“I can't say I feel relieved or satisfied, just the opposite, I am crushed. Only my goal is reached: I know what I have to know; I have understood all that has happened to me since January. The Nausea has not left me and I don't believe it will leave me so soon; but I no longer have to bear it, it is no longer an illness or a passing fit: it is I.”

Jean-Paul Sartre, *Nausea* (1938).

“The Dude abides.”

Jeffrey “The Dude” Lebowski, *The Big Lebowski* (1998).

University of Alberta

Molecular and cellular analysis of skeletal muscle and neuronal development in a necdin-null mouse model of Prader-Willi syndrome

by

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Abstract

Prader-Willi syndrome (PWS) is a recurrent microdeletion syndrome characterized by severe obesity, hyperphagia, hypotonia, and developmental delay, and is caused by the loss of expression of four protein-coding genes and set of small nucleolar RNAs on chromosome 15. *NDN*, encoding the protein necdin, is one of these genes, and a large body of literature supports the theory that necdin is important for the differentiation and survival of neurons. Given that necdin is also abundant in developing muscle and that hypotonia is a cardinal feature of PWS, I hypothesize that necdin promotes normal skeletal muscle development. I provide two lines of evidence demonstrating that loss of necdin impairs muscle development in mice. First, necdin interacts with the inhibitor of muscle differentiation EID-1 to relieve inhibition of MyoD-dependent transcription by sequestering this protein in the cytoplasm in over-expression assays. Unexpectedly, the presence of necdin increases EID-1 protein abundance in transfected cells and endogenous EID-1 is less abundant in *Ndn*-null embryonic mouse tissue compared to controls. Finally, conversion from MyoD+ to Myosin Heavy Chain+ cells is impaired in limb bud cultures from *Ndn*-null embryos, consistent with the hypothesis that loss of necdin impairs muscle differentiation by failing to relieve EID-1-dependent transcriptional inhibition. Second, loss of necdin impairs polarization of muscle progenitors *in vitro* and *in vivo* due to failed activation of the actin-myosin cytoskeleton, and reduces the proportional area of forelimb extensor muscles in *Ndn*-null mice at birth. This conclusion is supported by defective centrosome re-orientation due to impaired nuclear rearward

movement and failed Cdc42 activation in *Ndn*-null mouse embryonic fibroblasts (MEFs), impaired myosin activation in *Ndn*-null MEFs and cortical neurons, and excessive branching and failure of hippocampal neurons to polarize with respect to a growth factor. Additionally, PWS patient fibroblasts display centrosome re-orientation defects and impaired myosin activation identical to *Ndn*-null MEFs, indicating that loss of necdin produces a similar phenotype in both mice and humans. These results provide strong evidence that necdin is critical for both the migration and primary differentiation of skeletal muscle, and validates the *Ndn*-null mouse as a model for hypotonia in PWS.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ARHGEF6 – a Rho guanine-nucleotide exchange factor 6

AS – Angelman syndrome

ASD – Autism spectrum disorder

BBS – Bardet-Biedl syndrome

BDNF – Brain-derived neurotrophic factor

bHLH – basic Helix-loop-helix

BMB – Boerhinger Mannheim blocking solution

BP – Breakpoint

BSA – Bovine serum albumin

CBP – Cyclic AMP response element-binding protein

CCHS – Congenital central hypoventilation syndrome

cDNA – complementary DNA

CXCR – CXC chemokine receptor

CYFIP1 – cytoplasmic Fragile X syndrome protein interacting protein 1

DAPI - 4',6-diamidino-2-phenylindole

DIV – Days *in vitro*

DMEM – Dulbecco's modified Eagle's medium

DMSO – Dimethyl sulfoxide

DRG – Dorsal root ganglia

ECL – Enhanced chemiluminescence

EID-1 – E1A-like inhibitor of differentiation 1

ELC – Essential light chain of myosin motor

ERK – Extracellular regulated kinase

F-actin – Filamentous polymerized actin

FBS – Fetal bovine serum

FD – Familial dysautonomia

FMRP – Fragile X mental retardation protein

G-actin – Globular monomeric actin

GFP – Green fluorescent protein

GnRH – Gonadotropin-hormone releasing hormone

H&E – Hematoxylin and Eosin stain

HAT – Histone acetyltransferase

HBSS – Hank's buffered saline solution

HDAC – Histone deacetylase

HGF, HGFR – Hepatocyte growth factor, Hepatocyte growth factor receptor

HRP – Horseradish peroxidase

IC – Imprinting center

IQ – Intelligence quotient

LBGM – Limb bud growth medium

LPA – Lipophosphatidic acid

MAPK – Mitogen activated protein kinase

MAGE – Melanoma antigen gene

MEF – Mouse embryonic fibroblast

MEFC2 – Myocyte enhancing factor C2

MEGAP – mental disorder-associated GAP protein

MG132 - N-(benzyloxycarbonyl)leucinylleucinylleucinal

MHC – Myosin heavy chain

MHD – MAGE homology domain

MLC – Myosin light chain, also RLC

MRCK – Myotonic dystrophy kinase-related Cdc42-binding kinase

MRF – Muscle regulatory factor

MTOC – Microtubule organizing center

NBM – Neurobasal medium

Necdin (*NDN*, *Ndn*) – Neural expressed in embryonal carcinoma differentiated protein

NGF – Nerve growth factor

NIPA – Non-imprinted in Prader-Willi/Angelman syndrome

PBS, PBS-X – Phosphate-buffered saline, Phosphate-buffered saline with TritonX-100

P/CAF – p300/CBP associated factor

PFA - paraformaldehyde

PWP – Prader-Willi-like phenotype

PWS – Prader Willi syndrome

RB – Retinoblastoma protein

RLC – Regulatory light chain of myosin motor, also MLC

SCG – Superior cervical ganglia

SDF-1α – Stromal derived factor 1 alpha

SDS – Sodium dodecyl sulfate

SEM – Standart error of the mean

Shh – Sonic hedgehog

SMC – Structural Maintenance of Chromosomes

snoRNA – Small nucleolar RNA

TBST, TBSTM – Tris-buffered saline with Tween, Tris-buffered saline with Tween and milk

UPD – Uniparental disomy

VEGF – Vascular-endothelial growth factor

WS - Waardenburg syndrom

Chapter 1. Introduction

1.1 Prader-Willi syndrome

1.1.1 Prader-Willi syndrome – Diagnostic Criteria

Prader-Willi syndrome (PWS, also known as Prader-Labhart-Willi syndrome) is a polygenic disorder originally described in children with severe hypotonia, small hands and feet, short stature, obesity, cognitive impairment, and hypogonadism (Prader 1956). The consensus diagnostic criteria for the diagnosis of PWS were revised by Holm and others in 1993 to include major criteria of hypotonia, feeding problems during infancy, excessive weight gain between one and 6 years, characteristic facial features, hypogonadism, developmental delay, hyperphagia, and deletion of chromosome 15q11-q13 (Holm et al. 1993). Minor criteria include decreased fetal movement or infant activity, characteristic behavioral problems (obsessive/compulsive behavior, stealing or lying, manipulation, especially regarding food, and temper tantrums), sleep apnea or other sleep disturbances, short stature, small hands and feet, narrow hands, skin picking, esotropia or myopia, hypopigmentation, thick or viscous saliva, and speech articulation defects. Supportive findings include normal neuromuscular studies, scoliosis and/or kyphosis, osteoporosis, early adrenal maturation, infantile temperature instability or altered temperature sensitivity in children and adults, decreased vomiting, a high pain threshold, and an unusual ability to solve jigsaw puzzles. Major criteria are given a score of one point and minor criteria half a point, with no score but increased certainty with supportive findings. Children aged three years or younger require five points for diagnosis, four of which must

come from major criteria, and children over three years of age require a total score of eight points for diagnosis, five of which must come from major criteria.

After the genetic basis of PWS was discovered, which will be described later, the criteria were revised to facilitate diagnosis via molecular testing. Depending on the age of the patient, different features suggestive of PWS are required to prompt molecular testing: Hypotonia and weak suck from birth to two years of age; global developmental delay with a history of neonatal hypotonia and weak suck in early childhood; developmental delay, hyperphagia, with a history of neonatal hypotonia and weak suck in later childhood; or the combination of mild mental retardation with characteristic behaviors, hypothalamic hypogonadism, and hyperphagia in adults (Gunay-Aygun et al. 2001). Molecular testing for PWS based on hypotonia and weak suck, the only aspect of PWS consistently identifiable in neonatal patients, has resulted in earlier clinical intervention and, with approval of growth hormone treatment, is credited with dramatically improving PWS patient height and body composition.

1.1.2 Genetic basis of PWS

PWS occurs approximately once in 25,000 live births, irrespective of race or ethnicity, and is the most common genetic disorder causing life-threatening obesity (Butler 1990). PWS is most often caused by a sporadic microdeletion of the paternally inherited chromosome 15q11-q13 (Butler and Palmer 1983; Knoll et al. 1989; Ledbetter et al. 1981) (Figure1.1). This region of the genome is differentially imprinted, or silenced by methylation, during gametogenesis, resulting in maternal- or paternal-specific expression rather than bi-allelic

expression (Driscoll et al. 1992). The imprinting center (IC) locus determines whether the allele will express maternal or paternal transcripts (Gabriel et al. 1999). Four protein-coding genes, including *SNURF-SNRPN*, *MKRN3*, *NDN*, and *MAGEL2*, and a suite of small nucleolar RNAs (snoRNA) in this region are subject to genomic imprinting, which silences the maternal allele. Therefore, paternal deletion of chromosome 15q11-q13, maternal uniparental disomy (UPD) for chromosome 15 (Cassidy et al. 1992; Nicholls et al. 1989), or defects in the IC that control expression of this region (Buiting et al. 1995; Sutcliffe et al. 1994) result in PWS.

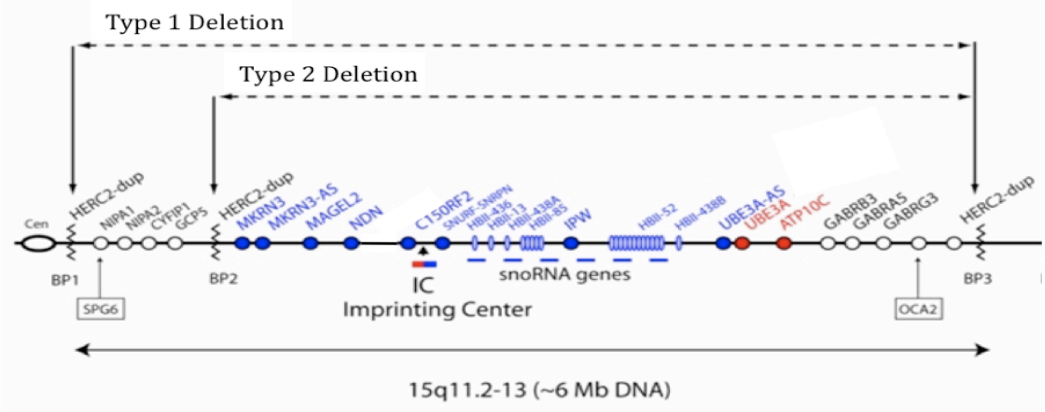

Figure 1.1. Organization of the Prader-Willi/Angelman syndrome region on chromosome 15. Gene names in blue are paternally expressed and expression is absent in people with PWS, while gene names in red are maternally expressed and expression is absent in people with AS. Adapted from http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=angelman.

The *SNURF-SNRPN* locus is a complex transcriptional unit, encoding 148 exons (Runte et al. 2001), including *SNURF*, which encodes a small nuclear ring finger protein (Moilanen et al. 1998), *SNRPN*, which encodes small nuclear RNA associated polypeptide SmN (Leff et al. 1992; Ozcelik et al. 1992), as well as introns that host a suite of snoRNAs (Cavaille et al. 2000; de los Santos et al. 2000). SnoRNAs are non-coding RNAs that modulate the processing of RNA transcripts, and PWS snoRNA *HBII-52* is known to regulate alternative splicing of the serotonin receptor 2C by binding and silencing exon Vb (Kishore and Stamm 2006). *MKRN3* (also known as ZNF127) encodes a RING zinc-finger protein (Jong, Carey et al. 1999; Jong, Gray et al. 1999), but the function of this protein is poorly understood. *NDN* and *MAGEL2* encode proteins that belong to the melanoma antigen (MAGE) homology protein family, characterized by the presence of the MAGE homology domain (MHD) (Barker and Salehi 2002). *NDN*, encoding necdin, was first identified as a gene whose expression is up-regulated in the P19 embryonal carcinoma cell line induced to differentiate into neurons via treatment with retinoic acid (Maruyama et al. 1991). Necdin promotes differentiation of neurons (Aizawa et al. 1992; Kobayashi, Taniura, and Yoshikawa 2002; Kuwajima, Nishimura, and Yoshikawa 2006; Maruyama et al. 1991; Takazaki, Nishimura, and Yoshikawa 2002), skeletal (Bush and Wevrick 2008; Deponti et al. 2007; Kuwajima et al. 2004) and smooth muscle (Brunelli et al. 2004), promotes cell-cycle exit (Hayashi et al. 1995; Kubota et al. 2009; Kuwako, Taniura, and Yoshikawa 2004; Liu, Elf et al. 2009; Taniura, Kobayashi, and Yoshikawa 2005; Taniura, Matsumoto, and Yoshikawa 1999; Taniura et al.

1998; Taniura and Yoshikawa 2002), and inhibits apoptosis (Andrieu et al. 2006; Deponti et al. 2007; Hasegawa and Yoshikawa 2008; Kurita et al. 2006; Kuwako et al. 2005; Kuwako, Taniura, and Yoshikawa 2004; Takazaki, Nishimura, and Yoshikawa 2002; Taniura, Matsumoto, and Yoshikawa 1999). Both necdin and MAGEL2 associate with centrosomal proteins (Lee et al. 2005); however, only necdin appears to be necessary for axonal or neurite extension (Kobayashi, Taniura, and Yoshikawa 2002; Lee et al. 2005; Liu, Wang et al. 2009; Pagliardini et al. 2005; Tennese, Gee, and Wevrick 2008). MAGEL2 mRNA is highly expressed in the hypothalamus (Lee et al. 2000; Lee, Walker, and Wevrick 2003) and its expression follows a circadian rhythm (Kozlov et al. 2007), although the function of MAGEL2 protein is not known. The extent to which loss of a particular gene contributes to the pathogenesis of PWS has been examined using mouse models, and will be described below.

Table 1. PWS candidate genes and known functions

Gene	Protein or transcript	Mouse Model	Function
MKRN3	MKRN3	-	RING finger protein (Jong, Carey et al. 1999; Jong, Gray et al. 1999)
MAGEL2	MAGEL2	*Magel2*-null mice display circadian, reproductive and behavioral defects (Kozlov et al. 2007; Mercer et al. 2009; Mercer and Wevrick 2009)	MAGE family protein expressed in a circadian rhythm most highly in the hypothalamus (Kozlov et al. 2007)
NDN	NECDIN	Neonatal lethal (Gerard et al. 1999), pain insensitivity (Kuwako et al. 2005), and increased skeletal muscle apoptosis (Deponti et al. 2007)	Necdin promotes neuronal differentiation (Aizawa et al. 1992; Kobayashi, Taniura, and Yoshikawa 2002; Kuwajima, Nishimura, and Yoshikawa 2006; Maruyama et al. 1991), skeletal and smooth muscle (Brunelli et al. 2004; Bush and Wevrick 2008; Deponti et al. 2007; Kuwajima et al. 2004), promotes cell cycle exit (Hayashi et al. 1995; Kubota et al. 2009; Kuwako, Taniura, and Yoshikawa 2004; Liu, Elf et al. 2009; Taniura, Kobayashi, and Yoshikawa 2005; Taniura, Matsumoto, and Yoshikawa 1999; Taniura et al. 1998; Taniura and Yoshikawa 2002), and inhibits apoptosis (Andrieu et al. 2006; Deponti et al. 2007; Hasegawa and Yoshikawa 2008; Kubota et al. 2009; Kurita et al. 2006; Kuwako et al. 2005; Takazaki, Nishimura, and Yoshikawa 2002; Taniura, Matsumoto, and Yoshikawa 1999)
SNURF-SNRPN	Small nuclear ribonucleoprotein polypeptide N	No phenotype {Yang, 1998 #901}	Neuronally enriched small nuclear ribonuclearprotein particle found in splicosomes (Ozcelik et al. 1992)

snoRNAs	HBII-85 HBII-52	MBII-85-null mice display growth retardation and have reduced weight gain despite over-eating (Ding et al. 2008; Skryabin et al. 2007)	HBII-52 regulates splicing of serotonin receptor subunit RNA (Kishore and Stamm 2006)

Angelman syndrome (AS) is a phenotypically unrelated syndrome caused by the loss of 15q11-q13 from the maternal allele (Knoll et al. 1989). Imprinting of *ATP10C* and *UBE3A* on the paternal allele prevents compensation, and people with AS are functionally null for these two genes (Albrecht et al. 1997; Rougeulle, Glatt, and Lalande 1997; Vu and Hoffman 1997). Mutations in *UBE3A* are sufficient to cause AS (Kishino, Lalande, and Wagstaff 1997; Matsuura et al. 1997).

