent myoblasts in addition to founder cells. Its structure is not entirely similar to SNS but it has a similar domain architecture prediction and associates with Duf/Kirre in heterotypic aggregates. Due to these reasons, I tested if
IrreC/Rst was able to substitute for SNS in precursor formation. The later phases of fusion requiring myoblast migration prior to adhesion, may be dependent on the unique longer cytoplasmic tail of SNS and interactions thereof. I generated double mutants of \textit{irrec[1]} with \textit{sns[s660]} and followed precursor myotube formation by EVE positive staining. At embryonic stages 15 and 16, when fusion is expected to have proceeded past precursor formation, both genotypes showed multiple EVE positive nuclei (Fig. 3.12A&B). Comparison of average EVE positive nuclei between the two showed no significant difference (Fig. 3.12C). Further, a comparison of percentage of hemisegments with multiple nuclei also showed no significant difference (Fig. 3.12D). Thus, precursor formation is not affected by removal of both SNS and IrreC/Rst in the fusion competent myoblasts. IrreC/Rst is not redundant with SNS and cannot substitute for it in the first phase of myoblast fusion.
Fig. 3.12: IrreC/Rst does not substitute for SNS in precursor formation.

EVE staining of homozygous mutants of sns[s660]/sns[s660] (A) and irrec/Y1; sns[s660]/sns[s660] (B). (C) shows a comparison of average EVE positive founder nuclei per hemisegment while (D) shows a comparison of percent hemisegments with multiple EVE nuclei for the DA1 muscle in both genotypes. All analyses show that there is no difference in DA1 in single and double mutants indicating IrreC/Rst does not substitute for SNS for precursor formation. An average of 3.8±nuclei for the DA1 founder were observed in irrec1; sns[s660] mutants compared to 3.2± in sns mutants alone.
3.3.6 Hbs substitutes for SNS in the first phase of myoblast fusion in DA1 and DO1 muscles

Hbris is highly similar to SNS in sequence and predicted domain architecture. It is expressed in atleast a subset of fusion competent myoblasts and colocalizes with SNS at the cell membrane in discrete punctae (Artero, Castanon et al. 2001), which signifies an association during myoblast fusion. Hbs also forms heterotypic aggregates with Duf/Kirre in S2 cells and can hence associate with cues from the founder cell. For these reasons, Hbs made an excellent candidate to substitute for SNS during the first phase of myoblast fusion. To test this possibility, I analyzed the EVE positive founder nuclei in recombinants of sns and hbs. Interestingly, homozygous embryos of sns[XB3],hbs[2593] showed an absence of precursor formation in most hemisegments (Fig. 3.13 C&D; Menon, Osman et al. 2005) compared to sns[XB3] where di- or tri-nucleated DA1 was observed (Fig. 3.13 A&B). The difference in average EVE positive founder nuclei were significantly different between the two genotypes and are graphically represented (Fig. 3.14A). Occasionally, I saw multiple EVE positive nuclei in DA1. To confirm the validity of the counts for average nuclei, I also determined the percentage of hemisegments with multiple EVE nuclei as a percentage and found that there was a significant difference (Fig. 3.14B).
Fig. 3.13: **Hbs substitutes for SNS in precursor formation in DA1.**

Anti-EVE (red) and anti-Pericardin (green) staining of homozygous mutants of sns[XB3] (A&B) and sns[XB3],hbs[2593] (C&D). There are multiple EVE nuclei in DA1 of sns[XB3] (A&B) but largely only single nuclei within DA1 of double mutants (red nuclei only in C&D). An average of $1.112 \pm 0.16$ EVE positive DA1 nuclei compared to $2.03 \pm 0.313$ in sns[XB3] was observed.
Fig. 3.14: Quantitation to show Hbs substitutes for SNS in the first phase of fusion in DA1.

A shows a comparison of average EVE positive nuclei per hemisegment while B shows a comparison of percent hemisegments with multiple EVE nuclei for the DA1 muscle in both genotypes. The differences are significant by calculation of P value (0.0001) for A and confidence intervals at 95% probability for B indicated as error bars. Both data show that in precursor formation for DA1 is absent in double mutants. While >80% hemisegments in the single sns mutants had multiple EVE positive nuclei in DA1, only 10% hemisegments displayed multiple nuclei. * indicates statistical significance.
This suggests Hbs substitutes for SNS during the first round of myoblast fusion in DA1. I wished to confirm the result in an independent muscle fiber to ensure this substitution is not specific to the DA1 muscle. I used Kr to label DO1 in sns\(\text{XB3}\) and sns\(\text{XB3},\ hbs[2593]\) mutants and found mono-nucleated DO1 in the latter alone (Fig. 3.15 C&D). The difference in the average DO1 nuclei in the single and double mutants was significant (Fig. 3.16A). Once again, I calculated the percentage of hemisegments with multiple Kr nuclei in the single versus double mutants and found a significant decrease from 91% to 33% (Fig. 3.16B). Thus, substitution by Hbs is consistent in the two dorsal muscle fibers tested.

Fig. 3.15: Hbs substitutes for SNS in precursor formation in DO1.
Anti-EVE (blue) and anti-Kr (green) and anti-MHC (red) staining of homozygous mutants of sns\(\text{XB3}\) (A&B) and sns\(\text{XB3},\ hbs[2593]\) (C&D). There are multiple Kr nuclei in DA1 and DO1 of sns\(\text{XB3}\) (A&B) but largely only single nuclei within DA1 and DO1 of double mutants (green nuclei only in C&D).
Fig. 3.16: Quantitation to show Hbs substitutes for SNS in the first phase of fusion in DO1.

A shows a comparison of average Kr positive nuclei per hemisegment for DO1 muscle while B shows a comparison of percent hemisegments with multiple Kr nuclei for the DO1 muscle in both genotypes. Compared to average DO1 nuclei of 2.27±0.39 in sns mutants, the double mutants had 1.33±0.26 nuclei. The differences are significant by calculation of P value (0.0001) for A and confidence intervals at 95% probability for B indicated as error bars. Both data show that in precursor formation for DO1 is absent in double mutants.
3.3.7 SNS and Hbs associate physically into hetero-oligomers

In fusion competent myoblasts expressing both SNS and Hbs, the two proteins colocalize into discrete punctae at the cell surface (Artero, Castanon et al. 2001). Due to the known genetic interaction between the two proteins, I tested for a physical interaction. I cotransfected constructs expressing \textit{UAS-hbs} and \textit{UAS-sns\textsc{FLAG}} in S2 cells, driven by a GAL4 vector. SNS-\textsc{FLAG} was efficiently immunoprecipitated using anti-\textsc{FLAG} antibody coupled resin from SNS-\textsc{FLAG} in transfections alone and cotransfection with Hbs (Fig. 3.17A Lanes 1&3). Hbs was not immunoprecipitated from extracts made from Hbs single transfections (Fig. 3.17A Lane 1). However, Hbs was co-immunoprecipitated in extracts from cotransfected cells expressing both SNS and Hbs (Fig. 3.17A Lane 3).

To confirm this association occurs in the embryonic mesoderm, I recombined \textit{UAS-hbs} and \textit{UAS-sns\textsc{V5}} and expressed them in the mesoderm of otherwise wild-type embryos using \textit{mef2GAL4}. Using anti-V5 coupled resin, SNS-\textsc{V5} was efficiently immunoprecipitated (Fig. 3.17B Lanes 2&4). Hbs, however, was only immunoprecipitated in lysates from embryos expressing both Hbs and SNS-\textsc{V5} but not from Hbs alone expressing lysates (Fig. 3.17B, Lane 3&4). Thus, SNS and Hbs are closely associated in an immunoprecipitable protein complex in S2 cells and the embryonic mesoderm. This finding does not eliminate that additional proteins are contained in the immunoprecipitable complex and required for association. This interaction is potentially in cis due to the expression of the proteins in the same cell types and a lack of reports of any heterotypic associations, although these results cannot eliminate an interaction in trans.
Fig. 3.17: Hbs and SNS associate into oligomers.
A) S2 cells were transiently transfected with single or cotransfections of SNS-FLAG and Hbs expressed using pWAGAL4. Lysates were prepared and immunoprecipitated with the anti-FLAG affinity matrix. B) Flies carrying transgenes UAS-sns-V5 and UAS-Hbs were recombined and induced to express protein using mef2GAL4. Lysates were prepared from 8-15 hr embryos and immunoprecipitated with the anti-V5 affinity matrix. Anti-Hbs western blotting of the eluates in each case show Hbs is co-immunoprecipitated with SNS (lane 3 in A and lane 4 in B) but not in its absence (lane 2 in A and lane 3 in B).
3.3.8 SNS associates into homo-oligomers

In wild-type embryos, SNS localizes at points of contact in discrete foci or puncta between a growing myofiber and adherent fusion competent myoblasts. This localization is lost in the absence of Duf/Kirre and IrreC/Rst and SNS becomes distributed throughout the myoblast membrane (Galletta, Chakravarti et al. 2004). However, punctae are still observed in these myoblasts although these are now spread around the membrane instead of localized to a single point of contact. In cell adhesion molecules such as Nephrin and C-cadherins, cis interactions have been proposed as a mandatory step for subsequent trans interactions (Gerke, Huber et al. 2003; Brieher, Yap et al. 1996). Thus, to test whether SNS is associated into homo-oligomers in cis, I cotransfected differently tagged forms of SNS in S2 cells. Using anti-HA coupled resin, SNS-HA was efficiently immunoprecipitated from single transfections and cotransfections with SNS-V5 (Fig. 3.18A Lanes 1&3) though SNS-V5 is only present in immunoprecipitations from SNS-HA cotransfected samples (Fig. 3.18A Lanes 2&3). A similar result was also obtained from cotransfection of SNS-FLAG and SNS-HA (Data not shown).

To confirm this association occurs in the embryonic mesoderm, I expressed both UAS-snsHA and UAS-snsV5 together in the mesoderm of otherwise wild-type embryos using mef2GAL4. SNS-V5 was efficiently immunoprecipitated from samples expressing UAS-snsV5 alone or co-expressing UAS-snsV5 and UAS-snsHA (Fig. 3.18B Lanes 2&4). SNS-HA was only co-immunoprecipitated from embryos expressing both SNS-HA and SNS-V5 and not from cells expressing SNS-HA alone (Fig. 3.18B Lanes 3&4). Thus, SNS associates in homo-oligomers potentially in cis as a trans interaction has not been
established in S2 cells and the embryonic mesoderm. As in the case with hetero-oligomers, it is undetermined if these complexes contain other proteins that are required for the association.

Fig. 3.18: SNS associates into homo-oligomers.