In addition to the imprinted genes found in the PWS/AS critical region, there are a number of non-imprinted genes. Consequently, PWS and AS patients with 15q11-q13 deletions are hemizygous for *C15orf2*, *GABARB3*, *GABARA5*, *GABARG3*, *OCA2*, and *HERC2* (Buiting and Horsthmke. Molecular Genetic Findings in Prader-Willi Syndrome in (Butler 2006)). Previously believed to be expressed only in the testis (Farber et al. 2000), monoalellic expression of *C15orf2* was subsequently detected in fetal brain (Buiting et al. 2007) and the use of antibody staining has detected C15orf2 protein, although the function remains unknown (Wawrzik et al. 2009). *Oca2* was identified as the gene mutated at the pink-eyed dilution locus, and mutations in *OCA2* are responsible for human type 2 oculocutaneous albinism (Gardner et al. 1992; Rinchik et al. 1993). Hypopigmentation in people with PWS is attributed to haploinsufficiency for *OCA2*. Polymorphisms in *HERC2* are responsible for differences in iris colour (Kayser et al. 2008; Sturm et al. 2008) and radiation-induced mouse mutants deficient for *Herc2* are runted, have neuromuscular defects, and the males are sterile (Walkowicz et al. 1999; Ji et al. 1999; Lehman et al. 1998). HERC2

functions to promote DNA repair (Bekker-Jensen et al. 2010), but how haploinsufficiency of *HERC2* might contribute to the PWS or AS phenotype is not clear. The genes encoding GABA receptor subunits, *GABARB3*, *GABARA5*, *GABARG3*, are not normally imprinted but show monoallelic expression in a subset of individuals with autism spectrum disorder (ASD) (Hogart et al. 2007). This is an interesting finding given that PWS patients and individuals with ASD share similar behavioral defects and cognitive impairment, and suggests that haploinsufficiency of GABA receptor subunit genes may contribute to the PWS phenotype (discussed below).

The deletion of paternally expressed genes on chromosome 15 can occur as the larger type 1 deletion, spanning from centromeric breakpoint (BP) 1 to telomeric BP 3, or the smaller type 2 deletion, which spans from centromeric BP 2 to telomeric BP 3 (Christian et al. 1995). Roughly 30-40% of PWS patients with a paternal deletion have the larger type one deletion and are hemizygous for four genes, *Non-imprinted in Prader-Willi/Angelman syndrome-1* (*NIPA-1*), *NIPA-2*, *CYFIP1*, and *GCP5*, normally encoded by the 550 kilobases between BP 1 and BP 2 (Chai et al. 2003). Mutations in *NIPA-1* (also known as *SPG6*) are found in patients with an autosomal dominant form of hereditary spastic paraplegia (Rainier et al. 2003), and both *NIPA-1* and *NIPA-2* encode magnesium transporters (Goytain et al. 2007; Goytain, Hines, and Quamme 2008). *GCP5* encodes a protein component of the γ-tubulin complex (Murphy et al. 2001) that is important for proper mitotic spindle assembly (Izumi et al. 2008). *CYFIP1* encodes a protein that interacts with the Fragile X mental retardation protein

(FMRP) (Schenck et al. 2001), and CYFIP1 has subsequently been shown to control neuronal connectivity in *Drosophila* (Schenck et al. 2003) and suppress epithelial cancer invasion (Silva et al. 2009). A recent publication described nine patients with a microdeletion between BP1 and BP2 who suffered from attention deficit hyperactivity disorder, autism, obsessive-compulsive behavior, dysmorphology, and speech and motor delay (Doornbos et al. 2009). These results support the theory that heterozygosity for the genes encoded between BP1 and BP2 contributes to increased cognitive impairment in PWS individuals with type 1 deletions (Bittel, Kibiryeva, and Butler 2006; Butler et al. 2004).

The genetic basis of PWS has been challenged recently by reports of individuals who have phenotypes similar to PWS, but have atypical genetic alterations on chromosome 15q11-q13 (Sahoo et al. 2008; Kanber et al. 2009; de Smith et al. 2009). These reports suggest that loss of the snoRNAs significantly contributes to obesity, developmental delay, and hypogonadism in patients with PWS. However, these authors have not explicitly ruled out the possibility that other PWS candidate genes could be dysregulated in relevant tissues, such as brain or muscle, as only patient lymphobast samples were used.

1.1.3 Hypotonia is a cardinal feature of PWS

Hypotonia in PWS infants is profound, and it is often this symptom that prompts molecular testing for PWS. PWS infants display poor suck reflex, resulting from hypotonia, and this leads to characteristic failure to thrive during the first year of life (Holm et al. 1993). Hypotonia also contributes to hypoventilation in PWS patients. Neonatal hypotonia in PWS is central rather

than peripheral, and is comparable to Werdnig-Hoffmann disease, Zellweger syndrome, or congenital myotonic dystrophy, but differs from these disorders in that PWS hypotonia is non-progressive (McCandless and Cassidy. Diagnostic Criteria for PWS in (Butler 2006)). Individuals with PWS have reduced lean muscle mass and abnormalities in muscle fiber type have been described in infants with PWS (Argov et al. 1984; Chitayat et al. 1989; Sone 1994; Miike et al. 1988), although patients typically have normal neuromuscular function (Holm et al. 1993). PWS infants are delayed in reaching motor milestones and, while there appears to be anecdotal evidence that particular motor difficulties might result from certain muscle groups being more severely affected than others (Goelz. Motor and Developmental Interventions in (Butler 2006)), specific muscle defects have not been thoroughly documented. The lack of evidence is presumably due to the profound overlying hypotonia that would make specific muscle defects difficult to detect. Results from mouse studies suggest that hypotonia in PWS likely results from a combination of intrinsic myopathy, neurological contribution (see below), as well as endocrine defects later in life; however, the cause(s) remain poorly understood.

1.1.4 Neurological aspects of PWS

Cognitive impairment in PWS varies from mild to moderate, with a mean full-scale intelligence quotient between 60-70 (Cassidy and Driscoll 2009). The IQ of PWS individuals is correlated with parental IQ (Malich et al. 2000), suggesting that variability in cognitive ability can be explained by genetic background. This finding is supported by a study that found little phenotypic

difference between PWS patients with typical deletions and PWS patients with maternal UPD of chromosome 15 (Webb et al. 2002). However, cognitive impairment is more severe in PWS patients with the larger type 1 deletion (Bittel, Kibiryeva, and Butler 2006; Butler et al. 2004), implicating the genes located between BP1 and BP2 as modifiers. Compared to others with intellectual disability and obesity, individuals with PWS strongly favor routines, hoard items, and will repeatedly share a particular thought or question with others (Clarke et al. 2002). However, this behavior is not consistent with activities observed in individuals with obsessive-compulsive disorder. Temper tantrums and impatience or impulsivity are very common in both children and adults with PWS, and roughly 50% of patients display angry or aggressive behavior (Dykens and Cassidy 1995). Psychotic episodes are present in approximately 10% of PWS patients and are far more frequent in those with maternal UPD or imprinting center defects, suggesting that defective imprinting of dosage-sensitive loci causes psychiatric illness in PWS (Soni et al. 2008). While this is an intriguing hypothesis, the gene (or set of genes) responsible and putative mechanism by which an imprinting defect might cause psychiatric illness remain to be determined. How specific defects in neuroanatomy might contribute to the PWS phenotype is not completely understood. One study reported that PWS individuals have enlarged ventricles, polymicrogyria, and incomplete closure of the cortical insula (Miller et al. 2007), cortical defects that are consistent with impaired cognitive function. Although the mouse is not the ideal model in which to study human brain development, detailed analysis of neuroanatomical defects

in the mouse models of PWS could provide insight into specific subtle defects visible upon closer inspection of patients.

1.1.5 Related disorders

There are a number of other genetic disorders with clinical symptoms that overlap with PWS, including obesity, developmental delay, and hypotonia. Bardet-Biedl syndrome (BBS) is an autosomal recessive disorder characterized by retinitis pigmentosa, obesity, renal dysfunction, polydactyly, and developmental delay (Beales et al. 1999). BBS results from the mutation of a BBS gene, and although the BBS genes are not related by nucleotide or amino acid sequence, they all appear to have shared roles in promoting normal cilia function (Zaghloul and Katsanis 2009). Cilia are grouped as motile, such as the flagella on sperm or on the epithelial cells lining the airways, or as non-motile primary cilia, which are found on most vertebrate cells and contain a high concentration of sensory proteins important for the interpretation of extracellular signals (Berbari et al. 2009). Necdin and MAGEL2 interact with BBS4 and co-localize around the basal body in transfected cells, suggesting that loss of necdin and MAGEL2 in PWS or BBS4 in BBS affects similar centriole-associated cellular processes resulting in phenotypic similarity between syndromes (Lee et al. 2005). Recent advances in understanding the function of proteins encoded by genes mutated in disorders related to BBS, including Meckel-Gruber syndrome (Smith et al. 2006; Kyttala et al. 2006), Joubert syndrome (Arts et al. 2007; Baala et al. 2007; Cantagrel et al. 2008; Delous et al. 2007; Sayer et al. 2006; Valente et al. 2006), Senior-Løken syndrome (Otto et al. 2005), and McKusick-Kaufman syndrome (Stone et al.

2000; Katsanis et al. 2000) have led to their classification as 'ciliopathies' (Badano et al. 2006). Descriptions of ciliary function in PWS have not been published, so it remains to be determined whether PWS can be considered a ciliopathy.

Disorders grouped under autism spectrum disorders (ASD) include autism, Asperger's syndrome, and pervasive developmental disorder-not otherwise specified, and are characterized clinically by persistent or repetitive behavior, reduced quality of communication, and severely impaired reciprocal socialization (Levy, Mandell, and Schultz 2009). People with PWS display persistent and repetitive behaviors that are, in some ways, similar to those with ASD, and individuals with PWS due to maternal UPD are thought to display slightly stronger ASD phenotypes (Veltman et al. 2004; Dykens, Sutcliffe, and Levitt 2004). This has led to the hypothesis that elevated levels of paternally imprinted *UBE3A* and *ATP10C* in maternal UPD PWS patients might contribute to the ASD phenotype in these individuals. Interestingly, the same flanking sequences that predispose the 15q11-q13 region to deletion also appear to promote duplications. Duplication of the PWS critical region is the most common chromosomal abnormality associated with autism and ASD, and a mouse model with an engineered duplication of the syntenic region on chromosome 7 displays social abnormalities (Nakatani et al. 2009). These transgenic mice showed elevated levels of mRNA for genes from the duplicated region and primary neuron cultures displayed an elevated response to serotonin receptor agonists, presumably due to elevated levels of *HBII-52* (Kishore and Stamm 2006).

It has been suggested that differences in the ratio of excitatory to inhibitory neurons may underlie neurological defects in people with autism and related disorders (Dykens, Sutcliffe, and Levitt 2004; Rubenstein and Merzenich 2003). The PWS critical region contains three genes encoding subunits of GABA receptors, which are expressed on inhibitory interneurons in the cortex. It is unlikely that changes in GABAergic interneuron activity result in phenotypic aspects shared between PWS and ASD patients, since PWS individuals with chromosomal deletions will carry half the normal number of GABA receptor subunit genes and ASD individuals with a duplication of the PWS critical region will carry one and a half the normal number. However, it is possible that increased or decreased magnitude of GABA receptor subunit expression is not as important, given the fact that interneurons may be dysregulated in both disorders. Furthermore, necdin promotes differentiation and survival of GABAergic neurons in mice (Kuwajima, Nishimura, and Yoshikawa 2006), suggesting that this neuronal population might be particularly sensitive to gene dosage in the PWS critical region.

Fragile X syndrome is caused by the silencing of *FMR1*, encoding the RNA-binding protein FMRP, and is characterized by cognitive impairment, elongated ears, a long face with pointed chin, macroorchidism, and autistic behaviors (Garber, Visootsak, and Warren 2008). There is a subpopulation of Fragile X patients with a Prader-Willi-like phenotype (PWP), which is characterized by obesity, small hands and feet, and sometimes hypotonia, in addition to developmental delay (de Vries et al. 1993; De Vries and Niermeijer

1994; Schrander-Stumpel et al. 1994). The molecular basis of PWP is heterogeneous and includes deletion of chromosome 1p36 (D'Angelo et al. 2006; Eugster, Berry, and Hirsch 1997; Tsuyusaki et al. 2010), interstitial deletions on chromosome 6 (Faivre et al. 2002; Gilhuis et al. 2000; Stein et al. 1996; Varela et al. 2006; Villa et al. 1995), monosomy of chromosome 6q1 (Turleau et al. 1988), monosomy of chromosome 10q26 (Lukusa and Fryns 2000), chromosome 14 maternal UPD (Berends et al. 1999; Hordijk et al. 1999; Cox, Bullman, and Temple 2004; Aretz et al. 2005; Mitter et al. 2006; Hosoki et al. 2009), and X chromosome aberrations (Chudley, Lowry, and Hoar 1988; Monaghan, Van Dyke, and Feldman 1998; Lammer et al. 2001; Florez, Anderson, and Lacassie 2003; Lachlan et al. 2004; Gabbett et al. 2008; Sanlaville, Schluth-Bolard, and Turleau 2009). Therefore, the genes affected in Fragile X patients with PWP likely encode proteins that function in pathways shared with those encoded by PWS candidate genes.

There are a small number of cases describing obese people with mutations affecting the genes encoding brain-derived neurotrophic factor (BDNF) or TrkB, the cognate receptor for this ligand. Mutations affecting *NTRK2*, encoding TRKB, were identified in four severely obese children with developmental delay (Gray et al. 2007; Yeo et al. 2004). Only one mutation was found to impair receptor function in an *in vitro* assay. A number of polymorphisms and chromosomal rearrangements affecting *BDNF* have been identified in obese individuals (Beckers et al. 2008; Friedel et al. 2005; Gray et al. 2006; Han et al. 2008). Other genes mutated in people with severe obesity include those encoding

leptin (Montague et al. 1997) and the leptin receptor (Clement et al. 1998), pre-pro-opiomelanocortin (Krude et al. 1998), melanocortin-4 receptor (Vaisse et al. 1998; Yeo et al. 1998), and *SIM1* (Holder, Butte, and Zinn 2000).

1.1.6 Mouse models of PWS

The multigenic nature of PWS makes it difficult to understand how loss of each gene contributes to the phenotype. Mice are suitable model organisms for the study of PWS, as organization of the PWS critical region on chromosome 15 in humans is similar to the syntenic region of mouse chromosome 7. Targeted gene disruption has produced several mouse models of PWS in which single or multiple PWS candidate genes have been eliminated. Studies of these mice have been useful in understanding how PWS candidate genes normally function and how a mammalian model system develops in their absence.

Three mouse models examined the effect of eliminating all paternal expression from the PWS critical region. The first took advantage of the *Is1CtX*-autosome mouse, in which the central portion of mouse chromosome 7, containing the PWS critical region, is translocated to the X chromosome (Cattanach et al. 1992). Females carrying an unbalanced translocation, which possess two copies of chromosome 7 plus the portion translocated to the X chromosome, were mated to males carrying a balanced translocation. Only male progeny were examined, as either normal or translocation-carrying X chromosomes were randomly inactivated in females. Male mice that inherited the paternal copy of chromosome 7 missing the central region, and both the normal chromosome 7 and the X chromosome carrying the translocation, were effectively

maternally disomic for the PWS critical region. Since these mice have no paternal contribution from the PWS critical region, they are genetically similar to PWS patients with maternal uniparental disomy. This was confirmed when the authors could not detect *Snrpn* expression via Northern blotting. Interestingly, these mice were smaller and failed to thrive, phenotypically resembling PWS infants. All mice missing the paternal copy of chromosome 7 either died or were euthanized 2-8 days after birth.

Six years later, targeted deletions were used to generate a pair of PWS mouse models, with the first targeting exons 5-7 of *Snrpn* and the second generating a 42 kb deletion of the putative IC and exons 1-6 of *Snrpn* (Yang et al. 1998). Analysis of paternally expressed genes from the PWS critical region, including *Ndn*, was normal in *Snrpn*-null mice but completely absent from IC deletion mice, confirming that the IC is necessary for normal paternal expression of PWS candidate genes. Snrpn-null mice displayed no overt phenotype, suggesting that loss of *SNRPN* is unlikely to contribute to the etiology of PWS. The IC deletion mice did not display embryonic lethality, but failed to gain weight despite nursing and died between 5 and 8 days after birth.

The third mouse model lacking expression from PWS critical region is the *TgPWS/AS(del)* mouse, in which the insertion of the Epstein-Barr virus Latent Membrane Protein 2A transgene deleted the entire PWS/AS homologous region (Gabriel et al. 1999). Mice that inherit the paternal transgene allele show reduced motor activity and typically die less than one week after birth. The striking phenotypic similarity among the maternal UPD, IC deletion, and *TgPWS/AS(del)*

mice implicates loss of paternally expressed PWS candidate genes in neonatal failure to thrive.

A mouse mutagenesis project identified the *p30Pub* allele, containing a deletion of chromosome 7 that extends from the pink-eyed dilution locus to a breakpoint in the PWS critical region (Ding et al. 2005). These authors report that *MBII-52* snoRNA expression is lost, but *MBII-85* snoRNA expression is unaffected by paternal loss of *p30Pub*. Given that mice expressing the paternal *p30Pub* allele are phenotypically normal, loss of *HBII-52*, the human orthologue of *MBII-52*, is unlikely to contribute to the PWS phenotype. Targeted deletion of paternally inherited snoRNA *MBII-85* (also known as *Snord116* or *Pwcr1*) in mice results in mild lethality (85% mutant survival) and growth retardation (Skryabin et al. 2007). In a second *MBII-85*-null mouse model, mutant mice do not display any lethality, but have reduced weight gain despite hyperphagic behavior (Ding et al. 2008). These *MBII-85*-null mice also display a delay in reproductive maturation, and mild neurological and motor learning defects reminiscent of PWS. These authors conclude that defects in *MBII-85*-null mice are largely due to defects in endocrine function, including dysregulation of growth hormone, gonadotropin hormone-releasing hormone, and ghrelin. The molecular mechanism(s) by which *MBII-85*/*HBII-85* normally regulates the physiological integration of these diverse signals remains to be determined.

Unlike the other PWS candidate genes, *Magel2* expression follows a circadian pattern and *Magel2*-null mice display circadian defects (Kozlov et al. 2007). While not a cardinal feature of PWS, excessive daytime sleepiness and

prolonged arousal have been described (Vgontzas et al. 1996). In addition to circadian defects, *Magel2*-null mice display abnormal obese body composition (Bischof, Stewart, and Wevrick 2007), fertility defects (Mercer and Wevrick 2009), altered brain volume, serotonin metabolism, and behavior (Mercer et al. 2009). *Magel2* is highly expressed in the hypothalamus (Lee et al. 2000; Lee, Walker, and Wevrick 2003), the region of the brain critical for regulation of circadian rhythm, appetite, and sexual maturation. Preliminary evidence suggests that Magel2 interacts with and regulates the activity of core circadian transcription factors (S. Weselake and R. Wevrick, unpublished data), indicating that Magel2 may function to regulate the homeostasis of circadian rhythms in the hypothalamus.

Among the PWS candidate genes, *NDN*, encoding NECDIN, is the best characterized. Four independent *Ndn*-null mice have been created, resulting in an unexpected spectrum of phenotypes. The first *Ndn*-null mouse (Ndn^{tm1Alb}) was created by replacing most of the *Ndn* coding sequence with a beta-galactosidase reporter, and was overtly normal, displaying none of the phenotypes associated with PWS (Tsai, Armstrong, and Beaudet 1999). The Ndn^{tm2Stw} mutant, described shortly thereafter, was created by replacing most of the *Ndn* coding sequence with a LacZ reporter cassette. The Ndn^{tm2Stw} mutant is the mouse model ("*Ndn*-null") used to obtain the results described in the data chapters of this thesis. Most Ndn^{tm2Stw} mutants die shortly after birth attributed to hypoventilation due to defects in neuromodulatory drive of the respiratory rhythm generating pre-Bötzinger complex (Gerard et al. 1999; Pagliardini et al. 2005; Ren et al. 2003). These *Ndn*-

null mice display defects in axonal outgrowth and fasciculation, with a reduced anterior commissural tract, and have ectopic axonal whorls in the hypothalamus, striatum, and internal capsule (Lee et al. 2005; Pagliardini et al. 2005). Reduced innervation of sympathetic nervous system targets, and impaired migration of the superior cervical ganglia, gonadotropin hormone-releasing hormone neurons, and GABAergic interneurons have also been reported (Kuwajima, Hasegawa, and Yoshikawa 2010; Miller, Wevrick, and Mellon 2009; Tennese, Gee, and Wevrick 2008).

Although the phenotypic differences between the *Ndn*^{tm2Stw} mutant and the *Ndn*^{tm1Alb} mutant were significant, the targeting vectors used to generate the mice were remarkably similar. Both constructs used a *BamHI* site near the 5' end of the *Ndn* coding sequence to insert the reporter construct, such that both mice express a fusion product containing the first thirty-one amino acids normally found on the N-terminus of necdin. The *Ndn*^{tm2Stw} mutant does not carry the neomycin selection cassette, as this sequence was flanked by Flox sequences and was excised after mating to a mouse ubiquitously expressing Cre recombinase. The *Ndn*^{tm1Alb} mutant carried the beta-galactosidase reporter as well as the neomycin resistance cassette. It is unclear what strain of mouse or breeding scheme was used by Tsai and others, but the difference in phenotypes is most likely due to strain-specific differences (Nicholls 1999). This notion is further supported by results from recent attempts to improve *Ndn*^{tm2Stw} mutant survival where out-breeding to the CD1 strain of mice has elevated survival to roughly 15% (J. Bischof and R. Wevrick, unpublished observations).

Two other *Ndn*-null mice have been described and neither of these mice displays the severe lethality of the *Ndn*^{tm2Stw} mutant. Muscatelli and colleagues used a loxP-flanked Neomycin construct inserted into the *Ndn* locus disrupting the first two thirds of the coding sequence to create the *Ndn*$^{tm1.1Mus}$ mutant (Muscatelli et al. 2000). Survival of mutant mice in which the Neo cassette was deleted was between 36-69%, with some *Ndn*$^{tm1.1Mus}$ mutant pups dying of respiratory distress shortly after birth. This observation confirms necdin as an essential protein during development of the respiratory control center (Pagliardini et al. 2005; Pagliardini et al. 2008; Zanella et al. 2008). Surviving *Ndn*$^{tm1.1Mus}$ mutant mice had reduced numbers of oxytocin-releasing and luteinizing hormone-releasing hormone neurons, and behavioral abnormalities reminiscent of PWS, including skin scraping and increased spatial learning (Muscatelli et al. 2000). These *Ndn*-null mice display increased levels of apoptosis in both developing neurons (Andrieu et al. 2006) and muscle (Deponti et al. 2007), supporting the hypothesis that necdin inhibits apoptosis or otherwise promotes survival.

Similar to the method employed by Gerard *et al*. and Tsai *et al*., Yoshikawa and colleagues used a Pgk1/Neomycin reporter cassette inserted into the *Ndn* coding sequence to disrupt *Ndn* expression using the *BamHI* site to create the *Ndn*^{tm1Ky} mutant (Kuwako et al. 2005). These mice display pain insensitivity due to diminished responsiveness to nerve growth factor (NGF) and consequentialy have increased apoptosis in dorsal root ganglia (DRG)(Kuwako et al. 2005), defects in the differentiation and migration of GABAergic neurons (Kuwajima, Hasegawa, and Yoshikawa 2010; Kuwajima, Nishimura, and

Yoshikawa 2006), elevated levels of apoptosis in neurons (Hasegawa and Yoshikawa 2008; Kurita et al. 2006), and replenish hematopoietic stem cells more rapidly following myelosuppressive injury (Kubota et al. 2009).

1.1.7 Necdin is a Multifunctional Protein

Ndn was originally identified in a subtraction screen of genes up-regulated in retinoic acid-treated P19 embryonal carcinoma cells (Maruyama et al. 1991) and is an intronless gene encoding a protein 325 amino acids long. Necdin belongs to the MAGE homology family of proteins, which are characterized by the presence of the poorly understood MAGE homology domain (Barker and Salehi 2002). *Ndn* mRNA was detected in both adult and developing mouse brain and immunostaining with the first antibody raised against necdin suggested that the protein was primarily located in the nuclei of differentiated neurons (Aizawa et al. 1992; Maruyama et al. 1991). Immunostaining with a second antibody demonstrated that necdin is present in both the nucleus and cytoplasm (Niinobe, Koyama, and Yoshikawa 2000). Over-expression of necdin led to cell cycle arrest (Hayashi et al. 1995) through interactions with E2F1 and p53 (Taniura, Matsumoto, and Yoshikawa 1999; Taniura et al. 1998), consistent with the hypothesis that necdin promoted differentiation via nuclear activities.

Interest in necdin function grew when *NDN* was identified as one of the genes deleted in people with PWS (Jay et al. 1997; MacDonald and Wevrick 1997; Sutcliffe et al. 1997) and led to the generation of *Ndn*-null mouse models which generally support the premise that necdin is important for neuronal differentiation and survival as described above (Gerard et al. 1999; Kuwako et al.

2005; Muscatelli et al. 2000; Tsai, Armstrong, and Beaudet 1999). While necdin has been shown to bind directly to DNA under permissive conditions *in vitro* (Matsumoto et al. 2001), necdin generally influences transcription by modulating either the subcellular localization of transcription factors or co-factors such as Msx2 or EID-1 (Brunelli and Cossu 2005; Bush and Wevrick 2008) or the co-activator/repressor function of transcription factors including Msx2, MyoD, ARNT2, HIF-1α, p53, E2F1, and Dlx2 (Brunelli et al. 2004; Deponti et al. 2007; Friedman and Fan 2007; Hasegawa and Yoshikawa 2008; Kurita et al. 2006; Kuwajima, Nishimura, and Yoshikawa 2006; Kuwajima et al. 2004; Miller, Wevrick, and Mellon 2009; Moon et al. 2005).

In addition to regulating transcription, necdin binds to and regulates signal transduction downstream of TrkA and p75 receptors (Bronfman et al. 2003; Ingraham and Schor 2009; Kuwako et al. 2005; Tcherpakov et al. 2002). Although these results invoke a new mechanism by which necdin functions, they are consistent with increased levels of apoptosis in *Ndn*-null mice. Necdin interacts with Fez1, BBS4, and Nogo-A outside of the nucleus to control neurite and axonal extension, processes that are defective in neurons from *Ndn*-null mice (Lee et al. 2005; Liu, Wang et al. 2009). In summary, necdin appears to possess multiple functions, many of which are relevant to PWS. These multiple functions strengthen the hypothesis that loss of necdin leads to impaired neuronal and muscle cell differentiation, increased neuronal apoptosis, and axonal extension defects, which in turn contribute to hypotonia and cognitive impairment in PWS.

1.2 Vertebrate Myogenesis

1.2.1 Somitic Origin of Skeletal Limb Muscle

Axial segmentation of the mesoderm along the rostral-caudal axis via somitogenesis (E8.0 in mouse) precedes the specification of skin, bone, and muscle progenitors in the vertebrate embryo. Cells that give rise to each tissue type obtain instructive cues in both a spatial and temporal manner. For example, sonic hedgehog (Shh) secreted from the notochord induces the medial half of the somite to form the sclerotome (Fan and Tessier-Lavigne 1994; Johnson et al. 1994), while Wnt and BMP secreted from the ectoderm and lateral mesoderm induce the dorsal-lateral portion of the somite to become hypaxial muscle (Marcelle, Stark, and Bronner-Fraser 1997). Subsequently, somites dissolve, with *Pax1* expression defining the disaggregated cells of the sclerotome (Wallin et al. 1994), while expression of *Pax3* and *Pax7* define the dermomyotome (Goulding et al. 1991; Jostes, Walther, and Gruss 1990), which is then a crescent-shaped tissue stratified into epithelial layers. *Pax3*-expressing muscle progenitors in the ventral-lateral tip delaminate from the dermomyotome and migrate ventrally where they will eventually differentiate into the intercostal and abdominal muscles. At the axial level of the forelimbs, a proportion of these cells respond to chemokines secreted by the mesenchyme of the limb buds and migrate distally into dorsal and ventral pools in the limb anlagen (Bober et al. 1994; Franz et al. 1993; Goulding, Lumsden, and Paquette 1994; Williams and Ordahl 1994). The dorsal and ventral pools of *Pax3*-expressing cells respectively give rise to the developing extensor and flexor muscles of the limb (Brohmann, Jagla, and

Birchmeier 2000; Gross et al. 2000). Expression of *Lbx1* specifies a subpopulation of laterally-migrating muscle progenitors and ablation of *Lbx1* in mice results in reduced or absent forelimb extensor muscles while flexor muscles are unaffected (Brohmann, Jagla, and Birchmeier 2000; Gross et al. 2000; Schafer and Braun 1999). These observations suggest that combinatorial transcription factor activity is required for the specification and development of different muscle groups in the forelimb, summarized in Figure 1.2, and will be discussed in the subsequent section.

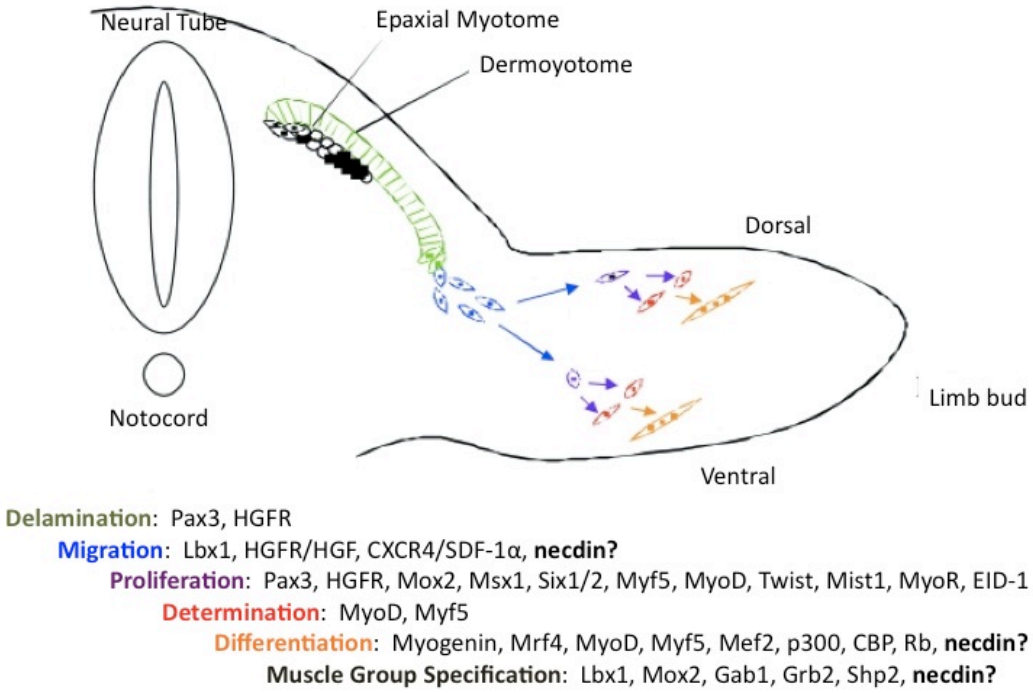

Figure 1.2. Development of limb musculature. Muscle progenitors delaminate from the ventral lip of the dermomyotome, proliferate while migrating into dorsal and ventral pools in the developing limb bud, and undergo primary differentiation to form multinucleate myotubes. Colour-coded cells represent different stages in muscle development and proteins important for each stage are listed below. Adapted from Buckingham and others (Buckingham et al. 2003).

Migratory muscle progenitors express receptors that respond to soluble chemokines secreted from the limb bud mesenchyme. Hepatocyte growth factor receptor (HGFR, also known as c-Met) and CXC chemokine receptor 4 (CXCR4) are two receptors known to mediate muscle progenitor migration into the developing limb (Bladt et al. 1995; Vasyutina et al. 2005). Pax3 drives the expression of *HGFR* in muscle progenitors (Yang et al. 1996; Epstein et al. 1996), and muscle progenitors in mouse mutants lacking *Pax3*, *HGFR*, or *HGF*, the cognate chemokine for HGFR, fail to migrate into the limb, and consequently do not form limb musculature (Dietrich et al. 1999). Similarly, genetic ablation of *CXCR4* reduces the number of muscle progenitors in the developing limb, and this defect is exacerbated when combined with the genetic ablation of *Gab1* (Vasyutina et al. 2005). Gab1 is a docking protein that is phosphorylated upon the activation of HGFR, and *Gab1*-/- mice have impaired muscle progenitor migration into the limb bud (Sachs et al. 2000). Similarly, *HGFR* mutations that impair interaction with the adaptor protein Grb2 (Maina et al. 1996; Maina et al. 2001) or a mutation in *Gab1* that impairs binding of the adaptor protein Shp2 (Schaeper et al. 2007) produce similar muscle progenitor migration defects. Thus, the proper distribution of limb musculature requires directed cellular migration of muscle progenitors.

1.2.2 Transcriptional Regulation of Myogenesis in the Limb

The differentiation of common progenitors into specialized cell types involves a hierarchical progression informed by spatial and temporal signals, which begins with the fertilization of an egg, proceeds past birth, and continues to

shape the organism throughout its life-time. Cell fate is controlled by DNA-binding transcription factors that act as molecular switches to regulate genetic expression in a combinatorial manner. With each type of cell expressing a common suite of genes, homologous populations can respond collectively to developmental cues to pattern tissues during embryogenesis. Differentiation of skeletal muscle has been well studied, providing insight into both the molecular mechanisms of transcription factors, as well as illuminating the complex origins of tissue during development. In 1988, Tapscott et al reported that the transfection of a single cDNA, encoding the basic helix-loop-helix (bHLH) gene *MyoD*, was sufficient to transform a fibroblast cell line into myogenic cells (Tapscott et al. 1988). MyoD was shown to associate with the retinoblastoma (RB) protein to promote cell cycle exit and allow muscle differentiation to occur (Gu et al. 1993). Subsequent studies identified a core group of muscle regulatory factors (MRFs), which include MyoD, Myf-5, MRF-4, and myogenin, whose collective activity during development highlights many of the nuances and subtleties involved in genetic regulation (Sartorelli and Caretti 2005).

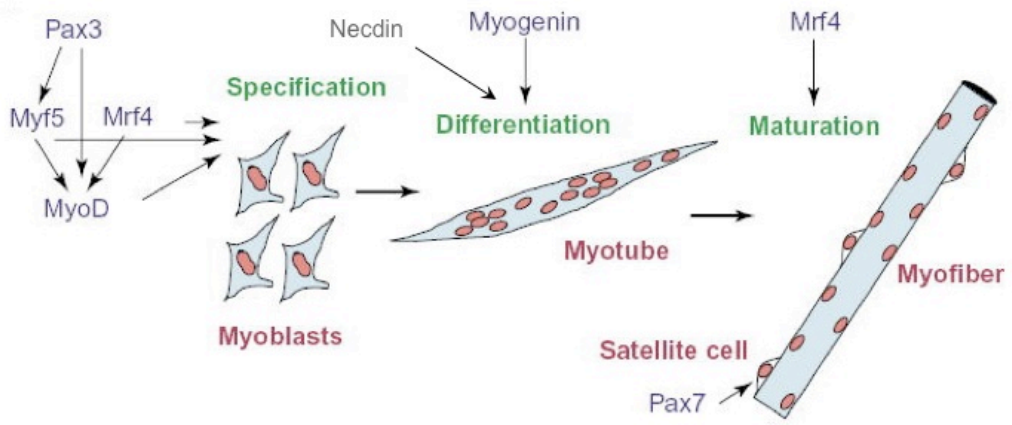

Figure 1.3. Summary of the temporal sequence of MRF activity. Pax3 acts to specify myoblasts, which express Myf5, MyoD, or Mrf4. Myogenin promotes cell-cycle exit and differentiation into multinucleate myotubes. Mrf4 is important for maturation to myofibres, while Pax7 is expressed in satellite cells located beneath the basal lamina. Necdin is hypothetically involved in the primary differentiation of myoblasts. Adapted from (Sartorelli and Caretti 2005).

The profound discovery that *MyoD*-/- mice are essentially normal and form muscle via prolonged, elevated *Myf-5* expression seemed paradoxical given the *in vitro* assessment of MyoD function (Rudnicki et al. 1992). Similarly, *Myf-5*-/- mice do not display a muscle phenotype (Braun et al. 1992). However, *MyoD*-/-;*Myf-5*-/- compound mutant mice do not develop skeletal muscle, demonstrating that MyoD and Myf-5 are largely functionally redundant (Rudnicki et al. 1993). Despite the apparent compensatory function, the MRFs are expressed in different spatial and temporal domains. For instance, in a normal mouse Myf-5 is active in forming the epaxial musculature of the trunk and body wall, while MyoD functions in forming the hypaxial musculature of the limbs (Kablar et al. 1997). Sequentially, *Myf-5* expression precedes that of MyoD, followed then by myogenin, with MRF-4 expression occurring primarily during postnatal development (Montarras et al. 1991).