A. S2 cells were transiently transfected with SNSHA and SNSV5 alone or cotransfected using pWAGAL4 for expression. Lysates were prepared and immunoprecipitated with the anti-HA affinity matrix. Anti-V5 western blotting of the eluate shows SNS-V5 is co-immunoprecipitated with the SNS-HA (lane 3). B. Flies carrying transgenes SNS-HA and SNS-V5 were recombined and induced to express protein using mef2GAL4. Western analysis of immunoprecipitates shows SNS-HA is co-immunoprecipitated with SNS-V5 and not by itself (Lanes 3&4).
3.3.9 Hbs does not rescue the myoblast fusion defects in sns mutants

In section 3.3.6, I showed that Hbs serves a redundant function with SNS during the first phase of myoblast fusion and substitutes for the function of the latter. In sns mutants, mature myofibers are not present, indicating that Hbs cannot substitute for SNS during the second phase of fusion. One possible explanation is that endogenous Hbs expression is insufficient to substitute for the second phase. Hbs is not present in all SNS expressing cells and further the levels may be low in those cells expressing Hbs such that it is unable to accomplish sufficient interactions as required for fusion. The second phase of fusion may require steps such as myoblast migration that depend on high levels of protein or strong interactions thereof lacking in Hbs. The first phase may require only adhesion between neighboring myoblasts to proceed to fusion.

I addressed the possibility that low levels of Hbs or lack of uniform Hbs in all fusion competent myoblasts results in the lack of substitution during the second phase. Hbs was overexpressed pan-mesodermally in sns mutant embryos to test for rescue of myoblast fusion. Driving expression of UAS-hbs using mef2GAL4 in sns mutants did not completely rescue the second phase of myoblast fusion. Myosin heavy chain staining however showed more fusion in the rescued embryos compared to sns mutants (Fig. 3.19 compare B &C) although mature muscles are unable to form. This suggests that Hbs can marginally rescue the second phase of myoblast fusion in sns mutants when overexpressed in all myoblasts, but still lacks the ability to take fusion to completion and form fully developed myofibers. Thus, the inability of Hbs to rescue the second phase is not simply due to lack of expression in all fusion competent myoblasts or the low levels
of Hbs in these cells. Another explanation for the observation that Hbs is not completely redundant with SNS and can only provide for a part of its true requirements could be due to differences in functional domains of SNS and Hbs. This is tested in the next section.

Fig. 3.19: Hbs is inefficient during later rounds of myoblast fusion.
Anti-MHC staining of A-A”) sns[XB3]/sns[Zf1.4], UAS-snsHA; mef2GAL4/+ B-B”) sns[XB3]/sns[Zf1.4], UAS-hbs; mef2GAL4/+ and C-C”) sns[s660] are presented for a comparison of extents of fusion. SNS-HA as expected rescues the myoblast fusion defect well and restores muscle pattern. Hbs on the other hand is unable to rescue the fusion defect completely. However, compared to sns mutants alone, there is more fusion in these rescued embryos (Compare B&B’ with C&C’).
3.3.10 Functional analysis of Hbs extracellular and cytodomains

Hbs substitutes for SNS function during the first phase but cannot substitute during the second phase of fusion even when expressed at high levels in all myoblasts coincident with myoblast fusion. Further, endogenous Hbs fails to substitute for SNS in the migration assay described in section 2.3.1. This could explain substitution during the first phase in the fusion competent myoblasts directly beneath the founder cell that do not require to migrate for adhesion but not in later rounds where the myoblasts are in layers further below and need to migrate prior to adhesion (Beckett and Baylies 2007). This could be due to specific associations of the SNS cytodomain to direct cytoskeletal changes required for myoblast migration which are lacking in the Hbs cytodomain. Alternatively, Hbs may be less efficient than SNS in mediating strong adhesion of the fusion competent myoblasts with the founder cell. Thus, the Hbs and SNS domains may have some common functional groups but Hbs is unable to perform all the same functions suggesting a difference in functional capabilities of the different domains of the two proteins. To test this, I generated chimeric constructs by swapping domains between the two proteins.

Hbs and SNS are highly similar in their extracellular domains (41% identity). Both bind ligand in heterotypic association when expressed on S2 cells (Dworak, Charles et al. 2001; Galletta, Chakravarti et al. 2004). To compare the potential of SNS and Hbs extracellular domains in vivo, I tested a chimera with Hbs extracellular and SNS transmembrane and cytodomain (UAS-HESTSC-HA) sequences for its ability to rescue the myoblast fusion defect in sns mutant embryos. Directing expression using mef2GAL4
rescued myoblast fusion to a large extent and definitely past precursor myotube formation in *sns* mutants (Fig. 3.20 A-A”). Unfused myoblasts were still present and occasionally muscles were missing in rescued embryos, indicating fusion was not following through to completion or that function was compromised. This construct is however more efficient in rescue of *sns* mutant embryos when compared to *UAS-hbs*. This suggests that the extracellular domain of Hbs is functionally comparable to that of SNS and the inability of full length Hbs to rescue *sns* mutants is not simply due to the differences in the extracellular domains.

While there are many conserved sequences, SNS and Hbs cytodomains share only 15% identity owing largely to the difference in sizes. The SNS cytodomain contains unique sequences as it is longer than the Hbs cytodomain with 200 residues outside of the region of extensive homology. The difference in SNS and Hbs could be due to differences in the cytodomains such that the latter is incapable of directing intracellular events for migration and/or fusion similar to SNS. To test this possibility, I generated a chimera of SNS extracellular and transmembrane domains and Hbs cytodomain (SESTHC-HA) and tested its ability to rescue the myoblast fusion defect in *sns* mutants. When expression was directed using *mef2GAL4*, the construct was able to largely rescue the myoblast fusion defect past precursor stage (Fig. 3.20 B-B”). There were however many unfused myoblasts and occasional missing muscles. The rescue was much better in comparison to *UAS-hbs* although the muscle pattern is not entirely wild-type. Hence, the reason for the lack of sufficient rescue with *UAS-hbs* is not simply due to differences in the cytodomain of Hbs and SNS. This suggests the cytodomain of Hbs is able to stimulate
the same signaling events required downstream of SNS in the fusion competent
myoblasts as evidenced from sequence requirements outlined in chapter II.

Fig. 3.20: Hbs:SNS chimera are functional during myoblast fusion.
Schematics and anti-MHC staining of rescues using SNS and Hbs chimeras. A-A")
sns[XB3]/sns[Zf1.4], UAS-HESTSC-HA; mef2GAL4/+ and B-B") sns[XB3]/ sns[Zf1.4],
UAS-SESTHC-HA; mef2GAL4/. Both chimera restore muscle pattern in mutants better
than UAS-hbs (compare to Fig. 3.19) although occasional defects are observed.
3.3.11 Hbs is phosphorylated on tyrosine residues

In the previous section, I observed that Hbs and SNS cytodomains can function in the fusion competent myoblast to direct myoblast fusion. Hbs cytodomain also contains protein:protein interaction motifs and phosphorylation sites that are conserved in SNS. In

Fig. 3.21: Hbs is phosphorylated on tyrosine residues.

*mef2GAL4* was used to express *UAS-snsHA* or *UAS-hbsHA* pan-mesodermally in embryonic stages. Immunoprecipitation with anti-HA from these and GAL4 control samples shows a phosphotyrosine immunoreactive band for SNS and Hbs (lanes 2&4) but not for GAL4 alone (Lane 1).
chapter II, I showed that SNS cytodomain undergoes tyrosine phosphorylation and these residues are critical for its function. I tested the possibility that Hbs is also similarly phosphorylated as it is able to substitute for SNS cytodomain function by performing the same analysis as outlined in section 2.3.10. *UAS-hbs-HA* was expressed pan-mesodermally using *mef2GAL4*. The protein was immunoprecipitated using anti-HA coupled resin and western blotting with anti-phospho-tyrosine antibody showed a band at the size expected for Hbs (Fig. 3.21 Lane4) not observed from control *mef2GAL4* embryos (Fig. 3.21 Lane1). Hbs is also phosphorylated on tyrosine residues.
3.4 Discussion

3.4.12 Mature myofibers form by two phase fusion with intermediate precursor myotube stage

*Drosophila* somatic mesoderm consists of two types of committed myoblast populations: the founder cells and the fusion competent myoblasts which fuse with each other to form fully developed mature muscles. 30 such fibers are present for each hemisegment and are specified by 30 individual founder cells. Each of these founder cells is characterized by the expression of specific identity genes which encode transcription factors localized to the nucleus such as *eve* and *kr* (Carmena, Gisselbrecht et al. 1998; Ruiz-Gomez and Bate 1997). As fusion competent myoblasts fuse with these founder cells, they adopt the fate of the growing myotube and their nuclei also stain for the founder cell markers.

This makes it convenient to discern the number of fusion events a growing syncitia has undergone by counting the number of nuclei within that myotube. Fusion occurs in 2 phases with the first resulting from a couple of rounds of fusion to form a bi- or tri-nucleated precursor myotube in the first phase of fusion. The precursor extends and spans the territory of the future muscle and recruits more fusion competent myoblasts in several rounds to form the mature myotube in a second phase of fusion. These steps have been visualized using the identity genes for counting nuclei within a myotube and different fusion mutants have been characterized recently for their requirements at these distinct phases (Rau, Buttgereit et al. 2001; Schroter, Lier et al. 2004; Menon, Osman et al. 2005; Schafer, Weber et al. 2007). I performed the same analysis on *sns* and *mbc* mutants and a deficiency that removes *duf/kirre* and *irrec/rst.*
In embryos lacking *duf/kirre* and *irrec/rst*, the founder cell of the DA1 marked with EVE, remained mono-nucleated indicating precursor myotubes could not form and fusion was completely blocked. However, in *sns* mutants, EVE staining showed multiple nuclei were observed within the DA1 muscle although these were fewer compared to wild-type (Menon, Osman et al. 2005). Allele specific differences were observed in different *sns* alleles tested but the result from the deficiency, *Df(2R)BB1*, removing *sns* locus, showed unambiguously that fusion was able to proceed to precursor myotube formation in the absence of *sns*. The lack of requirement for SNS during precursor formation was further confirmed in the DO1 muscle.

This 2 phase fusion occurs universally in ventral, lateral and dorsal muscles (Beckett and Baylies 2007). Whether all precursor myotube formation proceeds in the absence of SNS is not confirmed. Subsets of fusion competent myoblasts, such as those in the dorsal region may be independent of a requirement for SNS during the first phase of fusion, but this may not be true for all fusion competent myoblasts. Other markers specific for lateral and ventral muscles need to be used to resolve this. A difference in the requirement for SNS during the first and second phase of fusion suggested the presence of a second molecule in such subsets of myoblasts that can substitute for SNS function and was directly tested.
3.4.13 Hbs is a regulator of myogenesis and substitutes for SNS during first phase of myoblast fusion

Fusion to form precursors does not proceed in the absence of fusion competent myoblasts by spurious fusion between two adjacent founder cells. Precursor myotube further requires intracellular signaling molecules downstream of IgSF proteins such as *mbc*, *loner* and *blow* (this chapter, (Schroter, Lier et al. 2004; Beckett and Baylies 2007). MBC functions downstream of Duf/Kirre in the founder cell, to direct cytoskeletal changes required for myoblast fusion and is also required in the fusion competent myoblasts (Chen and Olson 2001; Balagopalan, Chen et al. 2007). One explanation is that in the absence of SNS another IgSF protein is able to regulate the same intracellular pathways.