As mentioned previously, cues such as Shh, the Wnts and BMPs instruct different regions of the somite to adopt specific tissue types, including cells fated to become muscle. Muscle progenitors of the dermomyotome express both *Pax3* and *Pax7*, related genes of the Paired domain homeobox family of transcription factors. Ectopic expression of *Pax3* is sufficient to drive *MyoD* expression, though homozygous *Splotch* (*Pax3* mutant) mice are able to form body muscle via Myf-5 instruction (Tajbakhsh et al. 1997). However, *Pax3* expression occurs at developmental time points and in developing structures where *MyoD* is not expressed, begging the question: How is *MyoD* expression delayed in Pax3-

expressing cells until these cells are in the proper location to differentiate into muscle?

Pax3, like all transcription factors, requires cooperation from a number of other proteins to effectively promote transcriptional activation. Additionally, the promoters of genes like *MyoD* are not simply permissive to activation, but rather are refractory to transcription via the residency of inhibitory transcription factors and repressive chromatin modifications. For example, Msx1 inhibits Pax3-dependent myogenesis in migrating muscle progenitors (Bendall et al. 1999) through direct binding of the MyoD promoter (Woloshin et al. 1995; Song, Wang, and Sassoon 1992) and by recruiting the repressive linker histone H1b to the core enhancer (Lee, Habas, and Abate-Shen 2004). By permitting *Pax3* expression but antagonizing Pax3-dependent MyoD activation, Msx1 maintains myoblasts in a proliferative state, poised to proceed with Pax3-dependent muscle differentiation. Msx1 represses its own expression, through inhibition of TATA-binding protein and p300 on its own promoter (Shetty et al. 1999), and through the expression of an antisense transcript (Blin-Wakkach et al. 2001). While the exact mechanism is not known, the transition from Msx1-positive myoblast to Msx1-negative myotube likely involves modulation of co-activators and co-repressors associated with both Msx1 and Pax3 to tip the balance in favor of myogenesis. Thus, it is not surprising that a number of other proteins known to influence muscle development are modulators of transcription.

Among the myriad of post-translational modifications that regulate transcription, the acetylation of histones and transcription factors are well

characterized (Kouzarides 2000). The acetylation of positively-charged lysine residues of histone tails neutralizes the residue, and is thought to decrease the affinity of histones for negatively-charged DNA. Loosening of DNA from histones facilitates sequence-specific binding of transcription factors, complexed with transcriptional co-activators, and the assembly of RNA polymerase machinery for robust transcription. Histone deacetylases (HDACs) repress transcription by associating with transcription factors to reverse histone acetylation and promote condensation of DNA around histones. Thus, histone acetylation is a dynamic mechanism by which cells regulate transcription.

Two related transcriptional co-activators, p300 and cyclic AMP response element-binding protein (CBP), have histone acetyltransferase (HAT) activity (Ogryzko et al. 1996; Bannister and Kouzarides 1996). Furthermore, direct acetylation of *MyoD* promotes transcriptional activity (Polesskaya et al. 2000). While p300 and CBP share both sequence and functional similarity, mice carrying a targeted deletion of either gene have different phenotypes, demonstrating that p300 and CBP are not redundant (Yao et al. 1998; Kung et al. 2000). For instance, acetyltransferase activity of p300, but not CBP, is critical for normal myogenic specification and development of muscle in the mouse (Roth et al. 2003). These results complement earlier studies demonstrating that the E1A viral protein blocks myogenic differentiation (Webster, Muscat, and Kedes 1988) by inhibiting the HAT activity of p300 and another myogenic co-activator p300/CBP associated factor (P/CAF) (Hamamori et al. 1999; Chakravarti et al. 1999).

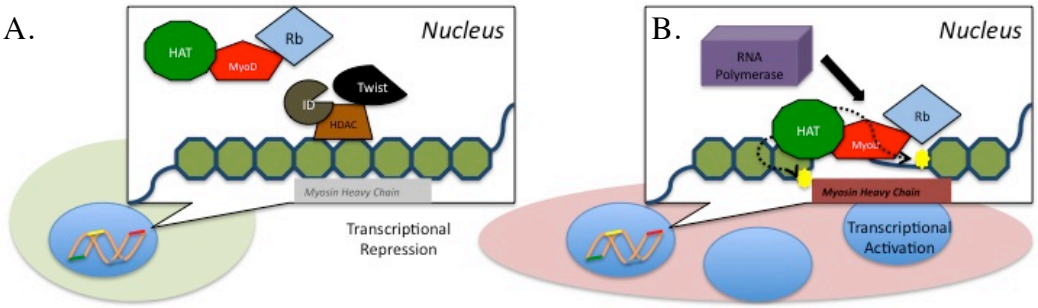

Figure 1.4. Chromatin regulation during muscle differentiation. (A) DNA (blue) of the Myosin Heavy Chain promoter is wrapped tightly around condensed chromatin (gray octagons) and histone acetylation is prevented by the presence of inhibitory proteins, such as Twist and HDACs. (B) Degradation of inhibitory proteins upon cell cycle exit allows histone acetylation (yellow stars), facilitating MyoD binding and assembly of RNA polymerase for robust transcription.

In addition to viral transformation, inhibition of histone acetylation occurs normally during the spatial and temporal regulation of muscle development. The protein Twist is a basic helix-loop-helix (bHLH) transcription factor that represses MyoD-dependent transcription by inhibiting requisite MyoD dimerization with E proteins (Hamamori et al. 1997), inhibiting the activity of the myocyte enhancing factor C2 (MEFC2) (Spicer et al. 1996), and inhibiting p300 HAT activity (Hamamori et al. 1999). There are a number of proteins functionally similar to Twist that inhibit myogenesis by interfering with DNA binding, dimerization with co-factors, or by inhibiting the activity of co-activators. Mist1 and MyoR are both bHLH transcription factors that inhibit myogenesis by binding to E-box DNA sequences, which are the consensus binding sites for MyoD. Mist1 additionally binds to MyoD, while MyoR binds to E proteins, thereby additionally inhibiting MyoD-dependent transcriptional activation (Lemercier et al. 1998; Lu et al. 1999).

The E1A-like inhibitor of differentiation (EID-1) is a developmentally regulated protein that inhibits p300-dependent HAT activity to inhibit MyoD-dependent muscle differentiation (MacLellan et al. 2000; Miyake et al. 2000), and provides an interesting example of how proliferating myoblasts differentiate into myotubes. *In vitro* experiments and evidence from non-muscle cells induced to differentiate revealed that the retinoblastoma protein (Rb) associates with EID-1 upon cell cycle exit. Rb simultaneously associates with the ubiquitin ligase MDM2 and promotes EID-1 ubiquitination, directing EID-1 to be degraded by the proteasome. In the absence of EID-1, the MyoD-p300 complex becomes a robust

transcriptional unit. Thus, the mechanism of EID-1 activity helps to explain how myoblasts expressing MyoD can resist MyoD-dependent muscle differentiation, and continue to proliferate until instructed to exit the cell cycle. While most studies typically focus on the interactions of only a few proteins *in vitro*, the mechanism by which muscle differentiation occurs *in vivo* clearly involves the co-ordination of many pathways and serves to highlight the complexity of transcriptional regulatory networks.

1.2.3 Transcription Factors and the Specification of Limb Muscle Groups

While the temporal transcriptional cascades necessary for myogenesis are well defined, the role transcription factors play in spatially defining muscle populations is relatively unknown. As mentioned previously, Pax3 regulates the expression of *HGFR*, which is critical for general migration of muscle precursors into the limb (Epstein et al. 1996; Yang et al. 1996). A number of transcription factors are expressed in the migratory pool of muscle precursors entering the limb bud. Mice in which these genes have been inactivated display a variety of phenotypes. Muscle progenitors fail to migrate into the dorsal domains of forelimb buds in *Lbx1*-/- mice, and these mice consequently fail to develop dorsally derived extensor muscles in the forelimbs (Brohmann, Jagla, and Birchmeier 2000; Gross et al. 2000; Schafer and Braun 1999). Expression of *CXCR4* is reduced in *Lbx1*-/- mice and migration of muscle progenitors into the limb of *CXCR4*-/- embryos is impaired (Vasyutina et al. 2005). However, *CXCR4*-/- mice do not phenocopy *Lbx1*-/- mice, suggesting that dysregulation of other Lbx1 target genes contributes to the migration defect.

Primary myogenesis is impaired in *Six1*-/- mice, which display defects in most skeletal muscles, although distal limb muscles are severely reduced (Laclef et al. 2003). A similar phenotype is observed in *Eya1*-/-:*Eya2*+/- embryos (Grifone et al. 2007). Compound *Six1*-/-:*Six4*-/- mice display a more severe defect, despite the observation that *Six4*-/- mice have no overt phenotype (Ozaki et al. 2001). Expression of *Pax3*, *Lbx1*, *c-Met*, *Myf-5*, *MyoD*, and *MRF4* is severely or completely lost in rostral somites in *Six1*-/-:*Six4*-/- embryos, suggesting that Six1 and Six4 co-operate very early to specify muscle cell identity (Grifone et al. 2005). The observation of defects in rostral but not caudal somites is consistent with observations that somitic cell identities are established not only by spatial cues, but also by axial identity (Alvares et al. 2003). *Mox2*-/- mice display a reduction in total limb muscle and are missing particular muscle groups, including several muscles of the flexor compartment (Mankoo et al. 1999). *Pax3* expression is significantly diminished the limbs of *Mox2*-/- embryos while *c-Met* expression is only slightly reduced, which is unusual given the loss of *c-Met* expression in *Splotch* homozygote embryos (Yang et al. 1996). *Myf-5* expression is reduced, but the expression of *MyoD* is unaffected in *Mox2*-/- embryos, indicating that Mox2, like Six1 and Six4, functions up-stream of Pax3 during myogenesis. Further studies are required to refine the molecular mechanisms by which limb muscle groups are specified.

1.2.4 Transcription Factors and Congenital Myopathies

Most congenital myopathies are due to mutations that affect the function or maintenance of muscle rather than the development. Myotonic dystrophy is

caused by a trinucleotide repeat expansion in the 3' untranslated region of *DMPK* and over-expression of this transcript interferes with myogenic transcription factor activity in both cell culture and in a mouse model (Amack, Reagan, and Mahadevan 2002; Storbeck et al. 2004). Therefore, defective regulation of myogenesis may be pathogenic in some myopathies. However, mutations to muscle regulatory factors do not appear to cause myopathy alone, but in one case act as a modifier in an individual with Becker muscular dystrophy (Kerst et al. 2000).

Mutations in *PAX3* result in haploinsufficiency and cause Waardenburg syndrome (WS) Type 1, a disorder characterized by sensorineural deafness, pigmentation abnormalities, and facial dysmorphology (Read and Newton 1997); however, a muscle phenotype for this syndrome has not been described. Yet, the clinical description of Klein-Waardenburg syndrome, also known as WS Type 3, includes severe presentation of WS Type 1 with upper arm or musculoskeletal anomalies (Goodman et al. 1982; Hoth et al. 1993; Tekin et al. 2001; Wollnik et al. 2003). This is the only genetic defect known to impair muscle migration in a mouse model that results in a developmental disorder with skeletal muscle involvement in humans.

1.3 Cell Polarity in Muscle and Neuronal Development

1.3.1 Initiating Cell Migration

The asymmetrical partitioning of intracellular components to establish polarity is an event critical to a number of developmental and homeostatic processes. Directed cell migration is important for vertebrate development, for

example during muscle development, and for an effective immune response. Neutrophils are the most abundant type of leukocyte in humans and play an integral role in mediating the innate immune response. While circulating through the vascular system, neutrophils undergo periodic "rolling" episodes where transient, reversible interactions between P-selectin receptors and glycoproteins on the vascular lumen slow the cells such that they can periodically monitor all parts of the body for fungal or bacterial infection (Johnston, Cook, and McEver 1989; Lawrence and Springer 1991). At sites of infection, neutrophils "brake" as the detection of chemokines causes weak and transient selectin interactions to be replaced by stronger integrin-mediated interactions. Subsequently, the activation of integrin receptors on the side of the neutrophil interacting with the vascular lumen polarizes the cell, permitting extravasation from the vasculature and migration into the surrounding tissue (Hickstein et al. 1989; Kishimoto et al. 1989).

The cellular responses of neutrophils to their interactions with the vascular lumen can be efficiently modeled in culture, monitored in tissue explants, or even a living animal. However, the complex interactions between surrounding tissue and neurons, muscle progenitors, or chondrocytes makes understanding the cellular mechanisms of migration more difficult in these other cell types. The processes of cellular migration are best understood through *in vitro* and *ex vivo* studies, which allow the perturbation and examination of sub-cellular events in a simplified context. These studies often employ the fibroblast, a highly motile cell of mesodermal origin that can be easily obtained and maintained in culture (Carrel

1923; Carrel and Ebeling 1926). Consequently, the mechanisms described by experiments using fibroblasts grown in culture are assumed to be relevant to developmental biology and the true significance of these discoveries, in most cases, remains to be determined.

Fibroblasts move *in vitro* under normal tissue culture conditions but their migration can be inhibited by the withdrawal of serum or by the close proximity of neighboring cells (Lipton et al. 1971; Stewart, Duley, and Allardyce 1979). These modes of inhibition are reversible and provide a system in which to study the initiation of migration. In a wound-healing assay, whereby a line of cells is removed from a confluent dish by scraping, neighboring fibroblasts begin to move into the 'wound'. The wound-healing assay is superior to following the stochastic migration of individual cells because wound-edge cells will always initially move in a path perpendicular to the wound. There are a number of other simple techniques used to measure migration, including the Boyden chamber and Matrigel assays (Kleinman and Jacob 2001). However, these assays are best suited for studying chemotaxis or steady-state migration, rather than initiation, and the wound-healing assay can be performed on coverslips and the cells imaged by fluorescent microscopes, permitting the observation of individual cells.

The first observed event during the initiation of wound-edge fibroblast migration is centrosome, or microtubule organizing center (MTOC), re-orientation, whereby the centrosome and associated Golgi apparatus moves to a position between the nucleus and the leading edge of the cell (Kupfer, Louvard, and Singer 1982) (Figure 1.5). Since re-orientation occurs before directed

migration, it is unlikely to be an effect secondary to migration itself. This establishment of polarity is thought to promote the trafficking of vesicles containing newly synthesized proteins to the growing leading edge of the cell. Centrosome re-orientation is not unique to fibroblasts, and occurs in cytotoxic T lymphocytes (Kupfer and Dennert 1984), macrophages (Nemere, Kupfer, and Singer 1985), astrocytes (Etienne-Manneville and Hall 2001), and in the amoeba *Dictyostelium discoideum* (Ueda et al. 1997) in response to a variety of polarizing stimuli. However, not all cell types undergo centrosome re-orientation (Yvon et al. 2002) and in cell types that do, only a proportion of wound-edge cells display this phenomenon. Thus, centrosome re-orientation is not absolutely required for migration but rather appears to be a measurable byproduct of more subtle essential cellular events occurring during the initiation of cell migration.

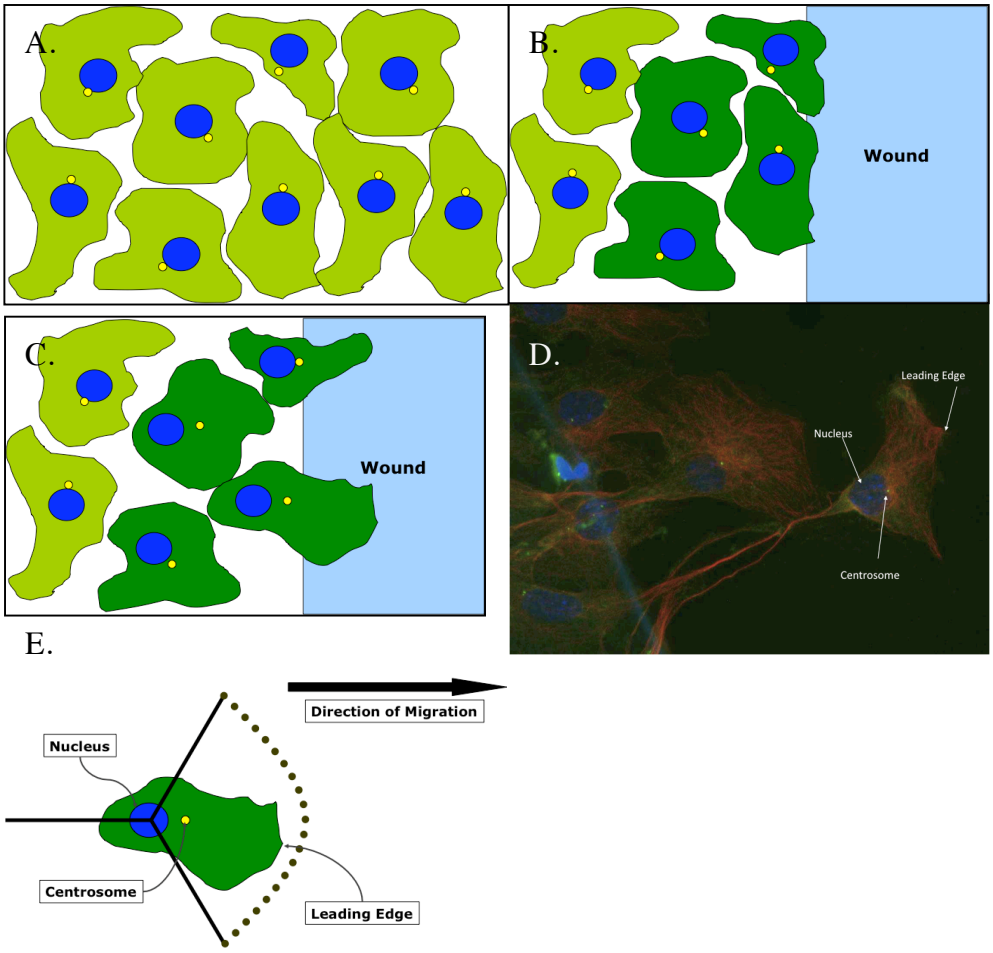
A.
B.
Wound
C.
Wound
D.
Leading Edge
Nucleus
Centrosome
E.
Direction of Migration
Nucleus
Centrosome
Leading Edge

Figure 1.5. Cartoon description of centrosome re-orientation during a wound-healing assay. (A) Cells are grown to confluence, (B) neighboring cells removed by wounding, (C) wound edge cells make protrusions towards the wound and re-orient their centrosome. Example of centrosome re-orientation at the edge of a wound. (A) MEFs grown to confluence, wounded, and allowed to migrate into the wound for 4 h were fixed and stained with antibodies to α-Tubulin (Red), γ-Tubulin (Green), and Hoescht dye (Blue). Scoring re-orientation. (E) Division of the cell with the nucleus as center into equal thirds with one-third facing the wound. Cells with their centrosome in this third are 'oriented', all others are 'not'.

1.3.2 GTPases and Cell Migration

Although centrosome re-orientation is not absolutely required for migration, it is not dispensable for directed cell movement, as cells with mutant forms or ablation of genes affecting centrosome re-orientation also have migration defects. The small Rho GTPase Cdc42 is required for both normal centrosome re-orientation and migration, and integrates extracellular signals to mediate changes to the actin and microtubule cytoskeletons (Gomes, Jani, and Gundersen 2005). GTPases are proteins that cycle between active GTP-bound and inactive GDP-bound states, differentially interacting with protein partners depending on the state of activity like molecular switches (Jaffe and Hall 2005). The large number of genes encoding GTPases and the diverse range of proteins regulating GTPase function underscore the importance of these proteins.

While functional redundancy, unexpected dominant-negative functions for mutant proteins, and discrepancies in the literature make assigning absolute roles for individual GTPases difficult, RhoA, Rac1, and Cdc42, have reasonably well characterized roles during fibroblast migration. In the Swiss 3T3 immortalized fibroblast cell line, RhoA mediates focal adhesions and actin stress fibers, while Rac1 activation promotes actin polymerization at the leading edge of the cell (Nobes et al. 1995). RhoA-dependent focal adhesions are integrin-rich signaling complexes through which the cell makes contact with the substrate (Horwitz et al. 1986; Tamkun et al. 1986) and stress fibers are contracting actin-myosin structures that link focal adhesions to generate traction (Badley et al. 1980; Kreis and Birchmeier 1980; Birchmeier et al. 1980). Rac1 activation stimulates actin

polymerization at the leading edge of cells resulting in the formation of lamellipodia, broad protrusions that allow directional extension of the cell (Nobes and Hall 1995). In the model proposed by Nobes and Hall (Nobes and Hall 1995), Cdc42 activation precedes RhoA or Rac1, and stimulates the formation of early adhesive complexes and finger-like filopodia. Co-ordination of new focal adhesion formation in filopodia, maturation of adhesions in growing actin-propelled lamellipodia, stress fiber contraction, and focal adhesion disassembly at the rear of the cell facilitates a treadmill-like mechanism of directed cell migration. Subsequent studies have elaborated on this model and have established additional roles for GTPases.

1.3.3 Regulation of the Cytoskeleton

In non-muscle motile cells such as fibroblasts, actin exists as globular monomers (G-actin) and filamentous polymers (F-actin), and the transition between the two forms requires accessory proteins. Furthermore, the mode in which actin polymerization occurs will either propel the growth of leading edge structures through the nucleation of G-actin to parallel single strands, forming filopodia, or to branched networks that form lamellipodia. Formins promote the linear growth of F-actin by promoting the addition of G-actin to the growing 'barbed' end (Evangelista et al. 2002; Pruyne et al. 2002; Sagot, Klee, and Pellman 2002; Sagot et al. 2002) and this activity is controlled by receptor-mediated activation of Rho family GTPases (Kohno et al. 1996; Evangelista et al. 1997; Watanabe et al. 1997). Capping of the barbed end inhibits F-actin growth (Fuchtbauer et al. 1983), resulting in a stalled fiber, which is then subject to

disassembly by severing proteins such as ADF (Norberg et al. 1979). The dynamic regulation of F-actin polymerization and depolymerization is known as actin treadmilling (Kirschner 1980) and participates in the protrusion and retraction of filopodia of migratory cells. GTPase-dependent activation of SCAR/WASp promotes Arp2/3 nucleation of G-actin from an existing F-actin filament (Machesky et al. 1999; Machesky and Insall 1998), resulting in the formation of a branched actin structure. Numerous F-actin branches form the cytoskeletal basis of the lamellipodium, the broad protrusion extending from migratory cells. The perpetual growth of F-actin at the leading edge generates a net retrograde actin flow that is visible in both fibroblasts (Theriot and Mitchison 1992) and the growth cones of neurons (Lin and Forscher 1995), and by interacting with stationary focal adhesions, actin polymerization provides one mechanism through which migrating cells produce forward movement.

Analogous to the highly ordered sliding filaments in skeletal muscle, F-actin fibers in motile cells are cross-linked by myosin motors typically specified as non-muscle myosins. Similar to those found in skeletal muscle, non-muscle myosin motors are multimeric complexes (reviewed in (Vicente-Manzanares et al. 2009)). Myosin heavy chain (MHC) proteins contain two domains, a globular actin-binding ATPase motor and a long, helical tail. MHC proteins dimerize via the helical tails to form a coiled-coil domain, and short hinge regions connect to the two globular head domains. ATP hydrolysis in the globular head forces contraction of the hinge region, and sequential alternating contraction of the globular domain allows for processive walking along actin filaments. MHC hinge

regions are stabilized by essential light chain (ELC) and their contractility is positively regulated by the phosphorylation of regulatory light chain (RLC; also commonly known myosin light chain, MLC). Pairs of MHC dimers interact through the coiled-coil domain to form antiparallel tetramers, with pairs of globular heads at either end of the helical rod. An MHC tetramer between two actin filaments can force the actin filaments to slide towards each other, thereby producing tension. Stress fibers, which are linear actin-myosin dense structures linking focal adhesions, are the major source of propulsion in motile cells.

The microtubule cytoskeleton plays an important role in establishing cell polarity in migrating fibroblasts (reviewed in (Siegrist and Doe 2007)). Like the actin cytoskeleton, microtubules are dynamic polarized structures assembled from monomeric subunits. However, microtubules are truly tubular filaments made up of repeating alpha- and beta-tubulin subunits that are typically nucleated by the centrosome at the 'minus' end and grow towards the cell cortex via addition to the 'plus' end. Microtubules experience dynamic instability and require accessory proteins to prevent depolymerization events known as microtubule catastrophe. While kinesin and dynein motors move along microtubules in a fashion similar to the way myosin motors move along actin filaments, microtubule-tracking motors tend to transport cargo rather than generate propulsive force in migrating cells. Distal transport of cell surface components by kinesin-dependent motors is important for synthesis and function of the leading edge. Rather, the microtubule cytoskeleton promotes directed cell migration through GTPases that initiate a molecular cascade that stabilizes interactions between microtubule tips and the

cell cortex, and by using dynein motors to create tension between the microtubule cytoskeleton and the cell cortex.

1.3.4 Mechanisms of Cell Polarity, Migration, and Axonal Extension in Neurons

Neurons share with fibroblasts similar cytoskeletal properties, including dynein and myosin motor activities, but require additional functions to form dendrites and to project axons. Consequently, the mechanisms by which neurons migrate and polarize differ from those of fibroblasts. Similar to studies of fibroblast migration, the mechanisms by which neurons migrate have been described by studies of cells *in vitro*. Initiation of neuronal migration involves the extension of a thin leading edge process in the direction of movement from the cell body, containing the nucleus. The centrosome translocates to an intermediate position between the leading edge and the nucleus inside the shaft of the process. The shaft then dilates, widening to accommodate the nucleus, which then moves to re-join the centrosome, and the trailing edge is retracted. Migrating neurons repeatedly undergo this characteristic worm-like movement and associated nucleokinesis until reaching their destination (Schaar and McConnell 2005). Both microtubule and actin cytoskeletons are required for this activity. Inhibition of microtubule dynamics in migrating neurons via incubation with nocodazole results in direct coupling of the centrosome and nucleus, rather than the salutatory process of the centrosome preceding the nucleus, while treatment with blebbistatin to inhibit myosin activity prevents forward nuclear movement and retraction of the trailing edge (Schaar and McConnell 2005). Mutations in *LIS1*, encoding a protein that interacts with dynein, cause the classical form of

lissencephaly (Reiner et al. 1993) and highlight the importance of dynein motor function during neuronal migration. Thus, as in migrating fibroblasts, co-ordination between the microtubule and actin cytoskeletons is critical for directed migration.

The event that constitutes polarity in neurons, whether the partitioning of cytoplasmic contents prior to axon extension, the ability to extend axons, or the directed projection of axons towards a growth factor, depends on the context of the study. *In vitro*, pyramidal cells such as mouse hippocampal neurons will position the centrosome at the base of the neurite destined to become the cell's axon (de Anda et al. 2005). The positioning of the centrosome at the base of the developing axon is thought to promote trafficking of vesicular and protein components to the rapidly growing axon, but axonal extension does not absolutely require centrosome positioning, as multipolar neurons form multiple axons without re-orientation of the centrosome.

The capacity of a neuron to form an axon depends in part on Cdc42 activation and actin polymerization at the growing end of the axon known as the growth cone. Hippocampal neurons cultured from conditional Cdc42-null mouse embryos form fewer axons than control neurons and the growth cones of these mutant neurons contain disorganized microtubules and abnormal phosphorylation of the actin polymerization regulator cofilin (Garvalov et al. 2007). Cdc42 and related Rho GTPases play additional roles during axonal extension, coupling the activation of receptors on growth cones that mediate both attraction and repulsion. For example, stimulation of the EphA receptor by Ephrin-A mediates axonal

repulsion and growth cone collapse by inhibiting Cdc42 and Rac1, while activating RhoA (Shamah et al. 2001). The cytoskeleton of the growth cone is highly dynamic, analogous to the leading edge of migrating fibroblasts (reviewed in (Geraldo and Gordon-Weeks 2009)). The base of the growth cone, known as the axonal wrist, contains bundled microtubules that become flattened and splayed in the growing process where they interact with F-actin and the cell cortex of the growth cone. The cytoskeletal components of the lamellar domain of the growth cone, in the form of filopodia and lamellipodia, are either actively stabilized or destabilized through receptor activation, with stabilized portions developing new focal adhesions through which the growth cone propels itself. Rho family GTPases, and the proteins regulating them, play integral though often overlapping functions during axonal extension and growth cone regulation (Pertz et al. 2008) and the body of literature describing these events continues to grow at a phenomenal rate.

1.3.5 Cytoskeletal Regulation during Cellular Polarization: Centrosome Re-Orientation Revisited

As mentioned above, centrosome re-orientation is observed during the initiation of cellular migration, thus predicting the direction of movement, and it provides a system in which to study how individual components, such as GTPases, molecular motors, and cytoskeletal elements co-operate to promote polarity. The hypothetical molecular mechanism driving centrosome re-orientation was refined in 2005 by Gundersen and colleagues (Gomes, Jani, and Gundersen 2005). Lysophosphatidic acid (LPA) is a serum component that acts

through the LPA receptor and is sufficient to promote polarization in immortalized fibroblasts (Gomes, Jani, and Gundersen 2005; Nagasaki and Gundersen 1996; Palazzo et al. 2001). Local enzymatic degradation of LPA by neighboring cells contributes to contact-mediated inhibition of migration (Nagasaki and Gundersen 1996), and removal of adjacent cells from a confluent dish in a wound-healing assay stimulates migration, at least in part, by increasing the concentration of LPA on the cell-free side of wound-edge cells. Wound edge cells do not re-orient their centrosomes or migrate in serum-free media (Palazzo et al. 2001), suggesting that the wound-healing and serum-stimulation responses in fibroblasts are analogous.

Stimulation with whole serum or LPA alone activates Cdc42 to initiate a cascade of events culminating in centrosome re-orientation and migration. Early studies using the actin depolymerizing drug cytochalasin D had no effect on LPA- or serum-dependent centrosome re-orientation (Palazzo et al. 2001), while disruption of dynein motor function was sufficient to prevent polarization (Etienne-Manneville and Hall 2001; Palazzo et al. 2001). These results suggested a model whereby local activation of dynein at the leading edge produced tension between the cell cortex and the microtubule cytoskeleton, effectively pulling the centrosome toward the leading edge (Figure 1.6).

Etienne-Manneville and Hall found that integrin-mediated activation of Cdc42 was critical for dynein-dependent centrosome re-orientation in astrocytes (Etienne-Manneville and Hall 2001). Given that integrins are distributed throughout the cell, it is unclear how non-specific activation of Cdc42 and dynein

could be sufficient to promote directed cell migration. Resolving how Cdc42 activation is required both locally and globally in a migrating cell required the introduction of new techniques. Live cell imaging allowed the visualization of different cytoskeletal elements during wound healing and led to the revelation that centrosome re-orientation is a misnomer. Gomes and colleagues generated NIH3T3 fibroblasts stably expressing GFP-tagged α-Tubulin to label microtubules, and observed these cells at wound edges in real time. Remarkably, the position of the centrosome with respect to the cell center (known as the cell centroid) did not change, while the nucleus moved away from the leading edge (Gomes, Jani, and Gundersen 2005). The authors noted that the rate of nuclear rearward movement was similar to microtubule retrograde flow, which is dependent upon actin retrograde flow (Salmon, Adams, and Waterman-Storer 2002; Waterman-Storer and Salmon 1997), and confirmed that actin retrograde flow is required for Cdc42-dependent nuclear rearward movement by observing defective centrosome re-orientation in cells treated with the myosin inhibitor blebbistatin (Gomes, Jani, and Gundersen 2005). Perturbation of the myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), which positively regulates myosin activation by phosphorylating MLC, produced the identical phenotype, thereby confirming the Cdc42-dependent myosin activation promotes nuclear rearward movement during centrosome re-orientation.

Interestingly, live cell imaging of wound-edge cells microinjected with an antibody to the dynein intermediate chain revealed that disruption of dynein resulted in centrosomes moving with the nucleus rather than being maintained at

the cell centroid (Gomes, Jani, and Gundersen 2005). This observation explains how the perturbation of Cdc42 function downstream of either LPA-induced directional activation or integrin-induced spatially unspecified activation impairs different components of the cytoskeleton while generating the same phenotype: a centrosome re-orientation defect. These results are summarized in Figure 1.6.

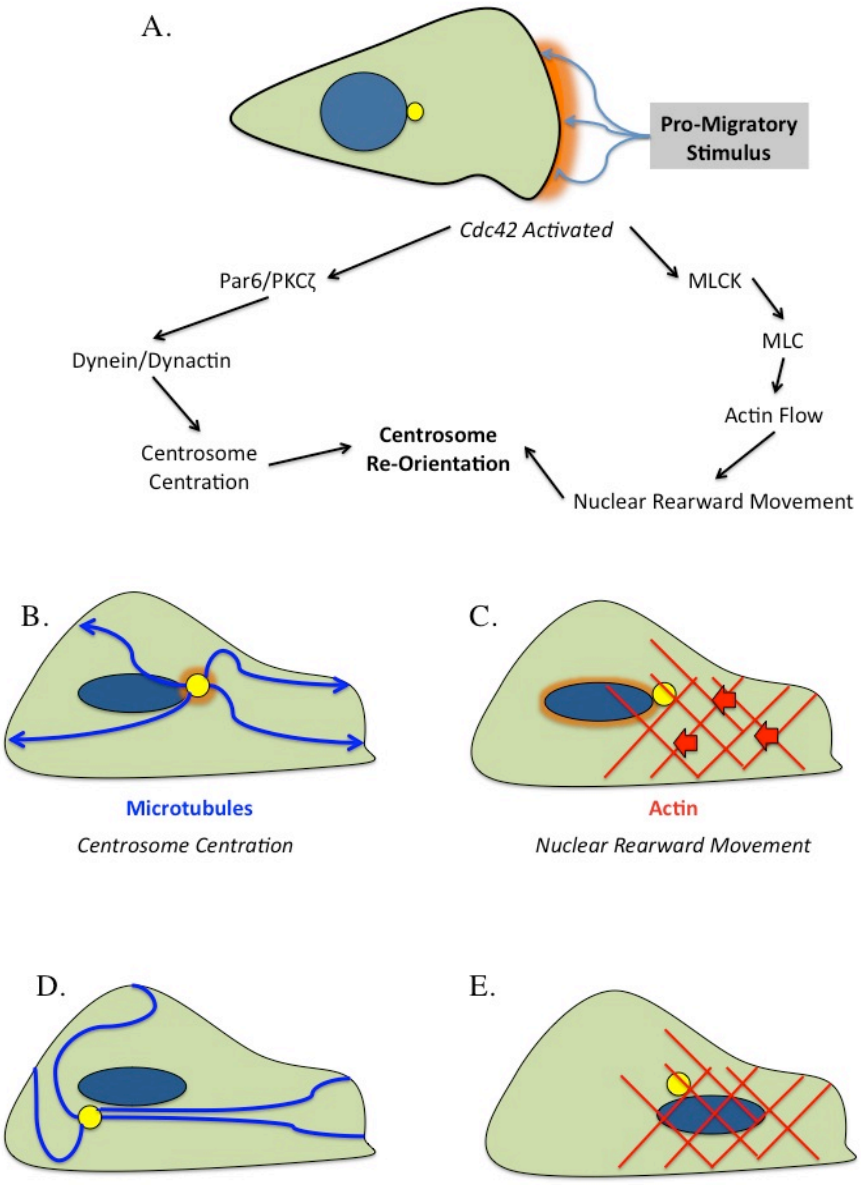

Figure 1.6. (A) Molecular cascade regulating microtubule- and actin-dependent properties of centrosome re-orientation. Activation of Cdc42 by pro-migratory stimuli, such as LPA, leads to dynein-dependent microtubule centrosome-centration (B) and myosin-dependent nuclear rearward movement (C). Centrosome re-orientation defects arise from (D) disruption of dynein function, resulting in loss of centrosome centration or (E) impaired myosin activation, resulting in failure to undergo nuclear rearward movement.