IrreC/Rst and Hbs are two such candidate cell adhesion molecules with known roles during myogenesis and with expression in fusion competent myoblasts. Both also interact heterotypically with Duf/Kirre and hence can mediate association of fusion competent myoblasts with founder cells (Dworak, Charles et al. 2001; Galletta, Chakravarti et al. 2004). In *sns, hbs* double mutants, the number of EVE and Kr nuclei decreased and were comparable to that observed in *mbc* mutants (this chapter; Menon, Osman et al. 2005). Such a difference was not observed in *irrec/rst; sns* double mutants in DA1. Hbs serves a redundant function with SNS during the first phase of myoblast fusion. The slower progression through the first phase in *sns* mutants may be due to the inefficiency of Hbs function which is consistent with other observations presented here.
3.4.14 The Hbs extracellular region and cytodomain function similar to those of SNS

Hbs substitutes for SNS during the first phase of myoblast fusion but is unable to substitute during later rounds of fusion to form mature myofibers. SNS is required to receive the attractant signal from Duf/Kirre and potentially directs the migration of fusion competent myoblasts toward the founder cell (section 2.3.1). Though Hbs is present in the sns mutant embryos, it does not mediate this migration. One possible explanation is that Hbs expression is inadequate. Even when overexpressed pan-mesodermally, Hbs is still unable to mediate fusion through multiple rounds to make fully mature myofibers, negating this possibility.

Alternatively, Hbs sequences may be unable to mediate the same interactions as SNS. I tested this possibility by generating chimera where regions of SNS were swapped with those of Hbs to check if this results in loss of or inefficient SNS function during myoblast fusion. Hbs mediates heterotypic aggregation in S2 cells with Duf/Kirre expressing cells like SNS (Dworak, Charles et al. 2001; Galletta, Chakravarti et al. 2004). However, the strength of this interaction in vivo is unknown. The chimeric construct HESTSC-HA shows the two extracellular domains can function similarly and the Hbs extracellular domain when substituted for that of SNS is still able to function during myoblast fusion and restore muscle fibers in sns mutants more efficiently than UAS-hbs. Further, this suggests that the lack of fusion competent myoblast migration in sns mutants to ectopic site of ligand expression is not due to lack of establishment of stable adhesive contacts.
Fusion competent myoblasts were recently shown to be not present in an entirely uniform monolayer. Some that lie directly beneath the founder fuse immediately with it while others present in lower layers presumably undergo cell migration hence fuse later (Beckett and Baylies 2007). Thus the idea that in the absence of SNS, Hbs can mediate fusion in the nearby fusion competent myoblasts but cannot direct migration of fusion in the farther cells appeared justified. This possibility was tested by the chimera swapping the Hbs cytodomain for that of SNS. Full length \textit{sns} cDNA rescues \textit{sns} mutants to restore wild-type muscle pattern. Specific deletions in the cytodomain result in lack of this ability to rescue. Interestingly, the chimera SESTHC-HA is able to function during myoblast fusion in \textit{sns} mutants to restore the muscle pattern albeit with few defects. The rescued embryos definitely progress past the rounds of fusion seen in rescue with \textit{UAS-hbs}.

Thus, the Hbs cytodomain is able to recapitulate the necessary interactions with intracellular molecules or pathways in the fusion competent myoblasts in the absence of SNS cytodomain. Consistent with this idea, Hbs is phosphorylated on tyrosine residues, which is a critical modification of the SNS cytodomain. This is also in agreement with the argument presented by Artero that the Hbs cytodomain is able to titrate downstream molecules of the SNS signaling pathway. However, the negative role of Hbs cytodomains observed by Artero is not observed here. It may have resulted from the nature of the truncation (Artero, Castanon et al. 2001). Further, a chimera of SNS extracellular domain with Hbs transmembrane and cytodomains does not have a dominant effect (Data not shown). The ability of SESTHC-HA to function during myoblast fusion presents the
questions why Hbs is unable to function in the myoblast migration assay in sns mutants when endogenously present.

These data also bring up another question. If both the extracellular and intracellular domains of Hbs in chimera are capable of functioning better than the full length, what makes Hbs inefficient in rescuing the myoblast fusion defect when present endogenously or overexpressed? One explanation is that the chimera are more efficient due to the presence of SNS sequences such that a stronger interacting SNS extracellular domain can make the weaker interactions of Hbs cytodomain function better or vice versa. Of note, several cell adhesion molecules of the IgSF associate with the cytoskeleton, and their adhesive strength critically depends on this interaction (Kamiguchi and Lemmon 2000; Gumbiner 2000). The cytodomain of SNS is not required for stabilization of adhesive contacts in S2 cells (Galletta, Chakravarti et al. 2004), but this may be essential in the embryonic mesoderm. In such a situation, the SNS cytodomain sequences may be better than Hbs cytodomain and the presence of the former stabilizes the adhesive contacts of the Hbs extracellular domain in the chimera making the latter more efficient.

As a corollary, ligand binding is shown to enhance cytoskeletal interactions of cell surface receptors. In Nephrin, ligand binding regulates association with the cytoskeleton (Verma, Kovari et al. 2006; Jones, Blasutig et al. 2006). Thus, in the chimera where only the Hbs cytodomain is switched for the SNS cytodomain, strong ligand binding by the SNS extracellular domain may enhance the interactions even from the weaker Hbs cytodomain hence making it function more efficiently. Such strong interactions are lacking in full length Hbs, since both domains of Hbs are slightly less
efficient in function and hence the intact protein itself can only provide for a few rounds of fusion but not completing the process. Note that both the chimera function less efficiently than full length sns cDNA indicating the sequences are indeed less efficient. Thus, one idea emerges that SNS and Hbs can both function similarly but SNS sequences mediate robust and more efficient interactions compared to Hbs sequences.

3.4.15 SNS associates into oligomers

SNS and Hbs colocalize at discrete points on the cell surface (Artero, Castanon et al. 2001). This could be directed by ligand binding or oligomerization and this differentiation awaits determination of mutants lacking one or the other association. Orthologous proteins in vertebrates, Neph1 and Nephrin can be co-immunoprecipitated when expressed on the same cell, suggesting a cis interaction (Barletta, Kovari et al. 2003). SNS and Hbs expressing cells do not form homotypic aggregates and hence their association is presumed to be in cis.

In addition, antiparallel trans interaction of dimers on opposing cells are suggested to require prior cis homodimerization or heterodimerization of Neph1 and Nephrin (Gerke, Huber et al. 2003). Such cis homodimerization is a mandatory step for subsequent trans interactions in C-cadherin-mediated cell contacts also (Brieher, Yap et al. 1996). Thus, SNS association into homo- or hetero-oligomers in cis may concentrate the protein to discrete points.

It is interesting to note that the Ig cell adhesion molecule Nectin utilizes different Ig domains for different functions. While the N-terminal Ig domain is essential for trans-
dimer formation, cis homodimerization, an essential step for subsequent trans homodimerization, is mediated by the second Ig domain of Nectin (Momose, Honda et al. 2002). Some extracellular Ig domains of SNS are absolutely essential while others are less critical (Kocherlakota, Hwang and Abmayr unpublished). Thus, differences in requirements for interactions in cis and trans may exist for these Ig domains and deletions can be tested for requirement of specific Ig domains for interactions in cis and trans by biochemical analyses.

3.4.16 SNS and Hbs are functionally related in the musculature

Dosage sensitive genetic interaction suggesting a functional relationship during myogenesis has been reported between SNS and Hbs (Artero, Castanon et al. 2001). The two appear to function in the same pathway during myoblast fusion potentially by regulating the same pathway components and indeed being redundant during the first phase of myoblast fusion. Overexpression of \textit{UAS-hbs} causes defects in the musculature. These are dominantly enhanced by removal of one copy of \textit{sns} leading the authors to suggest an antagonistic relationship (Artero, Castanon et al. 2001). These conclusions do not correlate with the data presented here which suggest Hbs is functioning partially redundantly with SNS and is capable of mediating the same processes only less efficiently.

The authors presented three possibilities to explain their suggestion of an antagonistic relationship between SNS and Hbs from the genetic interaction data. One possibility was that overexpression of Hbs titrates ligand by interaction with Duf/Kirre
on the founder cell and making it less available for SNS. If this were true, the overexpression of the extracellular domain alone should recapitulate the Hbs overexpression result. This was not the case and the authors found the cytodomain to be responsible for the dominant effect.

Another possibility enumerated by the authors is that Hbs and SNS may combine to form a “negative” receptor. In this scenario the Hbs/SNS coreceptor may respond differently to ligand than the “positive” SNS receptor. This possibility is supported by the observation of SNS:Hbs hetero-oligomers. However, I do not agree with the idea that the hetero-oligomer functions negatively. I do agree that the homo- and hetero-oligomers function differently and the latter are less efficient and hence unfused myoblasts and occasional missing muscles are observed when the hetero-oligomer is the predominant functional unit during myoblast fusion.

The physical interaction between SNS and Hbs can also be used to explain the genetic interaction data. Overexpression of UAS-hbs may result in higher numbers of less efficient Hbs:SNS hetero-oligomers or Hbs homo-oligomers (data not shown). This results in a decreased efficiency of ligand binding and/or cytoplasmic interactions thus leading to less efficient signaling and incomplete myoblast fusion. The removal of one copy of sns in these embryos further increases such less productive oligomers and hence enhances the phenotype. Such an argument is in agreement with the results of both Artero and those presented here and may be a better fit for the interaction and its implications.

Lastly, the authors propose Hbs and SNS may converge on an intracellular downstream target that plays a role in regulating fusion. This agrees with their data that the cytodomain in fact mediates the dominant effect of Hbs overexpression. It also fits
with our observation that the cytodomain of Hbs is able to function similarly to the SNS
cytodomain in a chimera during myoblast fusion. Such an interaction from the
overexpression of Hbs may result in titration of cytoplasmic components of migration
and/or fusion related pathway in less efficient Hbs cytodomain associations. These cannot
strictly be viewed as a negative interaction as they result in mediating the same events as
the SNS cytodomain albeit less efficiently and hence there is incomplete fusion. Our data
present a new and alternate view to the relationship of SNS and Hbs during myogenesis.
Chapter 4
Summary and perspectives

The process of cell fusion is central to several processes such as bone, muscle and placenta formation, fertilization, and viral-host cell entry. Recently, advances in stem cell biology have made it possible to reprogram adult somatic cells to attain any fate by fusion with embryonic stem cells (Cowan, Atienza et al. 2005). Cell fusion can also be used as a method of cell mediated gene therapy where healthy cells are fused to diseased or incapacitated cells to enable expression of critical factors for tissue repair (Blau, Dhawan et al. 1993). Cell-cell fusion is a fascinating process as it requires coordination of both inter- and intra-cellular events. In our laboratory, we study the process of myoblast fusion using the simple genetic tool, Drosophila melanogaster.