1.4 Relevance

Prior to the beginning of this project, very little was known about the role of necdin in muscle – or whether necdin was important for muscle development at all. Figure 1.7 summarizes the pathways through which necdin was known to act when I joined the Wevrick laboratory in September 2004. Two papers had described a mechanism whereby necdin promoted the differentiation of smooth muscle cells (Brunelli et al. 2004) and C2C12 myoblasts (Kuwajima et al. 2004) by relieving the repressive effects of the homeobox transcription factor Msx2. This model posits that the appearance of necdin upon differentiation co-operates with Msx2 in smooth muscle cells to positively regulate the expression of smooth muscle genes, whereas necdin co-operates with the related protein MAGE-D1 to antagonize the repressive effects of Msx2 on *Myosin Heavy Chain* (MHC) expression in C2C12 cells. In both scenarios, necdin is presumed to modulate the activity of transcriptional co-activators and/or co-repressors. The proposed mechanism is consistent with those of previous publications demonstrating that necdin is important in neurons for the transcriptional regulation of cell cycle exit (Taniura et al. 1998), apoptotic inhibition (Taniura, Matsumoto, and Yoshikawa 1999), and differentiation (Kobayashi, Taniura, and Yoshikawa 2002; Sasaki et al. 2002). However, this model is unsatisfactory, since necdin is expressed in developing skeletal muscle (Brunelli et al. 2004; Kuwajima et al. 2004) but Msx2 is not expressed in skeletal muscle precursors (Bendall et al. 1999). Thus, despite

the abundant expression of a PWS candidate gene in muscle, the prevailing view was that neuronal dysfunction was the cause of hypotonia in PWS.

This view was strengthened with the publication by Pagliardini and others reporting that motor neurons in *Ndn*-null mice were defasciculated, had abnormal projections, and dysmorphic axons (Pagliardini et al. 2005). Deponti and colleagues were the first to describe a muscle defect in a *Ndn*-null mouse and to show that over-expression of necdin could affect skeletal muscle regeneration *in vivo* (Deponti et al. 2007). While these authors had substantial animal model evidence and transactivation assays demonstrating that necdin positively modulated MyoD-dependent transcription, they did not identify the underlying molecular mechanism. The following year, I published my description of the interaction between necdin and the E1A-like inhibitor of differentiation 1 (EID-1), demonstrating that necdin relieved EID-1-dependent inhibition of MyoD-dependent transcription in over-expression assays, and that primary myoblasts from *Ndn*-null embryo limb buds have impaired differentiation into Myosin Heavy Chain-expressing myotubes in culture (Bush and Wevrick 2008). This study lacked *in vivo* data due to inadequate EID-1 antibodies, but provided a possible mechanism to explain the observations by Deponti and others.

The most recent publication describing a role for necdin in muscle posits that necdin protects differentiated muscle from apoptosis during tumor-induced wasting (Sciorati et al. 2009) and is consistent with similar studies in developing neurons (Hasegawa and Yoshikawa 2008; Kurita et al. 2006; Kuwako et al. 2005; Takazaki, Nishimura, and Yoshikawa 2002; Taniura, Matsumoto, and Yoshikawa

1999). It is unclear how to interpret this study in the context of the PWS phenotype, where infants present with profound hypotonia but gain strength with age and do not appear to suffer from muscle atrophy or injury in adulthood. Further research is required to clarify the role of necdin during muscle development, and to understand how loss of necdin contributes to hypotonia in PWS.

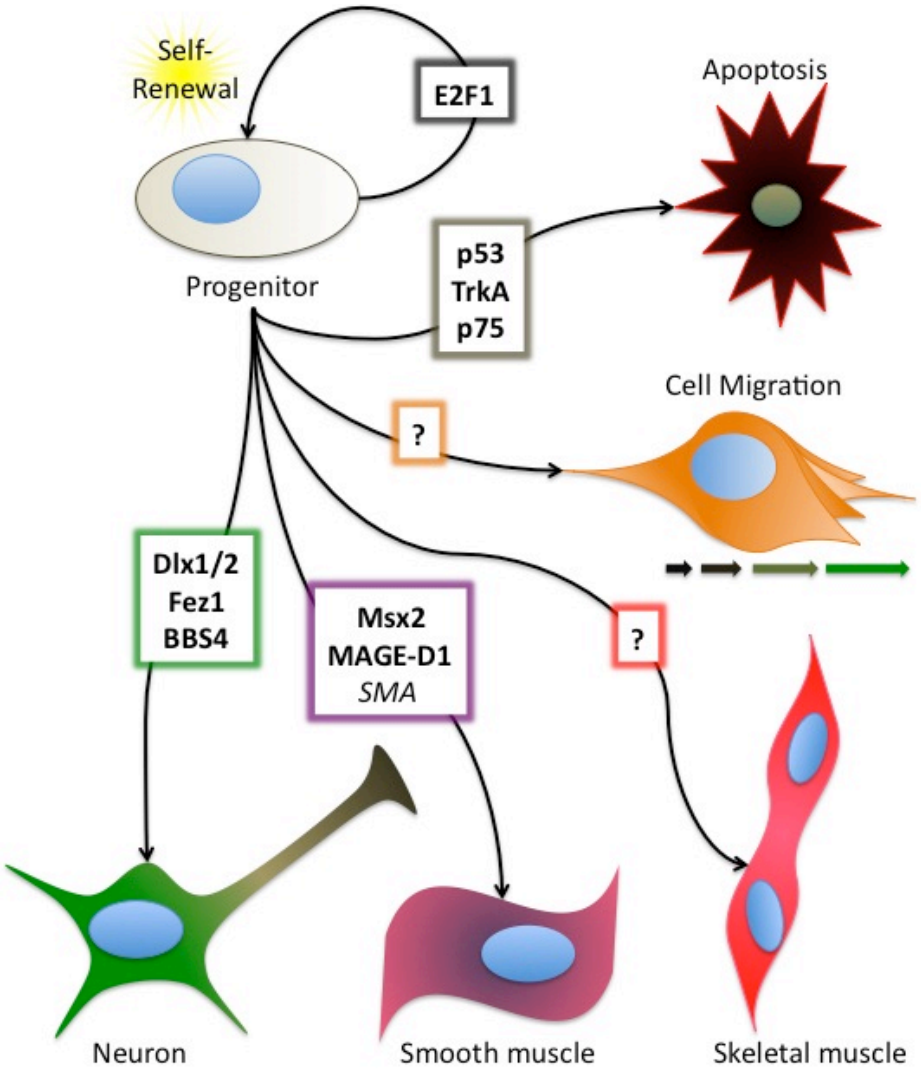

Figure 1.7. Summary of pathways involving necdin circa September 2004. Necdin promotes cell cycle exit via E2F1, inhibits apoptosis through interactions with p53, TrkA, and p75, interacts with Msx2 through MAGE-D1 to regulate *smooth muscle actin* (*SMA*) expression in C2C12 and smooth muscle cells, and regulates neuronal maturation by interacting with Dlx1/2 and associating with centrosomal proteins Fez1 and BBS4.

1.5 Hypothesis

I propose that infantile hypotonia in PWS can be largely explained by defects due to the loss of necdin in four discrete processes critical for normal skeletal muscle development: muscle precursor migration, primary myoblast differentiation, motor neuron development, and satellite cell differentiation. To promote normal muscle development, necdin interacts with proteins to both modulate transcription and mediate signal transduction. While the proportions of necdin found in the nucleus and cytoplasm can change depending on the presence of partner proteins, the broad cellular distribution suggests that necdin may influence transcription and signal transduction simultaneously. Despite the multifunctionality of necdin, loss of this protein leads to subtle developmental defects and relatively normal skeletal muscle in adults with PWS. Limb muscle development, initially impaired by altered pools of migrating precursors and the primary differentiation of these cells, is likely delayed in people with PWS and eventually the subsequent differentiation of primary and satellite myoblasts nearly corrects infantile hypotonia. Determining whether skeletal muscle development is simply delayed due to loss of necdin or if compensatory mechanisms are involved will require additional study. The work presented in this thesis documents the importance of necdin for cell polarization and primary differentiation during skeletal muscle development, and complements previous studies demonstrating that necdin is important for motor neuron development (Pagliardini et al. 2005) and satellite cell differentiation (Deponti et al. 2007). Together, these data

support the theory that loss of necdin produces small but significant defects in multiple stages of muscle development, and that the combination of these defects due to the loss of necdin produces the profound hypotonia observed in infants with PWS.

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Chapter 2. Materials and Methods

Parts of this chapter have been previously published.

Bush, J. R. and Wevrick, R. (2008). The Prader-Willi syndrome protein necdin interacts with the E1A-like inhibitor of differentiation EID-1 and promotes myoblast differentiation. *Differentiation* 76(9):994-1005.

2.1 Yeast two-hybrid screen.

To identify necdin-interacting proteins, full-length necdin was cloned into the pSOS vector of the cytoplasmic yeast two-hybrid CytoTrap system to form the bait vector pSOS-Ndn (Stratagene, La Jolla, CA). The target library was constructed from fetal brain cDNA in the pMyr vector and purchased from Stratagene (5 x 10^9 clones). An interaction screen of approximately one million target clones was carried out according to the manufacturer's instructions with appropriate controls.

2.2 Plasmids

Full-length necdin (325 amino acids) was cloned into pcDNA3.1 HisMaxC mammalian expression vector (Invitrogen, Carlsbad, CA) with an N-terminal Xpress epitope tag to form pcDNA3.1HisMaxXpressNdn (referred to as Xpress-Ndn) (Lee et al. 2005). Full length EID-1 cDNA was cloned into a pCI-HA vector with an N-terminal in-frame HA tag (Promega Corp., Madison, Wisconsin) to generate HA-EID-1. The expression plasmids EMSV-MyoD, 4RTK-Luc, and beta-MHC-Luc have been previously described (MacLellan et al. 2000; Ji et al. 2003). pRLTK was also from Promega Corp.

2.3 Animals

All animal studies procedures were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Health Sciences Animal Policy and Welfare Committee for the University of Alberta. Homologous recombination in mouse embryonic stem cells replaced

most of the *Ndn* coding sequence with a ß-galactosidase reporter in mice carrying the Ndn^{tm2Stw} transgene. These mice have been previously described (Gerard et al. 1999) and are maintained on a C57/BL6J background by breeding through the maternal line. Male offspring carrying the Ndn^{tm2Stw} allele are normal and are bred to C57/BL6J females to produce experimental embryos. Embryos that inherit the paternal Ndn^{tm2Stw} allele are functionally necdin null due to imprinting that silences the maternal allele (*Ndn*-null), while sibling embryos that inherit the paternal wild-type allele are normal (control). Necdin protein is undetectable in cell and tissue lysates from *Ndn*-null mice.

2.4 Cell lines – described in Table 2

Table 2

Name of cell line	**Source of cell line**	**Growth medium**	**Cell type**	**Species**
HEK293	American Type Culture Collection Repository (ATCC)	Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS)	Human Embryonic Kidney	Human
GT1-7	Dr. Pamela Mellon, University of California at San Diego, CA	DMEM with 10% FBS	Hypothalamic neuron	Mouse
U2OS	ATCC	DMEM with 10% FBS	Osteosarcoma tumor	Human
C2C12	ATCC	DMEM with 15% FBS	Muscle satellite cell	Mouse
FB5	NIGMS Human Genetic Cell Repository (HGCR), Camden, NJ	DMEM with 20% FBS	Fibroblast	Human
FB8	HGCR	DMEM with 20% FBS	Fibroblast	Human
FB10	HGCR	DMEM with 20% FBS	Fibroblast	Human
FB12	HGCR	DMEM with 20% FBS	Fibroblast	Human
FB14	HGCR	DMEM with 20% FBS	Fibroblast	Human
FB16*	Brain and Tissue Banks for Developmental Disorders, University of Miami, Miami, FL	DMEM with 20% FBS	Fibroblast	Human
FB17	HGCR	DMEM with 20% FBS	Fibroblast	Human
FB18	HGCR	DMEM with 20% FBS	Fibroblast	Human

PWS129*	Dr. Daniel Driscoll, University of Florida, Gainesville, FL	DMEM with 20% FBS	Fibroblast	Human
RCB1560/RIKEN*	RIKEN Bioresource Centre Cell Bank in Tsukuba, Japan	DMEM with 20% FBS	Fibroblast	Human

* Denotes cell lines derived from patients with PWS that have a paternal deletion on chromosome 15q11-q13 and are functionally necdin-null due to genetic imprinting on the maternal chromosome.

2.5 Cell culture

All cell culture reagents are from Gibco/Invitrogen, Carlsbad, CA/Grand Island, NY unless otherwise specified.

All cell lines and human fibroblasts were maintained as described in **Table 2**, and, with the exception of HEK293 cells, the cells were passaged by rinsing once with 37ºC Phosphate-buffered saline, pH7.0 (PBS), incubating with 0.05% Trypsin-EDTA at 37ºC for 5 minutes, then re-suspending in growth medium, and seeding new flasks. HEK293 cells were passaged as above but incubated with Trypsin at room temperature. C2C12 cells were maintained in DMEM with 15% FBS and induced to differentiate using DMEM with 2% sheep serum. U2OS cells were employed for EID-1 studies because the Rb pathway, which normally regulates EID-1 protein abundace, was preserved in this cell line and because the cells were flat, permitting better microphotography and immunofluorescent detection of transfected proteins.

Embryonic day 16.5 (E16.5) embryos were collected from timed-pregnant C57 mice, and mouse embryonic fibroblasts (MEFs) were prepared by decapitation and mincing of the body, incubation in 0.05% Trypsin-EDTA for 15 minutes, and dissociation by adding DMEM with 10% FBS plus penicillin and streptomycin and pipetting repeatedly. Control and *Ndn*-null embryos were identified by genotyping of spare tissue (Gerard et al. 1999), and the cell suspensions were combined by genotype. The suspensions were allowed to settle for five minutes and the supernatant was then used to seed flasks containing additional DMEM with 10% FBS plus penicillin and streptomycin. MEFs were

further maintained in this medium, and were passaged twice before experimental use to promote cellular homogeneity and were never used after the fifth passage.

Primary embryonic limb bud cultures were prepared from the forelimbs of E10.5 necdin-null and control littermates as described by Weston *et al*. (Weston et al. 2000). E10.5 embryos were collected, the forelimbs removed, placed as pairs in tubes containing Puck's saline containing glucose, and minced. The remaining embryonic tissue was used for genotyping. The limb buds were then centrifuged at 1000 xg for 5 minutes, re-suspended in Puck's saline containing 10% chick serum and 1 unit/ml Dispase, and allowed to mix end-over-end at 37ºC for 45 minutes. Limb bud cells were then centrifuged at 1000 xg for 5 minutes, re-suspended and pooled by genotype in 40% DMEM/60% F12 with 10% FBS (limb bud growth medium, LBGM). The number of cells in suspension was determined, the cells centrifuged at 1000 xg for 5 minutes, re-suspended in LBGM at a concentration of 10^7 cells/ml, and 10 μl of suspension plated drop-wise on a glass coverslip. Cells were allowed to settle for 1 hour before the addition of LBGM.

Neurons were prepared from E16.5 corticies or E18.5 hippocampi by incubating in Hank's Buffered Saline Solution (HBSS) with trypsin at 37ºC, and mixing end-over-end for 25 minutes. Trypsin was inactivated by adding Neurobasal medium containing B27 and N2 supplements, penicillin, and streptomycin (NBM). Trypsinized brain tissue was centrifuged at 200 xg for 5 minutes, pooled by genotype in NBM, and the tissue dissociated by repeated pipetting. Suspensions were allowed to settle for 5 minutes, and the supernatant

was plated on poly-L-lysine-coated coverslips or 6 well dishes such that neurons from each cortical or hippocampal lobe seeded two wells. Cortical and hippocampal neurons were maintained in NBM for up to 5 days.

2.6 Antibodies – described in Table 3

Table 3

Antigen/Name	Host Animal	Source	Application
Akt (#9272)	Rabbit Polyclonal	Cell Signaling Technology, Inc. Danvers, MA	Immunoblot
Phospho-Akt (Ser473; #9271)	Rabbit Polyclonal	Cell Signaling Technology, Inc. Danvers, MA	Immunoblot
Alpha-tubulin (ab18251)	Rabbit Polyclonal	Abcam, Inc., Cambridge, MA	Immunocytochemistry, Immunohistochemistry
Cleaved Caspase-3 (Asp175; #9661)	Rabbit Polyclonal	Cell Signaling Technology, Inc. Danvers, MA	Immunohistochemistry
Cdc42	Mouse Monoclonal	Thermo Scientific, Rockford, IL	Immunoblot
EID-1 (GN735/736)	Rabbit Polyclonal	Dr. W. Robb MacLellan, UCLA, Los Angeles, CA	Immunoblot, Immunoprecipitation
EID-1	Rabbit Polyclonal	Millipore/Chemicon/Upstate International, Billerica, MA	Immunoblot
ERK (K-23; sc-94)	Rabbit Polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA	Immunoblot
Gamma-Tubulin (T6557)	Mouse Monoclonal	Sigma, St. Louis, MO	Immunoblot, Immunocytochemistry
Gamma-Tubulin (ab16504)	Rabbit Polyclonal	Abcam, Inc., Cambridge, MA	Immunocytochemistry, Immunohistochemistry
HA	Mouse Monoclonal	Sigma, St. Louis, MO	Immunoblot, Immunoprecipitation
HA (SC-204)	Rabbit Polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA	Immunoblot, Immunocytochemistry
HGFR (AF527)	Goat Polyclonal	R&D Systems, Minneapolis, MN	Immunohistochemistry
ID2 (SC-489)	Rabbit Polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA	Immunoprecipitation
Ki67 (ab15580)	Rabbit Polyclonal	Abcam, Inc., Cambridge, MA	Immunohistochemistry
MF20	Mouse Monoclonal	Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA	Immunocytochemistry

Table 3 - continued

MLC2 (#3672)	Rabbit Polyclonal	Cell Signaling Technology, Inc. Danvers, MA	Immunoblot
Phospho-MLC2 (Ser19; #3671)	Rabbit Polyclonal	Cell Signaling Technology, Inc. Danvers, MA	Immunoblot
MyoD	Mouse Monoclonal	Dako Corporation, Carpinteria, CA	Immunoblot, Immunocytochemistry
Necdin	Rabbit Polyclonal	Millipore/Chemicon/Upstate International, Billerica, MA	Immunoblot, Immunoprecipitation
Phospho-p44/42 (Thr202/Tyr204; #9101)	Rabbit Polyclonal	Cell Signaling Technology, Inc. Danvers, MA	Immunoblot
Pax3	Mouse Monoclonal	Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA	Immunocytochemistry, Immunohistochemistry
Rac1 (23a8)	Mouse Monoclonal	Millipore/Chemicon/Upstate International, Billerica, MA	Immunoblot
RhoA (26C4; sc-418)	Mouse Monoclonal	Santa Cruz Biotechnology, Santa Cruz, CA	Immunoblot
Tau1 (mab3420)	Mouse Monoclonal	Millipore/Chemicon/Upstate International, Billerica, MA	Immunoblot, Immunocytochemistry
TFIID (N12)	Rabbit Polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA	Immunoblot
TrkB (H181; sc-8316)	Rabbit Polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA	Immunoblot
Phospho-Trk (Tyr490; #9141)	Rabbit Polyclonal	Cell Signaling Technology, Inc. Danvers, MA	Immunoblot
Vinculin (V9131)	Mouse Monoclonal	Sigma, St. Louis, MO	Immunocytochemistry
Xpress	Mouse Monoclonal	Sigma, St. Louis, MO	Immunoblot, Immunocytochemistry, Immunoprecipitation

2.7 Immunoprecipitation and immunoblot analysis

GT1-7 hypothalamic neuronal cells or C2C12 cells used for co-immunoprecipitation experiments were transfected using FuGene (Roche Applied Science, Laval, QC) as per the manufacturer's instructions and lysed in 150 mM NaCl, and 0.5% IGEPAL, 50 mM Tris-Cl, pH 8.0 with Complete Mini protease inhibitors (Roche). Lysates were added to an intermediate buffer such that the final solution was 100 mM NaCl, and 0.2% IGEPAL, 20 mM Tris-Cl, pH 8.0 ("wash buffer") with a final volume of 0.5 ml. The lysates were then pre-cleared with 17 μg Protein G-agarose slurry (Sigma-Aldrich Corporation) mixing end-over-end for 1 h at 4ºC. The resin was pelleted and the supernatant was incubated end-over-end with 2.3 μg of mouse monoclonal anti-HA antibody, 1.2 μg mouse monoclonal anti-Xpress, 5 μg of rabbit polyclonal anti-necdin antibody, 5 μg of rabbit polyclonal anti-EID1 antibody, or 5 μg rabbit polyclonal anti-ID2 antibody in a final volume of 0.5 ml overnight at 4ºC. The lysates were incubated with 43 μg Protein G-agarose slurry mixing end-over-end for 4 h at 4ºC. The immunocomplexed proteins were precipitated and washed 4 times in wash buffer at 4ºC. Bound proteins were eluted by boiling in sample buffer (20% glycerol, 4% SDS, 2% beta-mercaptoethanol, 0.13 M Tris-Cl, pH 6.8), resolved by SDS-polyacrylamide get electrophoresis, and transferred to Immobilon P polyvinylidene fluoride membranes. Membranes were blocked for 30 minutes with TBST (137mM NaCl, 0.1% Tween 20, 20mM Tris, pH 7.5) and 5% skim milk powder (TBSTM) and incubated overnight with the primary antibody in TBSTM at room temperature. Membranes were washed three times in TBST,

incubated with HRP-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ) in TBSTM for 1 h at room temperature, washed three times in TBST, and detected with ECL reagent (Millipore, Billerica, MA) as per the manufacturer's instructions with Kodak film or using a Kodak Image Station. Cells for immunoblot analysis without immunoprecipitation were collected in sample buffer and processed as described above. Antibodies were removed by denaturation at 67°C for 30 minutes in 100 mM beta-mercaptoethanol, 2% SDS, and 62.5 mM Tris-Cl, pH 6.8, and re-probed with anti-gamma-tubulin antibody to quantify protein loading. Blot intensities were quantified using Kodak Image Station Software and are reported as ratios of protein signal pixel intensity to gamma-tubulin pixel intensity. Proteasomal inhibition assays were completed by treating cells with 20 μM MG132 (Sigma-Aldrich Corp.) in DMEM with 10% fetal bovine serum (FBS) for 5 h, then collecting and analyzing protein levels as described above. To measure protein half-lives, cells were treated with 100 μg/ml cycloheximide (Sigma-Aldrich Corp.) in DMEM with 10% FBS, cell lysates collected as described above, and proteins were detected by immunoblot analysis. Protein levels were quantified as described above and plotted versus the collection times. Decay curves were fit to the data points and the protein half-life was determined using the equation of the line.

2.8 Transactivation assay

U2OS cells were transfected in 24 well plates then incubated for 48 h. Empty vector plasmids were co-transfected in order to keep the total amount of plasmid transfected constant across the transfections. Lysates were collected as

per manufacturer's instructions, and luciferase activity was measured using a TD-20/20 Luminometer (Turner Designs). Beta-MHC-Luc or 4RTKluc activities were normalized to an internal Renilla luciferase control. Three experiments were completed, with each experiment performed in triplicate. One set of results representative of the three experiments is presented, with values indicating the mean +/- the standard error of the mean (SEM) and compared for significance $p < 0.05$ using the Student t-test.

2.9 Immunofluorescence

Cells were cultured on cover slips in 6-well plates, transfected using FuGENE (Roche Applied Science), and incubated for 24 h. The cells were then washed once with PBS, fixed for 15 minutes with 2% paraformaldehyde, and washed 3 times with PBS containing 0.05% TritonX-100 (PBS-X). The cells were then blocked for 15 minutes with PBS-X with 2% Boerhinger Mannheim Blocking solution (BMB), incubated with primary antibody in PBS-X with 2% BMB for 1 h, washed twice with PBS-X, incubated with goat anti-rabbit Alexa 594 and goat anti-mouse Alexa 488 secondary antibodies (Invitrogen) in PBS-X with 2% BMB for 1 h, washed twice with PBS-X, and mounted using Vectamount containing DAPI (Vector Laboratories, Burlingame, CA). Cells were imaged using a Leica DMRE microscope and Northern Eclipse software and 58 to 102 cells were counted for each set of transfections. Primary limb bud cultures, MEFs, and primary embryonic neurons were prepared for immunofluorescence as above but without transfection.

For nuclear export inhibition, cells were transfected, incubated for 24 h, treated with 5 nM Leptomycin B (Sigma-Aldrich Corp.) in methanol or methanol alone for 5 h, and fixed as described above.

2.10 Cytoplasmic-Nuclear fractionation

HEK293 cells were transfected by calcium chloride as described above and expression was allowed to proceed for 48 h. Cells were washed once in PBS, collected in phosphate-buffered saline pH 7.3 (PBS) and centrifuged at 560 x g for 5 minutes. The cells were resuspended in 1 ml ice cold Buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl), centrifuged at 560 x g for 5 s, resuspended again in 1 ml Buffer A, and placed on ice for 10 minutes to lyse the cells. At 4°C, the suspensions were vortexed for 30 s, passed 6 times through a pestle B Dounce homogenizer, and centrifuged at 560 x g for 5 s. The supernatant collected contained the cytoplasmic fraction. At 4°C, the pellets were rinsed in 1 ml Buffer A, centrifuged for 5 s, resuspended in 25 µl Buffer B (10 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol), and incubated on ice for 15 minutes. The pellets were sonicated and centrifuged at maximum speed for 5 minutes at 4°C. The supernatant collected contained the nuclear fraction. Proteins were detected by immunoblot analysis as described, with the purity of the preparation monitored through detection of the nuclear protein TFIID.

2.11 Wound healing

Migration assays in MEFs were performed essentially as described (Gomes, Jani, and Gundersen 2005) with representative results from repeated experiments shown. MEFs were grown to confluence, scraped with a cell lifter (Fisher), then washed once with phosphate-buffered saline (PBS), fixed and stained with 0.2% Cresyl violet (Sigma) containing 10% methanol at the time points described. The migration distance was determined measuring the length between the edge of the wound and the migration front using a dissecting microscope (Leica). Wounds were measured at five different positions, and three individual plates were measured at each time point for each genotype. The experiment was repeated three times with MEFs prepared from different litters.

2.12 Centrosome orientation

Cells were plated on coverslips, grown to confluence, wounded by scraping with a pipette tip, and then processed for immunofluorescence as described above. For experiments using MEFs, three coverslips per time point were prepared, while only one coverslip was prepared for each human fibroblast cell line. Three fields containing at least five cells adjacent to the wound were captured per coverslip, and the images were analyzed with ImageJ, imaging processing and analysis software (NIH). Orientation towards the wound was determined by placing a "Y" at the center of the nucleus such that the open third facing the wound was perpendicular to the edge of the wound. Cells with gamma-tubulin staining inside the third facing the wound were considered 'oriented' and all other cells measured were considered 'not oriented'. Immediately after wounding and prior to any cellular response, the population of wound edge cells

will have centrosomes evenly distributed among all three thirds. Therefore, 33% of wound edge cells at any time point will be oriented by chance alone. The percentage of oriented cells at 4 hours was compared to the expected frequency of 67% (Palazzo et al. 2001) for each MEF genotype or human fibroblast line (n=38 to 266 cells for each cell line, PWS129 fibroblasts formed clumps and could not be used). The X-Y co-ordinates of the centrosome, center of the cell, and center of the nucleus were determined with the X-axis perpendicular to the wound. Wound edge cell centration was measured as the distance between the centrosome and the center of the cell, and nuclear rearward movement was measured as the difference in the X-value between the center of the nucleus and the center of the cell.

2.13 Serum starvation-stimulation

MEFs or human fibroblasts were grown to 50% confluence, starved in serum-free DMEM for 16 hours, stimulated with DMEM containing FBS, then prepared for immunoblot analysis or immunofluorescence. MEFs starved and stimulated to study focal adhesion and protrusion formation were labeled with rhodamine-conjugated phalloidin (Sigma) and anti-vinculin antibodies, then the average cell area, focal adhesion size and number, and the number of lamellipodial ruffles determined using ImageJ.

2.14 Cdc42/Rac1/RhoA activation assay

MEFs were serum starved and stimulated, collected and lysed in ice-cold H-buffer (20 mM HEPES, 60 mM NaCl2, 5 mM MgCl2, 0.2% NP-40, pH 7.4) for 5 minutes, vortexed for 10 seconds, and then centrifuged for 10 minutes at

10,000xg. Lysates were incubated with glutathione beads and either GST-Pak1 to detect activated Cdc42 and Rac1, or GST-Rhotekin (GST-Rhot) to detect activated RhoA for 1 hour at 4ºC mixing end-over-end. Samples were washed three times with ice-cold H-buffer, and the beads were then processed for immunoblot analysis as described for immunoprecipitation assays.

2.15 Growth factor-induced polarization

Human hepatocyte growth factor (HGF), stromal-derived factor-1α (SDF-1α), or brain-derived neurotrophic factor (BDNF) (Peprotech, Rocky Hill, NJ) was added to 4% low melting point agarose cooled to 37ºC, with final concentration 1 ng/μl for HGF and SDF, and 5 ng/μl for BDNF. For muscle progenitor assays, a 10 μl growth factor-containing agarose drop was pipetted onto each coverslip, with 10 μl of agarose alone at the opposite end. For hippocampal neuron cultures, the agarose drops were placed in the well of a 6-well plate. Limb bud culture cells settled in the center of the coverslips for 1 hour before addition of 2 ml of media, incubation, and processing for immunofluorescence. Orientation of Pax3-positive cells was determined by centrosome position with respect to the growth factor-containing agarose drop using the centrosome orientation method, after 4 hours or 8 hours. For hippocampal neuron polarity assays, cells were allowed to adhere for 3-4 hours before coverslips were transferred to 6-well plates containing agarose drops. Two ml of Neurobasal media was added to release the growth factor, and neurons processed for immunofluorescence after 8 hours. Hippocampal neuron polarity was determined by scoring Tau-1-positive single-axon neurons that projected

from the third of the cell body facing the growth factor. A finding that significantly more than 33% of Pax3-positive cells or Tau-1-positive single-axon neurons were polarized indicates a response to the growth factor (35-126 cells per genotype per experiment).

2.16 Immunohistochemistry and Hematoxylin & Eosin staining

E9.5 and E10.5 embryos were fixed in 4% PFA in PBS, cryoprotected in 30% sucrose, 10% DMSO in PBS at 4ºC overnight, rinsed once in PBS, then frozen in Shandon Cryomatrix (Thermo Scientific) and stored at -80ºC until cryosectioning. Mice were genotyped by LacZ staining of spare tissue (Gerard et al. 1999). Embryos were cryosectioned at a transverse angle and a thickness of 20μm, baked for 30 minutes at 42ºC, post-fixed in 4% PFA in PBS for 7 minutes, rinsed three times in PBS, permeabilized in PBS-X for 30 minutes, blocked for 1 hour in PBS-X with 5% BSA and 10% goat serum (Vector), and then incubated with the primary antibody overnight in PBS-X with 5% BSA and 1% goat serum. The sections were washed three times in PBS-X, blocked for 1 hour in PBS-X with 5% BSA and 10% goat serum, incubated with secondary antibodies in PBS-X with 5% BSA and 1% goat serum for 1 hour, washed once in PBS-X, stained with Hoecsht (Molecular Probes) in PBS-X for 10 minutes, washed twice in PBS-X, and then mounted with 1:3 glycerol:PBS. The sections were visualized using a Leica DMRE microscope and Northern Eclipse software. Forelimbs from E18.5 *Ndn*-null and control embryos were processed as above, but processed without post-fixing for Hematoxylin & Eosin (H&E) staining using standard techniques.

2.17 Statistical Analysis

Continuous data were compared using one-tailed or two-tailed Student t-test in Excel. Categorical data were compared using a Fisher's exact or chi-square analysis in GraphPad Prism. For all types of analyses, $p<0.05$ was deemed significant.

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Chapter 3. The Prader-Willi syndrome protein necdin interacts with the E1A-like inhibitor of differentiation EID-1 and promotes myoblast differentiation

Parts of this chapter have been previously published.

Bush, J. R. and Wevrick, R. (2008). The Prader-Willi syndrome protein necdin interacts with the E1A-like inhibitor of differentiation EID-1 and promotes myoblast differentiation. *Differentiation* 76(9):994-1005.

With the exception of the yeast two-hybrid screen, Jason Bush completed all of the experiments presented in this chapter.

3.1 Introduction

NDN, encoding necdin, is one of four protein-coding genes genetically inactivated in individuals with Prader-Willi Syndrome (PWS). PWS is marked by global developmental delay, hypoventilation, childhood-onset hyperphagia, severe obesity, and hypogonadism (Gunay-Aygun et al. 2001). Hypotonia of central origin is present prenatally, and reduced lean muscle mass with abnormal proportions of type 2 muscle fiber subtypes have been described in infants with PWS (Argov et al. 1984; Chitayat et al. 1989; Sone 1994; Miike et al. 1988). Necdin belongs to the MAGE (melanoma-associated antigen) family of proteins characterized by the presence of a MAGE homology domain (MHD) (Barker and Salehi 2002; Sasaki, Hinck, and Watanabe 2005), and is most highly expressed in the nervous system, muscle and skin. The expression of necdin in neural precursor cells promotes and accelerates their differentiation (Hayashi et al. 1995; Uetsuki et al. 1996), and the loss of necdin in PWS could contribute to the neurological component of the phenotype. Necdin associates with centrosomal proteins during axonal outgrowth in sympathetic neurons (Lee et al. 2005), and facilitates TrkA signaling to promote survival of nerve growth factor-dependent nociceptive neurons (Kuwako et al. 2005; Salehi et al. 2000). In muscle satellite cells, necdin promotes differentiation after injury and protects myoblasts from apoptosis (Deponti et al. 2007). On some strain backgrounds, most mice carrying a genetic inactivation of gene encoding necdin (*Ndn*-null) die of a neonatal central respiratory defect (Gerard et al. 1999) and surviving *Ndn*-null mice have decreased sensitivity to pain (Kuwako et al. 2005).

While a number of proteins interact with necdin, the translation of these interactions into a molecular model explaining the physiological defects observed in PWS is still forthcoming. Necdin interacts with and modulates the activity of two homeobox transcription factors, antagonizing the repressive action of Msx2 on the differentiation of mesenchymal progenitor cells, and potentiating gene activation by Dlx proteins during GABAergic neuronal differentiation (Brunelli and Cossu 2005; Brunelli et al. 2004; Kuwajima et al. 2004; Masuda et al. 2001; Sasaki et al. 2002). Necdin interacts with the cell cycle regulatory transcription factors E2F1 and E2F4 (Kurita et al. 2006; Taniura et al. 1998), both of which are targets of retinoblastoma tumor suppressor protein (pRb) (Nevins 1992). Together, these studies suggest that necdin may be important in the differentiation of multiple cell types through its interactions with DNA-binding transcription factors (Yoshikawa 2000).

Skeletal muscle formation requires the activation of a myogenic program by the sequential activity of bHLH transcription factors and associated chromatin remodeling proteins, and is dependent on pRb. The E1A-like inhibitor of differentiation-1 (EID-1, also known as CRI-1) was originally identified in two separate screens as a pRb-interacting protein (MacLellan et al. 2000; Miyake et al. 2000) and is implicated in transcriptional regulation during myogenesis. EID-1 inhibits MyoD-dependent transactivation by suppression of the acetyltransferase activity of p300, a transcriptional co-activator required for myogenic differentiation. EID-1 is degraded upon cell-cycle exit by ubiquitination and proteasomal degradation in an Rb-dependent manner (MacLellan et al. 2000;

Miyake et al. 2000), a process that is antagonized by the activity of the RET finger protein RFP (Krutzfeldt et al. 2005). EID-1 is ubiquitously expressed in human tissue with greatest expression in cardiac and skeletal muscle, and brain (MacLellan et al. 2000; Miyake et al. 2000), and has similar expression in mouse tissues (Bavner et al. 2002).