4.1 Molecular aspects of SNS function

In Drosophila, body wall muscles required for locomotion are formed by fusion between founder and fusion competent myoblasts. Myoblast fusion proceeds through distinct steps such as cell-cell recognition, actin cytoskeletal rearrangements, migration, adhesion, membrane alignment, recruitment of fusion related machinery and fusion. Although several key molecules have been identified, the mechanism of fusion has not been completely understood yet, suggesting aspects and pathways that need to be yet unraveled (reviewed in Abmayr 2005).
The studies so far have shown several interesting properties of the process. There are several similarities as well as differences between the properties and processes within the two populations of myoblasts. Differentially expressed cell surface and intracellular proteins have been identified as essential for myoblast fusion. SNS and Duf/Kirre form a ligand-receptor pair providing the basis for the directionality of fusion. These proteins mediate intracellular events in either myoblast population for fusion to proceed (Chen and Olson 2001; Chen, Pryce et al. 2003; Kim S 2007).

Surprisingly, some intracellular events and key molecules appear to be common in the two myoblasts. Formation of F-actin foci which potentially direct myoblast cell shape change and migration are coincident in the two myoblasts prior to fusion. On the surface, these actin rearrangement events appear to be the same. However, these may serve different functions and it will be interesting to dissect such differences. For example, in the fusion competent myoblasts, these may regulate migration while in the founder cells, these may regulate shape change for extension and attachment to specific sites in the ectoderm.

Intracellular pathways in the two myoblasts also show similarities and differences. Some proteins such as MBC, Kette and Rac1 are common to both populations while others such as Ants/Rols and Loner are only present in the founder cell and still others such as Blow and Sltr/D-WIP are present only in fusion competent myoblasts (Balagopalan, Chen et al. 2007; Chen and Olson 2001; Chen, Pryce et al. 2003; Luo, Liao et al. 1994; Kim S 2007). It is enticing to think the common proteins in the two cells may be involved in recruitment of fusion machinery which has to occur on both sides of the juxtaposed membranes in the myoblasts. The unique subset of
intracellular proteins, on the other hand, may mediate separate events such as migration in the fusion competent myoblast and cell shape change in the founder cell. Such considerations make the cytodomains of the cell surface proteins very interesting and a thorough understanding of the interactions thereof is critical to understanding these differences.

I show in Chapter II that the SNS cytodomain has multiple functional sequences. It requires consensus PXXP putative SH3 binding sites and tyrosine residues in its cytodomain for function during myoblast fusion. The latter sequences undergo phosphorylation in the embryonic mesoderm and this may function as an important molecular switch to regulate downstream events. These critical sequences may regulate interactions with intracellular proteins to regulate the events during myoblast fusion. The results indicate that SNS is capable of interactions with SH3, SH2 and PTB domains in intracellular proteins for signaling. Identification of such interactions will reveal pathways downstream of SNS similar to those identified for Duf/Kirre signaling.

It has been shown recently that SNS indeed interacts with D-Crk, an SH2-SH3 domain containing adaptor, and recruits Sltr/D-WIP in cultured cells (Kim S 2007). D-Crk interaction may be dependent on PXXP in SNS via its SH3 domain or tyrosines via its SH2 domain. These differences can be tested using the mutagenized constructs to determine nature of interaction. It is further enticing to imagine a situation where neither mutation completely abolishes D-Crk binding but both function in an additive manner for efficient interaction. This is consistent with the two mutagenized transgenes being less efficient at rescue but not completely losing ability to rescue the myoblast fusion defect.
The recruitment of Sltr/D-WIP has interesting implications for SNS function. Sltr/D-WIP regulates F-actin foci formation which may regulate cell migration. The actin rearrangements have additionally been implicated in trafficking of fusion related machinery suggesting a role for SNS in their recruitment. Other intracellular proteins may also be similarly recruited downstream of SNS. Such changes in subcellular localization of proteins have been studied using antibodies and structures such as FURMAS have been described which are dependent on SNS (Kesper et al 2007). It will be interesting to observe any changes in these subcellular localizations in response to mutations in the SNS cytodomain.

A mass spectrometric analysis of affinity purifications of SNS alongside the mutations and deletions generated can help identify new interactions with intracellular pathway components. Further, a tandem purification of SNS using SNS and phosphotyrosine antibodies compared to affinity purifications of tyrosine mutant transgenes of SNS will reveal interactions that are dependent on the tyrosine phosphorylation. Protein binding may be enhanced or decreased in the presence of phosphorylation. The former has been observed for Nck interaction with Nephrin where phosphorylation of specific residues in the cytodomain of Nephrin result in enhanced Nck binding (Jones, Blasutig et al. 2006). This interaction is critical in signaling actin reorganization within the cell.

Since mutagenesis of tyrosine and proline residues only compromises protein function, it is likely that interacting partners of these sites may also only have mild phenotypic effect. Such molecules may have escaped identification in genetic screens and
can be identified by such a biochemical approach looking for interaction partners downstream of SNS.

4.2 Relationship between SNS and Hbs

In chapter III, I showed that the first round of fusion occurs even in the absence of SNS. Hbs, a closely related molecule substitutes for this function. However, Hbs is only partially redundant with SNS and does not substitute for SNS completely during myoblast fusion indicating a complex relationship. I addressed a few possibilities for such partial redundancy. One was that Hbs expression is insufficient and that Hbs may only be able to substitute for SNS in those fusion competent myoblasts where the expression overlaps. Pan-mesodermal overexpression also showed that Hbs was inadequate during the second phase of fusion. Another possibility was that Hbs sequences were less efficient at interactions. This could be the case as the chimera I generated for the study were less efficient than SNS in ability to rescue myoblast fusion in sns mutants. The chimera also showed the individual Hbs domains function similarly to SNS and not antagonistically as previously suggested.

The results raised another question. If IrreC/Rst does not substitute for SNS, what is the implication of its presence in fusion competent myoblasts. Do these different and overlapping patterns of SNS, IrreC/Rst and Hbs divide the fusion competent myoblasts into distinct subsets which may differ in their fusion capabilities? Do these proteins control and regulate the fusion potentials of these distinct fusion competent myoblasts?
I also observed a physical interaction between SNS with itself and SNS and Hbs. What is the role of oligomerization? It may serve to increase local protein concentration for strong adhesive contacts. It may also be a pre-requisite for trans interaction with Duf/Kirre. Determining the domain(s) of SNS which mediates such associations and generating mutants thereof is required for such differentiation. This will also help determine if the domain requirements differ for homo- and hetero-oligomer formation?

An interesting idea regarding SNS oligomerization comes from studies in Nephrin. Oligomerized Nephrin associates into lipid rafts although it is unknown if oligomerization is a required for lipid raft association (Simons, Schwarz et al. 2001). A large number of signaling events have been shown to occur in the lipid raft environment. This opens up the possibility that SNS forms microdomains by similar association where signaling events are concentrated and future fusion occurs. These might be similar to the FURMAS (Kesper, Stute et al. 2007). To test such possibilities, mutants affecting oligomerization are required.

Hetero-oligomerization may have a role in the genetic interaction between SNS and Hbs. It will be further interesting to determine whether the physical and genetic interactions between Hbs and SNS are observed in other tissues and what roles they serve in development. If the proteins are partially redundant in the musculature, they may be partially or fully redundant in other tissues precluding observation of obvious phenotypic effects except in double mutants.

Some SNS cytodomain sequences have been identified to be required although not absolutely essential for function. Some explanations have been discussed where these data are presented. Additionally, an association with Hbs may also explain the reduced
efficiencies of mutants in rescue of myoblast fusion in sns mutants. SNS and Hbs are phosphorylated on tyrosine residues. The two cytodomains are capable of functioning in a similar manner although Hbs sequences are inefficient compared to SNS. If the compromised transgenes of SNS form hetero-oligomers with Hbs, in the absence of the residues in the cytodomain of SNS, the Hbs sequences may be able to substitute albeit weakly for interactions. This may explain the lowered efficiency of rescue by these transgenes rather than an absolute effect. If these transgenes were expressed in an sns, hbs double mutant background, an absolute requirement may become obvious.

The current studies have shed light on certain aspects of SNS and Hbs function. For the first time, I have demonstrated phosphorylation of SNS and a requirement for tyrosine residues in its cytodomain for function during myoblast fusion. SNS also requires proline rich regions in its cytodomain for function. These sequences may regulate interactions with intracellular proteins. Endogenous Hbs substitutes for SNS in the first phase of myoblast fusion. The studies presented here with Hbs suggest a redundant and/or synergistic relationship with SNS.
Bibliography


Appendix A

Molecular dissection of the SNS transmembrane domain function

Most eukaryotic cells retain their distinct character as mononucleated compartments although interactions with nearby cells are common and required to maintain tissue stability and structure. Occasionally, pairs or groups of cells are required to completely fuse their plasma membranes, allowing exchange and mixing of cytoplasmic components becoming syncytia, wherein distinct nuclei are maintained within a single cytoplasm and plasma membrane. Interactions of cell surface proteins need to overcome the forces between two opposing cells to establish membrane proximity (Cohen and Melikyan 2004). Biological fusion events are strongly regulated by fusion proteins to prevent non specific fusion events from destroying tissue structure and integrity (Chen, Grote et al. 2007).

Fusion of cell membranes is mediated by integral membrane proteins in several cases. It has been shown that in these cases, the transmembrane (TM) domains of such integral membrane proteins are specifically required. Membrane fusion can be divided into early and late steps. At the early step, membranes are brought into close proximity by the action of extracellular domains of the fusion proteins. TM domains may support this early step by enhancing the stability via multimerization as seen in viral (Doms and Helenius 1986) or SNARE-based (Margittai, Otto et al. 1999) fusion complexes.

Lipid mixing between the two membranes is a late step and may be facilitated by structurally flexible TM domains. Conformational flexibility appears to be one important
structural property of membrane-active fusion protein domains. β-branched and/or Glycine residues are overrepresented in the TMs of SNAREs (Langosch, Crane et al. 2001) and viral fusion proteins (Cleverley and Lenard 1998). One possible explanation provided for viral fusion proteins is that requirement for membrane bend following fusion of the outer monolayers requires integral membrane proteins to allow membrane flexibility. For this bending, the TM domains at sites of fusion should contain amino acids with small flexible side chains. Glycine is able to allow protein kinking and hence membrane bending and is preferred in the TMs of fusion proteins.

Functional studies demonstrated that mutagenizing Glycine residues within the TM of viral fusion proteins such as human immunodeficiency virus (HIV), influenza hemagglutinin (HA) (Melikyan, Markosyan et al. 2000), the vesicular stomatitis virus (VSV) G protein (Cleverley and Lenard 1998), or the reovirus fusion associated small transmembrane protein (Shmulevitz, Salsman et al. 2003) drastically reduced the fusogenicities of these full-length proteins in cellular assays. Specifically mutating the Glycine residues in the TM of HIV or VSV G to another small side chain containing Alanine resulted in a complete loss of activity showing a specific requirement for the Glycine. Protein kink is also allowed by the presence of proline although to a lesser extent. Fusion capabilities of TM of viral fusion proteins were reduced on substitution with Proline.

Fusion of the myoblast membranes is a central requirement for the formation of multinucleate syncitial muscle fibers. The cell adhesion molecules SNS, Duf/Kirre and IrreC/Rst mediate attraction bringing the cell membranes in close proximity. Until fusion
occurs, the proteins Duf/Kirre and SNS are found to remain in discrete puncta maintaining the sites of contact between the two cells.

In SNS, a replacement of transmembrane and cytodomain sequences by a GPI anchor resulted in a lack of ability to rescue myoblast fusion defect in sns mutants. The truncated form is comparable to full length in directing heterotypic aggregates with Duf/Kirre in transfected S2 cells (Galletta, Chakravarti et al. 2004). The myoblasts are however unable to migrate and make contacts with the founder due to the absence of the cytodomain sequences and hence cytoplasmic interactions mediating cell migration. The lack of myoblast migration precludes the observation of a function for SNS and specifically, the TM domain in membrane fusion. Such a function has been observed in TM of influenza HA or of the VSV G protein where a replacement with GPI anchor allows for outer monolayer fusion but blocks removal of the inner monolayers for full fusion (Kemble, Danieli et al. 1994).

To further investigate the role of the TM sequences of SNS specifically, domain swaps and mutational analysis has been undertaken and the preliminary results are presented here. The TM domain of SNS has two conserved Glycine residues. The second Glycine (G1091) is centrally placed such that it can provide the flexibility required for membrane bending during outer monolayer fusion and may thus have a critical role in SNS function. This residue is present in Nephrin TM domain but not in Hbs or Syg-2 TM domains. To ascertain a role for this G1091 for SNS function, I mutagenized the residue and the preliminary results are also presented here.
Domain swap of SNS TM domain

To determine whether there is a specific sequence requirement for SNS TM domain, I substituted the TM domains of two orthologous proteins Hbs and Syg-2 and generated chimera with SNS. The TM domains are not highly conserved between these proteins with 56% identity between SNS and Hbs and 14% between Syg-2 and SNS TM domains. The rescue assay described in chapter II was used to test the effect of such domain swaps on SNS function.

Both Hbs and Syg-2 TM domain swaps still resulted in rescue of myoblast fusion in sns mutants (Fig A.1B&C). Muscle fiber formation was restored although several defective and/or missing muscles were observed. The rescue is not comparable to that of full length sns cDNA (Fig A.1A).

In the generation of these domain swaps, convenient restriction enzyme sites were placed in the coding region before and after the TM domain. These sites change the amino acid sequence by one residue on either side and may hence result in the reduced ability of the domain swaps to function during myoblast fusion. This parent construct needs to be tested similarly as a control to determine whether the inefficiency to direct fusion is a result of the Hbs and Syg-2 TM domains being less efficient or due to the amino acid changes resulting from the cloning sites. These results suggest that the TM domain of SNS may not have a highly unique function that cannot be substituted by TM domains from other related proteins. To determine whether any TM domain can suffice, as long as membrane targeting is unaffected, TM from an unrelated protein with no known function in cell fusion must be tested.
Fig. A.1: Substitution of Hbs and Syg-2 TM domains for SNS in chimera.

(A) Rescue using full length cDNA of sns, sns[XB3]/sns[Zf1.4], UAS-snsHA; mef2GAL4/++; (B) Rescue using TM domain swap with Hbs, sns[XB3]/sns[Zf1.4], UAS-SEHTSC-HA; mef2GAL4/++; (C) Rescue using TM domain swap with Syg-2, sns[XB3]/sns[Zf1.4], UAS-SESyTSC-HA; mef2GAL4/+. Several muscle fibers form and the Hbs and Syg-2 TM domain chimera are able to mediate fusion.
G1091 mutagenesis

To determine if the conserved Glycine residue in the center of the TM domain of SNS has a requirement for allowing membrane flexibility, mutagenesis was undertaken to substitute four different residues at this position. Mutagenesis to a hydrophilic charged Aspartic Acid residue (G1091D) completely abolished the ability of the protein to function during myoblast fusion (Fig A.2A) presumably by disrupting the helical structure of the TM domain.

Proline was chosen for its known ability to also allow protein kinking with the idea that it should substitute for membrane flexibility at this position. UAS-G1091P allows partial rescue of the myoblast fusion defect (Fig A.2B). Alanine and Leucine substitutions should mimic the hydrophobicity at this position but not allow membrane flexibility. In viral fusion peptides, such substitutions have resulted in lack of fusion activity. UAS-G1091A expression resulted in partial rescue of the myoblast fusion defect (Fig A.2C) with several missing muscles and unfused myoblasts. Replacing the Glycine with Leucine (UAS-G1091L) which has a comparatively bigger side chain rescued the myoblast fusion defect in sns mutants considerably with few minor defects (Fig A.2D).

These data indicate that a specific requirement for this position may exist and can be provided for by Leucine. This position is occupied by a Valine in the TM of Hbs and Syg-2. Hence, the position may require a hydrophobic amino acid with certain properties for a specific function. One possibility is that the TM domain of SNS may have a role in homo- or hetero-oligomerization of SNS as has been described for other viral proteins. Glycine and Leucine may be better at mediating such an interaction compared to Alanine or Proline. This possibility can be tested by using the Glycine mutant constructs for
efficiency of such interactions to determine whether the differences in the rescue capabilities are due to the absence of such interactions. Alternatively, these residues may also regulate interactions with other myogenic membrane proteins.

---

**Fig. A.2: Mutagenesis of Glycine residue in the SNS TM domain.**

(A) sns[XB3]/sns[Zf1.4], UAS-[G109D]HA; mef2GAL4/+
(B) sns[XB3]/sns[Zf1.4], UAS-[G1091P]HA; mef2GAL4/+ 
(C) sns[XB3]/sns[Zf1.4], UAS-[G1091A]HA; mef2GAL4/+ 
(D) sns[XB3]/sns[Zf1.4], UAS-[G1091L]HA; mef2GAL4/+. Complete lack of fusion is observed with substitution by hydrophilic amino acid (A). Partial rescue is observed with mutations to proline and alanine (B&C). Substitution with leucine does not have a dramatic effect on the ability of the protein to rescue myoblast fusion (D).

These data are preliminary and need to be verified with multiple transgenes and quantitation of protein expression to confirm the differences in rescue efficiencies.

Preliminary analysis shows the mutagenized transgenes are targeted to the membrane although a quantitation of the protein targeted to the membrane is more appropriate for such an analysis.
### Appendix B

**Supplemental data tables**

Table B.1: Results of pairwise comparison of number of unfused myoblasts from different transgenic rescue results. Pairs marked yellow are same set comparison showing high correlation. Pairs marked blue are comparisons between dissimilar genotypes with higher numbers of unfused myoblasts.

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Difference</th>
<th>Lower bound</th>
<th>Upper bound</th>
<th>Adjusted p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆1233(2)-∆1233(1)</td>
<td>3.882352941</td>
<td>-21.18386474</td>
<td>28.94857062</td>
<td>0.999738119</td>
</tr>
<tr>
<td>2xPXXP- ∆1233(1)</td>
<td>-14.4375</td>
<td>-39.79217617</td>
<td>10.91717617</td>
<td>0.651130486</td>
</tr>
<tr>
<td>S17HA(1)- ∆1233(1)</td>
<td>-91.85714286</td>
<td>-116.0230212</td>
<td>-67.6912645</td>
<td>0</td>
</tr>
<tr>
<td>S17HA(2)- ∆1233(1)</td>
<td>-94</td>
<td>-118.5727399</td>
<td>-69.42726008</td>
<td>0</td>
</tr>
<tr>
<td>SNSHA- ∆1233(1)</td>
<td>-106.947368</td>
<td>-131.5201083</td>
<td>-82.37462851</td>
<td>0</td>
</tr>
<tr>
<td>Y14HA(1)- ∆1233(1)</td>
<td>-27.21052632</td>
<td>-51.78326623</td>
<td>-2.6377864</td>
<td>0.018969506</td>
</tr>
<tr>
<td>Y14HA(2)- ∆1233(1)</td>
<td>-25.61538462</td>
<td>-52.07135083</td>
<td>0.840581604</td>
<td>0.06516432</td>
</tr>
<tr>
<td>2xPXXP- ∆1233(2)</td>
<td>-18.31985294</td>
<td>-40.22791202</td>
<td>3.588206139</td>
<td>0.174022648</td>
</tr>
<tr>
<td>S17HA(1)- ∆1233(2)</td>
<td>-95.7394958</td>
<td>-116.2600495</td>
<td>-75.21894206</td>
<td>0</td>
</tr>
<tr>
<td>S17HA(2)- ∆1233(2)</td>
<td>-97.88235294</td>
<td>-118.8805194</td>
<td>-76.88418646</td>
<td>0</td>
</tr>
<tr>
<td>SNSHA- ∆1233(2)</td>
<td>-110.8297214</td>
<td>-131.8278878</td>
<td>-89.83155488</td>
<td>0</td>
</tr>
<tr>
<td>Y14HA(1)- ∆1233(2)</td>
<td>-31.09287926</td>
<td>-52.09104574</td>
<td>-10.09471277</td>
<td>0.000307665</td>
</tr>
<tr>
<td>Y14HA(2)- ∆1233(2)</td>
<td>-29.49773756</td>
<td>-52.671464</td>
<td>-6.32401111</td>
<td>0.003476902</td>
</tr>
<tr>
<td>S17HA(1)-2xPXXP</td>
<td>-77.41964286</td>
<td>-98.29157282</td>
<td>-56.54771289</td>
<td>0</td>
</tr>
<tr>
<td>S17HA(2)-2xPXXP</td>
<td>-79.5625</td>
<td>-100.9041806</td>
<td>-58.2208194</td>
<td>0</td>
</tr>
<tr>
<td>SNSHA-2xPXXP</td>
<td>-92.50986842</td>
<td>-113.851549</td>
<td>-71.16818782</td>
<td>0</td>
</tr>
<tr>
<td>Y14HA(1)-2xPXXP</td>
<td>-12.77302632</td>
<td>-34.11470691</td>
<td>8.568654281</td>
<td>0.590953928</td>
</tr>
<tr>
<td>Y14HA(2)-2xPXXP</td>
<td>-11.17788462</td>
<td>-34.66332547</td>
<td>12.30755624</td>
<td>0.823274622</td>
</tr>
<tr>
<td>S17HA(2)-S17HA(1)</td>
<td>-2.142857143</td>
<td>-22.05761056</td>
<td>17.77189627</td>
<td>0.99997763</td>
</tr>
<tr>
<td>SNSHA-S17HA(1)</td>
<td>-15.09022556</td>
<td>-35.00497898</td>
<td>4.824527851</td>
<td>0.282874902</td>
</tr>
<tr>
<td>Y14HA(1)-S17HA(1)</td>
<td>64.64661654</td>
<td>44.73186313</td>
<td>84.56136996</td>
<td>4.84E-14</td>
</tr>
<tr>
<td>Y14HA(2)-S17HA(1)</td>
<td>66.24175824</td>
<td>44.0450016</td>
<td>88.43851488</td>
<td>7.63E-14</td>
</tr>
<tr>
<td>SNSHA-S17HA(2)</td>
<td>-12.94736842</td>
<td>-33.359182</td>
<td>7.459181355</td>
<td>0.515748933</td>
</tr>
<tr>
<td>Y14HA(1)-S17HA(2)</td>
<td>66.78947368</td>
<td>46.38292391</td>
<td>87.19602346</td>
<td>4.32E-14</td>
</tr>
<tr>
<td>Y14HA(2)-S17HA(2)</td>
<td>68.38461538</td>
<td>45.74558105</td>
<td>91.02364971</td>
<td>6.12E-14</td>
</tr>
<tr>
<td>Y14HA(2)-SNSHA</td>
<td>79.73684211</td>
<td>59.33029233</td>
<td>100.1433919</td>
<td>0</td>
</tr>
<tr>
<td>Y14HA(2)-SNSHA</td>
<td>81.33198381</td>
<td>58.69294948</td>
<td>103.9710181</td>
<td>1.37E-14</td>
</tr>
<tr>
<td>Y14HA(2)-Y14HA(1)</td>
<td>1.5951417</td>
<td>-21.04389263</td>
<td>24.23417603</td>
<td>0.999998788</td>
</tr>
</tbody>
</table>
Table B.2: Results of pairwise comparison of percentage of hemisegments with defects in different transgenic rescue results.

$\Delta C$ (1) and (2) = 2 independent transgenes of $\Delta 1233-1263HA$. $\Delta A = \Delta 1113-1163HA$, $\Delta A+B+C+D = \Delta 1113-1278HA$, $\Delta A+C+D$ (1) and (2) = 2 independent transgenes of $\Delta 1113-1163+1233-1278HA$. Pairs marked yellow are same set comparison showing high correlation. Pairs marked blue are comparisons between dissimilar genotypes with higher percentages.

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Difference</th>
<th>Lower bound</th>
<th>Upper bound</th>
<th>Adjusted P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta C$ (2)-$\Delta C$ (1)</td>
<td>0.93056</td>
<td>-0.19431</td>
<td>2.05542</td>
<td>0.23106</td>
</tr>
<tr>
<td>$\Delta A+B+C+D$-$\Delta C$ (1)</td>
<td>1.70833</td>
<td>0.81438</td>
<td>2.60229</td>
<td>4.09E-08</td>
</tr>
<tr>
<td>$\Delta A$-$\Delta C$ (1)</td>
<td>-1.5644</td>
<td>-2.3617</td>
<td>-0.76709</td>
<td>1.50E-08</td>
</tr>
<tr>
<td>$\Delta A+C+D$ (1)-$\Delta C$ (1)</td>
<td>1.70833</td>
<td>0.83432</td>
<td>2.58235</td>
<td>1.74E-08</td>
</tr>
<tr>
<td>$\Delta A+C+D$ (2)-$\Delta C$ (1)</td>
<td>1.70833</td>
<td>0.91103</td>
<td>2.50564</td>
<td>3.78E-10</td>
</tr>
<tr>
<td>$5XPDZ$-$\Delta C$ (1)</td>
<td>-1.4792</td>
<td>-2.3731</td>
<td>-0.58521</td>
<td>4.63E-06</td>
</tr>
<tr>
<td>$2XPXXP$ (1)-$\Delta C$ (1)</td>
<td>0.58333</td>
<td>-0.59926</td>
<td>1.76592</td>
<td>0.92499</td>
</tr>
<tr>
<td>$2XPXXP$ (2)-$\Delta C$ (1)</td>
<td>0.34469</td>
<td>-0.45260</td>
<td>1.14199</td>
<td>0.97291</td>
</tr>
<tr>
<td>$s17HA$ (1)-$\Delta C$ (1)</td>
<td>-1.7917</td>
<td>-2.8681</td>
<td>-0.71520</td>
<td>3.90E-06</td>
</tr>
<tr>
<td>$s17HA$ (2)-$\Delta C$ (1)</td>
<td>-1.4641</td>
<td>-2.1924</td>
<td>-0.73574</td>
<td>5.76E-09</td>
</tr>
<tr>
<td>$SNSHA$-$\Delta C$ (1)</td>
<td>-1.8089</td>
<td>-2.5372</td>
<td>-1.0805</td>
<td>7.26E-13</td>
</tr>
<tr>
<td>$Y14HA$ (1)-$\Delta C$ (1)</td>
<td>0.67803</td>
<td>-0.0222</td>
<td>1.37830</td>
<td>0.06871</td>
</tr>
<tr>
<td>$Y14HA$ (2)-$\Delta C$ (1)</td>
<td>0.60833</td>
<td>-0.4681</td>
<td>1.68479</td>
<td>0.81824</td>
</tr>
<tr>
<td>$\Delta A+B+C+D$-$\Delta C$ (2)</td>
<td>0.77777</td>
<td>-0.51255</td>
<td>2.06808</td>
<td>0.74116</td>
</tr>
<tr>
<td>$\Delta A$-$\Delta C$ (2)</td>
<td>-2.4949</td>
<td>-3.7203</td>
<td>-1.2696</td>
<td>3.41E-09</td>
</tr>
<tr>
<td>$\Delta A+C+D$ (1)-$\Delta C$ (2)</td>
<td>0.77777</td>
<td>-0.4988</td>
<td>2.05435</td>
<td>0.72670</td>
</tr>
<tr>
<td>$\Delta A+C+D$ (2)-$\Delta C$ (2)</td>
<td>0.77777</td>
<td>-0.44755</td>
<td>2.00310</td>
<td>0.66730</td>
</tr>
<tr>
<td>$5XPDZ$-$\Delta C$ (2)</td>
<td>-2.4097</td>
<td>-3.7000</td>
<td>-1.1194</td>
<td>9.44E-08</td>
</tr>
<tr>
<td>Compound</td>
<td>ΔC (1)</td>
<td>ΔC (2)</td>
<td>ΔC (3)</td>
<td>ΔC (4)</td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>2XPXXP (1)</td>
<td>-0.34722222</td>
<td>-1.85196985</td>
<td>1.157525403</td>
<td>0.999953795</td>
</tr>
<tr>
<td>2XPXXP (2)</td>
<td>-0.58585859</td>
<td>-1.81119039</td>
<td>0.639473218</td>
<td>0.940180771</td>
</tr>
<tr>
<td>S17HA (1)</td>
<td>-2.72222222</td>
<td>-4.14507839</td>
<td>-1.29936606</td>
<td>3.92E-08</td>
</tr>
<tr>
<td>S17HA (2)</td>
<td>-2.39463602</td>
<td>-3.57625375</td>
<td>-1.21301829</td>
<td>4.14E-09</td>
</tr>
<tr>
<td>SNSHA</td>
<td>-2.7394636</td>
<td>-3.92108133</td>
<td>-1.55784587</td>
<td>9.10E-12</td>
</tr>
<tr>
<td>Y14HA (1)</td>
<td>-0.25252525</td>
<td>-1.41705845</td>
<td>0.912007948</td>
<td>0.999977498</td>
</tr>
<tr>
<td>Y14HA (2)</td>
<td>-0.32222222</td>
<td>-1.74507839</td>
<td>1.100633943</td>
<td>0.999962775</td>
</tr>
<tr>
<td>ΔA-ΔA+B+C+D</td>
<td>-3.27272727</td>
<td>-4.29020737</td>
<td>-2.25524718</td>
<td>4.71E-13</td>
</tr>
<tr>
<td>ΔA+C+D (1) -ΔA+B+C+D</td>
<td>1.78E-15</td>
<td>-1.0786436</td>
<td>1.078643603</td>
<td>1</td>
</tr>
<tr>
<td>ΔA+C+D (2) -ΔA+B+C+D</td>
<td>0</td>
<td>-1.01748009</td>
<td>1.017480092</td>
<td>1</td>
</tr>
<tr>
<td>2XPXXP (1)</td>
<td>-1.125</td>
<td>-2.46592995</td>
<td>0.215929948</td>
<td>0.211680964</td>
</tr>
<tr>
<td>2XPXXP (2)</td>
<td>-1.36363636</td>
<td>-2.38111646</td>
<td>-0.34615627</td>
<td>0.000711752</td>
</tr>
<tr>
<td>S17HA (1)</td>
<td>-3.5</td>
<td>-4.74833784</td>
<td>-2.25166216</td>
<td>5.08E-13</td>
</tr>
<tr>
<td>S17HA (2)</td>
<td>-3.17241379</td>
<td>-4.13680384</td>
<td>-2.20802374</td>
<td>4.45E-13</td>
</tr>
<tr>
<td>SNSHA</td>
<td>-3.51724138</td>
<td>-4.48163143</td>
<td>-2.55285133</td>
<td>3.81E-13</td>
</tr>
<tr>
<td>Y14HA (1)</td>
<td>-1.03030303</td>
<td>-1.97368274</td>
<td>-0.08692332</td>
<td>0.018288169</td>
</tr>
<tr>
<td>Y14HA (2)</td>
<td>-1.1</td>
<td>-2.34833784</td>
<td>0.148337845</td>
<td>0.151259521</td>
</tr>
<tr>
<td>ΔA+C+D (1) -ΔA</td>
<td>3.272727273</td>
<td>2.272722767</td>
<td>4.272731778</td>
<td>4.52E-13</td>
</tr>
<tr>
<td>ΔA+C+D (2) -ΔA</td>
<td>3.272727273</td>
<td>2.339023504</td>
<td>4.206431041</td>
<td>3.90E-13</td>
</tr>
<tr>
<td>5XPDZ-ΔA</td>
<td>0.085227273</td>
<td>-0.93225282</td>
<td>1.102707365</td>
<td>1</td>
</tr>
<tr>
<td>2XPXXP (1)-ΔA</td>
<td>2.147727273</td>
<td>0.869200733</td>
<td>3.426253813</td>
<td>2.97E-06</td>
</tr>
<tr>
<td></td>
<td>ΔA</td>
<td>ΔB</td>
<td>ΔC</td>
<td>ΔD</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>2XPXXP (2)-ΔA</td>
<td>1.909090909</td>
<td>0.975387141</td>
<td>2.842794678</td>
<td>2.87E-09</td>
</tr>
<tr>
<td>S17HA (1)-ΔA</td>
<td>-0.22727273</td>
<td>1.40832495</td>
<td>0.9537795</td>
<td>0.999994505</td>
</tr>
<tr>
<td>S17HA (2)-ΔA</td>
<td>0.10031348</td>
<td>-0.77523501</td>
<td>0.975861969</td>
<td>0.999999991</td>
</tr>
<tr>
<td>SNSHA-ΔA</td>
<td>-0.24451411</td>
<td>-1.1200626</td>
<td>0.631034382</td>
<td>0.999609634</td>
</tr>
<tr>
<td>Y14HA (1)-ΔA</td>
<td>2.242424242</td>
<td>1.390073216</td>
<td>3.094775269</td>
<td>5.19E-13</td>
</tr>
<tr>
<td>Y14HA (2)-ΔA</td>
<td>2.172727272</td>
<td>0.991675045</td>
<td>3.3537795</td>
<td>1.60E-07</td>
</tr>
<tr>
<td>ΔA+C+D (2)-ΔA+C+D (1)</td>
<td>1.78E-15</td>
<td>1.00000451</td>
<td>1.000004505</td>
<td>1</td>
</tr>
<tr>
<td>5XPDZ-ΔA+C+D (1)</td>
<td>-3.1875</td>
<td>-4.2661436</td>
<td>-2.1088564</td>
<td>5.17E-13</td>
</tr>
<tr>
<td>2XPXXP (1)-ΔA+C+D (1)</td>
<td>-1.125</td>
<td>-2.45271849</td>
<td>0.202718493</td>
<td>0.198564873</td>
</tr>
<tr>
<td>2XPXXP (2)-ΔA+C+D (1)</td>
<td>-1.36363636</td>
<td>-2.36364087</td>
<td>-0.36363186</td>
<td>0.000505426</td>
</tr>
<tr>
<td>S17HA (1)-ΔA+C+D (1)</td>
<td>-3.5</td>
<td>-4.73413559</td>
<td>-2.26586441</td>
<td>5.14E-13</td>
</tr>
<tr>
<td>S17HA (2)-ΔA+C+D (1)</td>
<td>-3.17241379</td>
<td>-4.11834796</td>
<td>-2.22647963</td>
<td>4.23E-13</td>
</tr>
<tr>
<td>SNSHA-ΔA+C+D (1)</td>
<td>-3.51724138</td>
<td>-4.46317554</td>
<td>-2.57130722</td>
<td>3.80E-13</td>
</tr>
<tr>
<td>Y14HA (1)-ΔA+C+D (1)</td>
<td>-1.03030303</td>
<td>-1.95480752</td>
<td>-0.10579854</td>
<td>0.014086831</td>
</tr>
<tr>
<td>Y14HA (2)-ΔA+C+D (1)</td>
<td>-1.1</td>
<td>-2.33413559</td>
<td>0.134135587</td>
<td>0.138877351</td>
</tr>
<tr>
<td>5XPDZ-ΔA+C+D (2)</td>
<td>-3.1875</td>
<td>-4.20498009</td>
<td>-2.17001991</td>
<td>5.02E-13</td>
</tr>
<tr>
<td>2XPXXP (1)-ΔA+C+D (2)</td>
<td>-1.125</td>
<td>-2.40352654</td>
<td>0.15352654</td>
<td>0.152844862</td>
</tr>
<tr>
<td>2XPXXP (2)-ΔA+C+D (2)</td>
<td>-1.36363636</td>
<td>-2.29734013</td>
<td>-0.4299326</td>
<td>0.000115742</td>
</tr>
<tr>
<td>S17HA (1)-ΔA+C+D (2)</td>
<td>-3.5</td>
<td>-4.68105223</td>
<td>-2.31894777</td>
<td>5.14E-13</td>
</tr>
<tr>
<td>S17HA (2)-ΔA+C+D (2)</td>
<td>-3.17241379</td>
<td>-4.04796228</td>
<td>-2.2968653</td>
<td>3.82E-13</td>
</tr>
<tr>
<td>SNSHA-ΔA+C+D (2)</td>
<td>-3.51724138</td>
<td>-4.39278987</td>
<td>-2.64169289</td>
<td>3.80E-13</td>
</tr>
<tr>
<td>Y14HA (1)-ΔA+C+D (2)</td>
<td>-1.03030303</td>
<td>-1.88265406</td>
<td>-0.177952</td>
<td>0.004352005</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-----------</td>
<td>-------------</td>
</tr>
<tr>
<td>Y14HA (2)-ΔA+C+D (2)</td>
<td>-1.1</td>
<td>-2.28105223</td>
<td>0.08105227</td>
<td>0.097435993</td>
</tr>
<tr>
<td>2XPXXP (1)-5XPDZ</td>
<td>2.0625</td>
<td>0.721570052</td>
<td>3.403429948</td>
<td>3.33E-05</td>
</tr>
<tr>
<td>2XPXXP (2)-5XPDZ</td>
<td>1.823863636</td>
<td>0.806383544</td>
<td>2.841343729</td>
<td>3.87E-07</td>
</tr>
<tr>
<td>S17HA (1)-5XPDZ</td>
<td>-0.3125</td>
<td>-0.94930384</td>
<td>0.379476257</td>
<td>1</td>
</tr>
<tr>
<td>S17HA (2)-5XPDZ</td>
<td>0.015086207</td>
<td>-1.29413143</td>
<td>0.63464867</td>
<td>0.096810994</td>
</tr>
<tr>
<td>SNSHA-5XPDZ</td>
<td>0.63464867</td>
<td>0.63464867</td>
<td>0.999883368</td>
<td></td>
</tr>
<tr>
<td>Y14HA (1)-5XPDZ</td>
<td>2.15719697</td>
<td>1.213817256</td>
<td>3.100576683</td>
<td>1.77E-11</td>
</tr>
<tr>
<td>Y14HA (2)-5XPDZ</td>
<td>2.0875</td>
<td>0.839162155</td>
<td>3.335837845</td>
<td>3.40E-06</td>
</tr>
<tr>
<td>2XPXXP (2)-2XPXXP (1)</td>
<td>-0.23863636</td>
<td>-1.5171629</td>
<td>1.039890176</td>
<td>0.99996186</td>
</tr>
<tr>
<td>S17HA (1)-2XPXXP (1)</td>
<td>-2.375</td>
<td>-3.84391516</td>
<td>-0.90608484</td>
<td>8.89E-06</td>
</tr>
<tr>
<td>S17HA (2)-2XPXXP (1)</td>
<td>-2.04741379</td>
<td>-3.28410799</td>
<td>-0.81071959</td>
<td>4.55E-06</td>
</tr>
<tr>
<td>SNSHA-2XPXXP (1)</td>
<td>-2.39224138</td>
<td>-3.62893558</td>
<td>-1.15554718</td>
<td>2.59E-08</td>
</tr>
<tr>
<td>Y14HA (1)-2XPXXP (1)</td>
<td>0.09469697</td>
<td>-1.12568398</td>
<td>1.315077918</td>
<td>1</td>
</tr>
<tr>
<td>Y14HA (2)-2XPXXP (1)</td>
<td>0.025</td>
<td>-1.44391516</td>
<td>1.49391516</td>
<td>1</td>
</tr>
<tr>
<td>S17HA (1)-2XPXXP (2)</td>
<td>-2.13636364</td>
<td>-3.31741586</td>
<td>-0.95531141</td>
<td>2.86E-07</td>
</tr>
<tr>
<td>S17HA (2)-2XPXXP (2)</td>
<td>-1.80877743</td>
<td>-2.68432592</td>
<td>-0.93322894</td>
<td>1.86E-09</td>
</tr>
<tr>
<td>SNSHA-2XPXXP (2)</td>
<td>-2.15360502</td>
<td>-3.0291535</td>
<td>-1.27805653</td>
<td>8.82E-13</td>
</tr>
<tr>
<td>Y14HA (1)-2XPXXP (2)</td>
<td>0.333333333</td>
<td>-0.51901769</td>
<td>1.18568436</td>
<td>0.988636846</td>
</tr>
<tr>
<td>Y14HA (2)-2XPXXP (2)</td>
<td>0.263636364</td>
<td>-0.91741586</td>
<td>1.444688591</td>
<td>0.999968489</td>
</tr>
<tr>
<td>S17HA (2)-S17HA (1)</td>
<td>0.327586207</td>
<td>-0.80804877</td>
<td>1.46322118</td>
<td>0.999446733</td>
</tr>
<tr>
<td>SNSHA-S17HA (1)</td>
<td>-0.01724138</td>
<td>-1.15287635</td>
<td>1.118393594</td>
<td>1</td>
</tr>
<tr>
<td>Residue position</td>
<td>Netphos</td>
<td>PONDR</td>
<td>Disphos</td>
<td>Prosite PKC</td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>-------</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>S1131</td>
<td>0.116</td>
<td></td>
<td>0.27</td>
<td>N</td>
</tr>
<tr>
<td>S1132</td>
<td>0.996</td>
<td></td>
<td>0.225</td>
<td>N</td>
</tr>
<tr>
<td>S1143</td>
<td>0.69</td>
<td>N</td>
<td>0.601</td>
<td>CKII</td>
</tr>
<tr>
<td>S1144</td>
<td>0.973</td>
<td>N</td>
<td>0.558</td>
<td>N</td>
</tr>
<tr>
<td>S1146</td>
<td>0.996</td>
<td>N</td>
<td>0.705</td>
<td>N</td>
</tr>
<tr>
<td>S1149</td>
<td>0.779</td>
<td>N</td>
<td>0.805</td>
<td>CKII</td>
</tr>
<tr>
<td>S1151</td>
<td>0.991</td>
<td>N</td>
<td>0.804</td>
<td>CKII</td>
</tr>
<tr>
<td>S1153</td>
<td>0.986</td>
<td>N</td>
<td>0.83</td>
<td>CKII</td>
</tr>
<tr>
<td>S1157</td>
<td>0.011</td>
<td>N</td>
<td>0.495</td>
<td>CKII</td>
</tr>
<tr>
<td>S1158</td>
<td>0.983</td>
<td>N</td>
<td>0.595</td>
<td>N</td>
</tr>
<tr>
<td>S1172</td>
<td>0.954</td>
<td></td>
<td>0.241</td>
<td>PKA</td>
</tr>
<tr>
<td>S1179</td>
<td>0.048</td>
<td>N</td>
<td>0.449</td>
<td>PKA</td>
</tr>
<tr>
<td>S1215</td>
<td>0.008</td>
<td></td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>Residue position</td>
<td>NetPhos</td>
<td>PONDR</td>
<td>DisPhos</td>
<td>Kinase</td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>-------</td>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>T1115</td>
<td>0.181</td>
<td>X</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>T1116</td>
<td>0.631</td>
<td>X</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>T1122</td>
<td>0.090</td>
<td>X</td>
<td>0.577</td>
<td></td>
</tr>
<tr>
<td>T1124</td>
<td>0.069</td>
<td>X</td>
<td>0.046</td>
<td></td>
</tr>
</tbody>
</table>
Table B.5: Oligonucleotides and restriction enzymes used to generate transgenic constructs.

<table>
<thead>
<tr>
<th>Construct name</th>
<th>Oligo name</th>
<th>Sequence</th>
<th>Restriction sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNSHA</td>
<td>XHASNSR</td>
<td>GCTCTAGACTAGGCGTAGTCGGGACGTCGTAGGGTTATA CGAGGTTGTCGTCCGC</td>
<td>AfII-XbaI</td>
</tr>
<tr>
<td>SNSV5</td>
<td>C-V5-SNS-R</td>
<td>GATCCCTCTAGACTAGGCTAGTCAGACCTAAGCCGCGGT TCQGGAATCGTTACCTACGAGGTGTCGTCCGC</td>
<td>AfII-XbaI</td>
</tr>
<tr>
<td>SNSFLAG</td>
<td>C-FLAG-SNS-R</td>
<td>GATCCCTCTAGACTACTTTGTGTCATCGTCTTTGGTAGTCTAAC GAGGTGTCGTCCGC</td>
<td>AfII-XbaI</td>
</tr>
<tr>
<td>20-5HA</td>
<td>sns20-5HAR</td>
<td>GCTCTAGACTAGCGTGATGTCGGGCACGTCGTAGGGGAATATATTTCCCGGTTGCTGCTGCC</td>
<td>Xhol-XbaI</td>
</tr>
<tr>
<td>Δ1113-1278HA</td>
<td>sns3867-3884R</td>
<td>GGTTGTCGACACTTTCTTCTGCGGCCCTG</td>
<td>AflII-XbaI</td>
</tr>
<tr>
<td>Δ1113-1163HA</td>
<td>sns3870_4038F</td>
<td>CGCCGCAAGAAAGTGGAGGAGGCAGCGCAAGAACAGCG</td>
<td>Xhol-XbaI</td>
</tr>
<tr>
<td>Δ1164-1232HA</td>
<td>sns4023_4245F</td>
<td>CAACCGGAATATTTATCTTTTCTCCTTGCTCCTCG</td>
<td>Xhol-XbaI</td>
</tr>
<tr>
<td>Δ1233-1263HA</td>
<td>sns4230_4338F</td>
<td>ATCAGCAAAGGCGGTCTCCACGCGCCCTGAGACTCCAC</td>
<td>Xhol-XbaI</td>
</tr>
<tr>
<td>Δ1263-1278HA</td>
<td>KSK-SNCC-del4756-4800F</td>
<td>GATCTTTGTCCTATCCGCAAAATTAGAATCTGCAACCCCATGACGTTGACCTGCTG</td>
<td>Xhol-XbaI</td>
</tr>
<tr>
<td>Δ1113-1232HA</td>
<td>BstEII_1278R</td>
<td>GGCCAGCGAGGTCAGCGTCAGGTGCTGACCTTCG</td>
<td>Xhol-XbaI</td>
</tr>
<tr>
<td>Δ1164-1278HA</td>
<td>MluI_1233_F</td>
<td>CTCCCTGGTCAACGCGTCGCTGGCTGCTGCTGAGTTCCTTCTGCTG</td>
<td>Mlu-BstEII</td>
</tr>
<tr>
<td>Δ1113-1163+1233-1278HA</td>
<td>MluI_1164_F</td>
<td>CTCCTGGTCAACGCGTCGCTGGCTGCTGCTG</td>
<td>Mlu-BstEII</td>
</tr>
<tr>
<td>S17-A17HA</td>
<td>sns-s1141a-f</td>
<td>GAGACGCTGGCGCCCGGCTTGGCTG</td>
<td>Xhol-XbaI</td>
</tr>
<tr>
<td></td>
<td>sns-s1149c-f</td>
<td>GAGAAAGACGAGGAGGATACGAGAATGAGGGCCAGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sns-s1155a-f</td>
<td>GAATAGGGCCCGCCCGCCAACCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sns-s1236a-f</td>
<td>GTTTATATTTCCCGGCCCGCCCTCCGCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sns-s1236c-f</td>
<td>GCGTTTATATTTCCCGGAGCCCGGAACCGCGCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sns-s1246a-f</td>
<td>CCAGATTGGGCCCTACTACAAAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sns-s1252a-f</td>
<td>CAACATGAAACGCTGACAGATAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sns-s1257a-f</td>
<td>CAGATACTTGGCTATCCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sns4639F</td>
<td>GCGCCAGTGGCTATTAATGACAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sns4696F</td>
<td>AAGGCCGGAGCCCTAGCAATGAGGGCCAGCGCCCAACGCGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sns_4050_F</td>
<td>AAGAAGGGCCCGCCACGTATTCGTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sns_4312_F</td>
<td>GACAGATACTTGGCTATCCGCAATG</td>
<td></td>
</tr>
<tr>
<td>Y14-F14HA</td>
<td>Y1153,1162F_F</td>
<td>GAGTCGTTCTCCAATGAGGGCAGCAGCCAACCGGAATTATTGAC</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Y1153F_F</td>
<td>AGCGAGTCTGTTCTCCAATGAGGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1162F_F</td>
<td>AGCCAACCGGAATTATGAGGAGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1186F_F</td>
<td>CCGCACCAGATTTCCAAGAGGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1234F_F</td>
<td>AGCAAACCGGAATTATTGACGAGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1248,1256F_F</td>
<td>GGGTCTCTTCTAAACATGAAACAGTGAAGATTTCTTGTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1248F_F</td>
<td>CCACCAAGATGGGTCTCTTCAAACTGAATGGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1256F_F</td>
<td>CACGAGACATATTGCTCTCTATCCGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KSK-Y526F-F</td>
<td>GCAAACATCGAGTGTTCCGACGAGCAGCCAGCTTTCGTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KSK-Y661F-F</td>
<td>CCCGCAAATGGAATCCCAGCAGCCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KSK-Y872F-F</td>
<td>GCATACCGGTGTCCTACAGTGACGAGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KSK-Y531F-F</td>
<td>CGCCGCACTTGGTCATAGACACAGGGTACCTAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KSK-Y572F-F</td>
<td>GGGGCGTCCAGCTTCTAGTGAGGAGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KSK-Y594F-F</td>
<td>CACCTGCGAGTCAATTCCAAGATAATGAGGATGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AflII-XbaI</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2xPXXP</th>
<th>4246MUT1snsF</th>
<th>TATATTGCATCCCGCATCTGACAGCGGCTGAGCATGTTGCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2xPXXP</td>
<td>pUAST-SNS3</td>
<td>CACTGAAATGGGAGTCTGCGGACGACCACCACACGTTTGGCGCGACGAGCAGGG</td>
</tr>
<tr>
<td>SNS-5PDZ</td>
<td>SNSAlaR</td>
<td>CGTCTAGACTAGGGCGGCGGCGGCAGCTTCATCTGG</td>
</tr>
<tr>
<td>SNS-5PDZ</td>
<td>SNS-PDZ-Int.F</td>
<td>GCCGACCGCGCTGGAGAAGGGGAGTGGCTTGG</td>
</tr>
<tr>
<td>SNS-5PDZ</td>
<td>SNS-PDZ-3-F</td>
<td>GGGTATGCGGCGCCAGGGCTGGCATATGAGGAGGGCAGCAGCT</td>
</tr>
<tr>
<td>SNS-5PDZ</td>
<td>SNS-PDZ-4+5-F</td>
<td>AATACCGGCCAGCCAGGAGGCGCGGCTGG</td>
</tr>
<tr>
<td>HESTSC-HA</td>
<td>Hbs2876F</td>
<td>TGGTGCACACTGCCCTGAAATAG</td>
</tr>
<tr>
<td>HESTSC-HA</td>
<td>sns3819_hbs3553R</td>
<td>GAATGCCCAGAGAGATGCTCAGGAGGATAGGAGGATGAGGAGGATGAGGAGGATGAGGAGGATGAGG</td>
</tr>
<tr>
<td>SESTHC-HA</td>
<td>sns3867_hbs3619F</td>
<td>CAGGGCCGCAAGAAGGATGCTCAGAGCGAAGGAGGAGGAGGATGAGGAGGATGAGGAGGATGAGG</td>
</tr>
<tr>
<td>SESTHC-HA</td>
<td>hbs3636_sns3882R</td>
<td>CTCCGCTTTTCGCTGCCACATCTTCTCTCCTGCT</td>
</tr>
<tr>
<td>SESTHC-HA</td>
<td>hrsHA-1</td>
<td>GCTCTAGAGGATCCCTAGGCGTAGTCGGGCACGTCGTAGGGGTAGTGATCCCGGAGAGGAGGATGAGGAGGATGAGG</td>
</tr>
<tr>
<td>G1091A-HA</td>
<td>KSK-G489A-F</td>
<td>CTGCCGCGCATTCGCTCCCTCTGG</td>
</tr>
<tr>
<td>G1091P-HA</td>
<td>KSK-G489P-F</td>
<td>CTCTGCGCGCATTCGCTCCCTCTGG</td>
</tr>
<tr>
<td>G1091L-HA</td>
<td>KSK-G489L-F</td>
<td>CTCTGCGCGCATTCGCTCCCTCTG</td>
</tr>
<tr>
<td>G1091D-HA</td>
<td>KSK-G489D-F</td>
<td>CTCTGCGCGCATTCGCTCCCTCTG</td>
</tr>
<tr>
<td>XhoI-XbaI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XhoI-XbaI</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix C

MUSCLE MORPHOGENESIS: THE PROCESS OF EMBRYONIC MYOBLAST FUSION

The following is a reference that reviews the process of myoblast fusion in *Drosophila* embryo with particular emphasis on key molecules involved in the process, to which I contributed. It is being presented here by the kind permission of Springer Science and Business Media.

VITA

Kiranmai S Kocherlakota

EDUCATION

2001-2007  PhD  Cell and Developmental Biology  Pennsylvania State
           Huck Institutes of Life Sciences  University

1998-2000  MSc  Biomedical Sciences  Delhi University, India
           Ambedkar Center for Biomedical Research

1995-1998  BSc  Microbiology  Osmania University, India
           Bharatiya Vidya Bhavans Degree College

PUBLICATIONS

Kocherlakota KS and Abmayr SM. Molecular dissection of the cytodomain of
Drosophila cell adhesion molecule, Sticks-and-Stones (SNS) reveals domains that play a
role in embryonic myogenesis. Manuscript in preparation.

Abmayr SM, Kocherlakota KS. Muscle morphogenesis: the process of embryonic
New York, N.Y: Landes Bioscience/Eurekah.com ;Springer Science+Business
Media;2006:p.1-12

Qadar Pasha MA, Kocherlakota KS, Khan AP, Norboo T, Grover SK, Baig MA,
Selvamurthy W and Brahmachari SK. Arterial oxygen saturation under hypoxic
environment of high altitude associates with routine physical activities of natives. Current
Science 2003; 85(4): 502-506

ABSTRACTS AND PRESENTATIONS

Kiranmai S Kocherlakota, Erika Geisbrecht and Susan M Abmayr. An
investigation of the oligomerization properties of SNS during myoblast fusion. 47th
Joshua Wunderlich, Erika Geisbrecht, Kiranmai Kocherlakota, David Ash, Mei-Hui
Chen, Selene Swanson, Laurence Florens, Michael Washburn, Susan Abmayr, Jeffrey
Haug. High throughput method for the identification of in vivo protein interactions and
modifications during D. melanogaster embryogenesis. ISAC XXIII International

Kiranmai S Kocherlakota and Susan M Abmayr. Essential tyrosine residues in its
cytodomain direct SNS-mediated myoblast fusion. 46th Annual Drosophila Research
Conference (2005).

Kiranmai S Kocherlakota, Rakhee Banerji and Susan M Abmayr. A requirement
for the cytodomain in SNS-mediated myoblast migration and fusion. Society for