In this study, we identified EID-1 as a necdin-interacting protein in a yeast two-hybrid screen. We demonstrated that necdin antagonizes the repressive effect of EID-1 at two muscle-specific promoters. Consistent with a model whereby necdin normally de-represses EID-1 during muscle differentiation, we found reduced numbers of myosin heavy chain expressing cells in limb bud muscle cultures from *Ndn*-null mice. Our results suggest that necdin contributes to the normal myogenic differentiation program through interactions with the EID-1 inhibitor of differentiation.

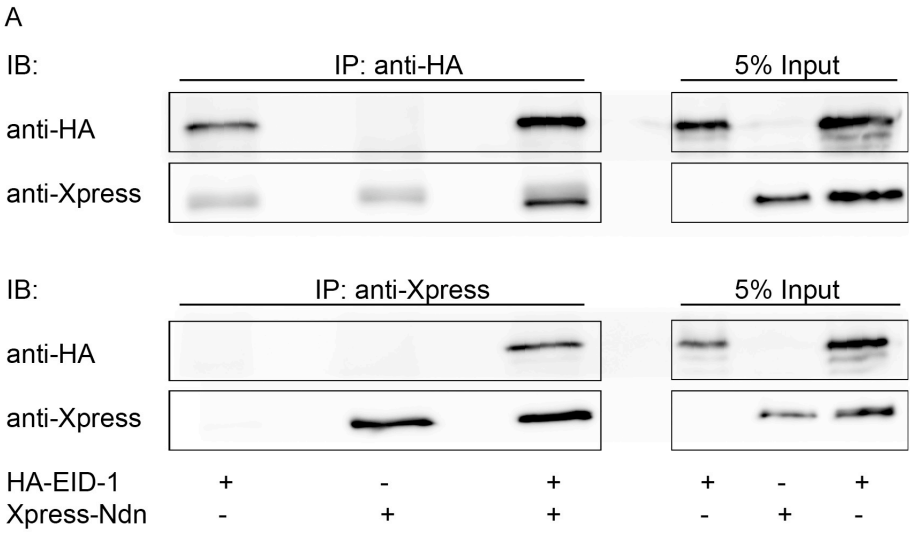
3.2 Results

3.2.1 Necdin interacts with EID-1

We used necdin as the bait protein together with a target library constructed from fetal brain cDNA in a yeast two-hybrid interaction screen (Broder, Katz, and Aronheim 1998; Lee et al. 2005). The CytoTrap library system is based on cytoplasmic interactions that activate the Ras signaling system, inducing cell growth. This methodology was used because transcriptional regulators can create false positives in conventional nuclear yeast two-hybrid screens. One of the novel proteins that we found to robustly interact with necdin in yeast corresponded to the full-length 187 amino acid EID-1 protein.

We tested the interaction between necdin and EID-1 in mammalian cells by co-immunoprecipitation of full-length epitope-tagged proteins in a transient transfection assay (Figure 3.1A). Lysates of C2C12 cells transfected with plasmid expression cDNAs encoding Xpress-tagged necdin (Xpress-Ndn) and HA-tagged EID-1 (HA-EID-1) were immunoprecipitated with an anti-Xpress monoclonal antibody. HA-EID-1 was then detected by anti-HA immunoblot analysis of the immunoprecipitated protein. In reciprocal experiments, immunoprecipitation of identically co-transfected C2C12 cell lysates with a monoclonal anti-HA antibody revealed the presence of Xpress-Ndn upon immunodetection with an anti-Xpress antibody. Parallel co-transfections of plasmid expression constructs encoding Xpress-Ndn or HA-EID-1 with expression constructs encoding irrelevant epitope-tagged proteins confirmed the interaction between necdin and EID-1 was specific (data not shown). We then validated the interaction by co-immunoprecipitation of transiently transfected HA-EID-1 with endogenous necdin in neuronal cells (Figure 3.1B). Lysates of GT1-7 (immortalized neuronal) cells transfected with plasmid expression cDNAs encoding HA-EID-1 or a control (HA-PITX2) were immunoprecipitated with an anti-necdin polyclonal antibody. HA-EID-1, but not HA-PITX2, was detected by anti-HA immunoblot analysis of the immunoprecipitated protein. In reciprocal experiments, immunoprecipitation of identically co-transfected GT1-7 cell lysates with an anti-HA antibody revealed the presence of necdin upon immunodetection with an anti-necdin antibody only in the HA-EID-1 transfected cells. These results suggest that endogenous necdin interacts with EID-1. Unfortunately, an interaction between the endogenous

proteins could not be confirmed because cross-reactivity of both the mouse and human anti-EID-1 antibodies with other proteins precluded their use in immunoprecipitation experiments for endogenous EID-1.



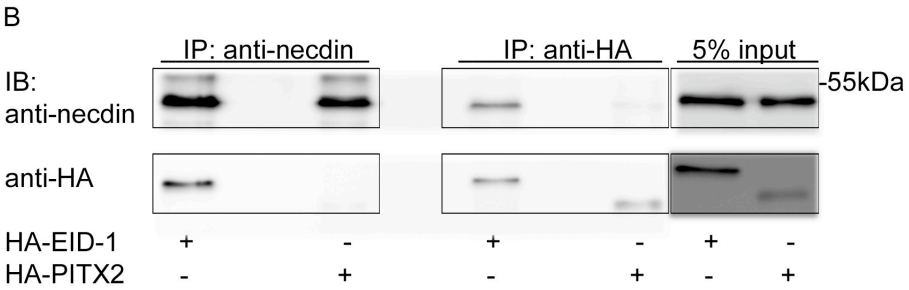


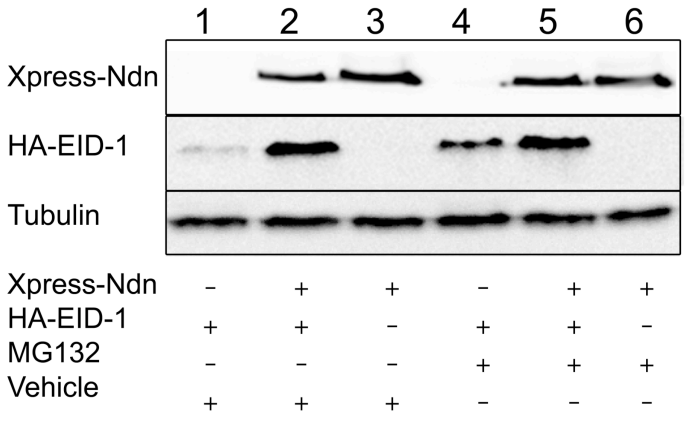
Figure 3.1. Necdin interacts with EID-1. (A) C2C12 cells were transfected with an expression plasmid encoding HA-EID-1, Xpress-Ndn, or both plasmids. Protein complexes were immunoprecipitated with either anti-Xpress or anti-HA antibodies, and then immunoprecipitates detected by immunoblotting with either anti-Xpress or anti-HA. 5% of the volume of cell lysate used for immunoprecipitation was immunoblotted to confirm the presence of the proteins. (B) GT1-7 cells were transfected with an expression plasmid encoding either HA-EID-1 or HA-PITX2. Protein complexes were immunoprecipitated with either anti-necdin or anti-HA antibodies, and then immunoprecipitates detected by immunoblotting with either anti-necdin or
anti-HA.

3.2.2 Necdin increases EID-1 stability

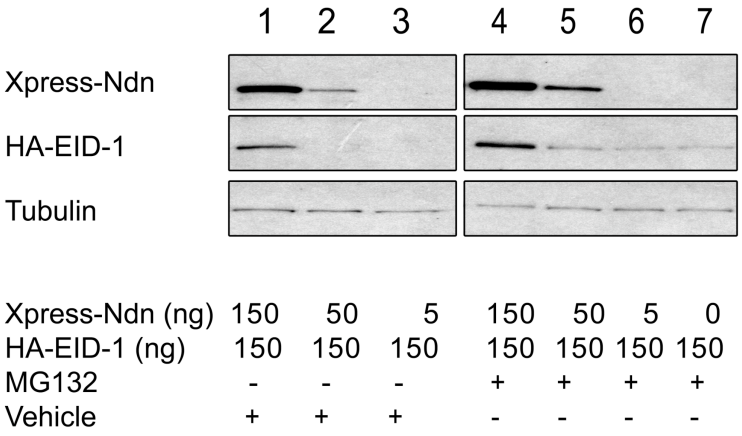
The retinoblastoma protein (Rb)-binding Ret finger protein RFP physically associates with EID-1, stabilizes, and extends the protein half-life of EID-1 in cycling cells (Krutzfeldt et al. 2005). We noted that the steady-state levels of EID-1 were higher in the presence of necdin than in the absence of necdin in cycling cells co-transfected with HA-EID-1 and Xpress-Ndn. To quantify this effect, HEK293 cells were transiently transfected with Xpress-Ndn, HA-EID-1, or both cDNAs. The recombinant proteins were detected by immunoblot analysis of cell lysates and the signal intensities normalized to levels of gamma tubulin (Figure 3.2A, lanes 1-3). EID-1 became 5.3 fold more abundant when co-expressed with necdin (lane 2) than when EID-1 was expressed with the control plasmid (empty vector) (lane 1). We then treated transfected cells with MG132 to inhibit proteasomal degradation, or sham-treated with vehicle alone (Figure 3.2A). Proteasomal inhibition increased the abundance of EID-1 (lane 4) 2.3 fold compared to DMSO treatment alone (lane 1), confirming that EID-1 is subject to proteasomal degradation. Steady-state levels of HA-EID-1 in the presence of necdin were comparable without or with proteasomal inhibition (lanes 2 and 5). Thus, the presence of necdin increases steady state levels of EID-1 above that simply expected from protection from proteasomal degradation. Neither proteasomal inhibition nor EID-1 co-expression had an effect on necdin stability. The increased levels of EID-1 in the presence of necdin were reproducible in subsequent experiments in HEK293 cells and were consistent with increased steady-state levels of HA-EID-1 in transfected C2C12 undifferentiated

and differentiated mouse myoblast cells (Figure 3.2B), in transfected U2OS osteosarcoma cells (data not shown), and endogenous EID-1 protein was less abundant in *Ndn*-null mouse embryo whole brain extracts compared to the levels observed in extracts from control embryos (Figure 3.3).

A


1 2 3 4 5 6
Xpress-Ndn
HA-EID-1
Tubulin
Xpress-Ndn - + + - + +
HA-EID-1 + + - + + -
MG132 - - - + + +
Vehicle + + + - - -

B


1 2 3 4 5 6 7
Xpress-Ndn
HA-EID-1
Tubulin
Xpress-Ndn (ng) 150 50 5 150 50 5 0
HA-EID-1 (ng) 150 150 150 150 150 150 150
MG132 - - - + + + +
Vehicle + + + - - - -

C

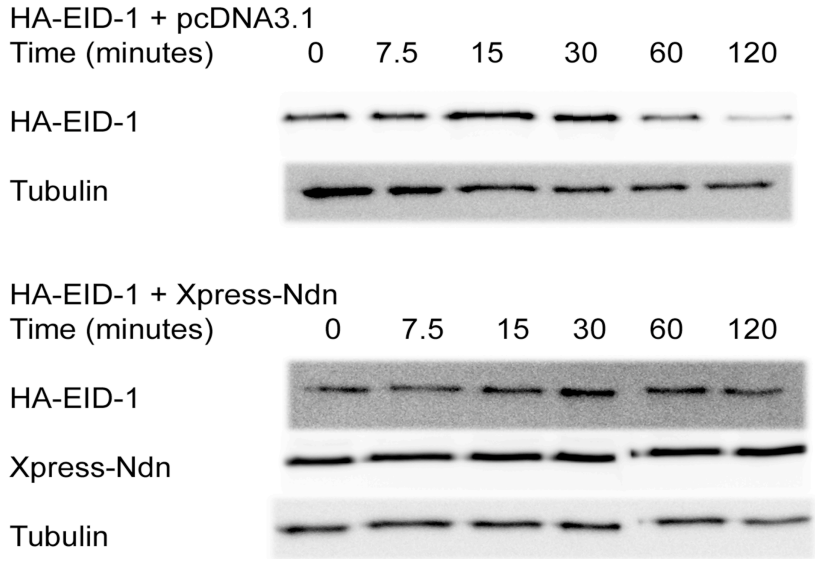

HA-EID-1 + pcDNA3.1
Time (minutes) 0 7.5 15 30 60 120
HA-EID-1
Tubulin
HA-EID-1 + Xpress-Ndn
Time (minutes) 0 7.5 15 30 60 120
HA-EID-1
Xpress-Ndn
Tubulin

Figure 3.2. Co-expression of necdin increases the abundance of EID-1 independently of proteasomal inhibition in HEK293 cells and the C2C12 myogenic cell line. (A) HEK293 cells were transfected with expression plasmids encoding Xpress-Ndn, HA-EID-1, or with both expression plasmids as indicated, then treated with either DMSO (lanes 1-3), or with a proteasomal inhibitor, MG132, in DMSO (lanes 4-6) for 5 h. Protein levels were detected by immunoblotting with the indicated antibody then normalized to levels of tubulin. (B) C2C12 cells were transfected with varying amounts of the expression plasmids encoding Xpress-Ndn, a constant amount of HA-EID-1, or with both expression plasmids in the amounts indicated at the bottom of the Figure. The cells were differentiated in culture for 3 d, then either treated with DMSO vehicle (lanes 1-3), or with the proteasomal inhibitor MG132, in DMSO (lanes 4-7) for 5 h before collection of cell lysates. Empty Xpress vector was co-transfected making up the difference in total plasmid transfected. Protein levels were detected by immunoblotting with the indicated antibody then normalized to levels of tubulin. (C) HEK293 cells were transfected with HA-EID-1 alone or HA-EID-1 and Xpress-Ndn, treated with 100 ug/ml cycloheximide, and lysates collected at the described time points. Protein levels were detected by immunoblot analysis, normalized to levels of tubulin, and the values used to generate protein decay curves. One representative set of experiments is shown.

The increased steady-state level of EID-1 induced by necdin led us to question whether EID-1 is degraded at a slower rate in the presence of necdin, as it is in the presence of RFP (Krutzfeldt et al. 2005). HEK293 cells were transiently transfected with HA-EID-1, Xpress-Ndn, or both cDNA constructs. After 24 h, the cells were treated with cycloheximide to inhibit *de novo* protein synthesis. The relative amounts of epitope-tagged proteins remaining were measured at intervals over 2 h by quantification of protein levels on immunoblots, as described above. The half-life of HA-EID-1 was 24 minutes, consistent with the previously measured half-life of 20 minutes for endogenous EID-1 (Krutzfeldt et al. 2005), but increased to 81 minutes when necdin was co-expressed (Figure 3.2C). Conversely, the half-life of Xpress-Ndn was comparable with or without co-expression of EID-1. Ideally, simulataneous co-expression of a third protein by transient transfection would show that necdin specifically promotes stability of EID-1 protein. We were unable to reproducibly determine whether levels of EID-1 were reduced in limb or brain tissue homogenates from mice or in lysates from a human fibroblast cell line from an individual with PWS and congenital inactivation of the necdin gene because of low levels of EID-1 in these samples and cross reactivity of the EID-1 antibodies.

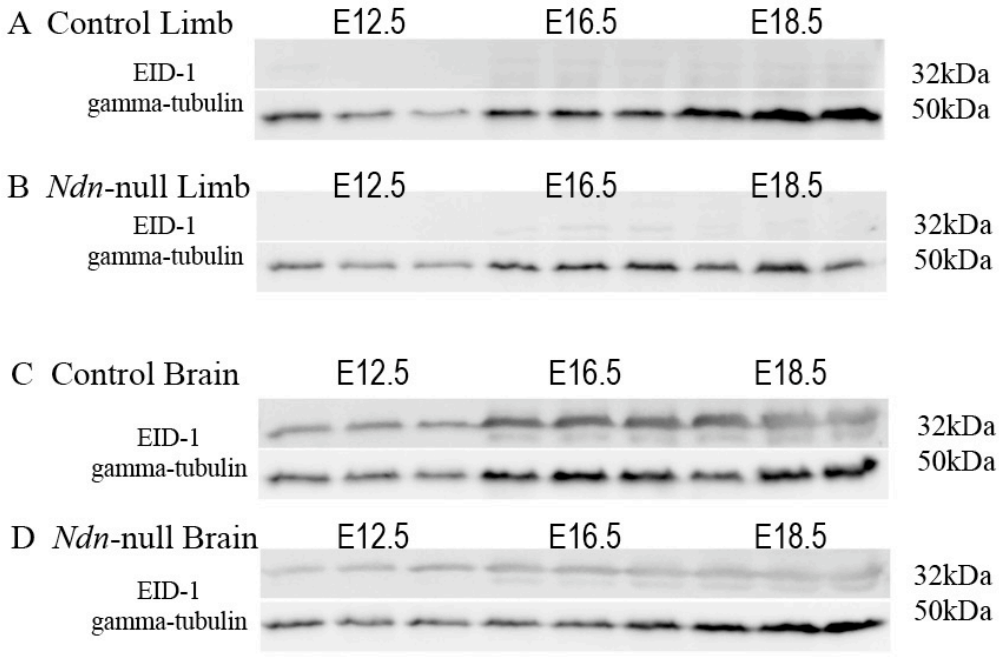


Figure 3.3. Endogenous EID-1 is less abundant in *Ndn*-null mouse embryo tissue. Tissue samples are from three individual embryos of either genotype at each developmental time point. Rabbit polyclonal anti-EID-1 (GN735/736) weakly detects endogenous EID-1 in limb muscle (A, B) and more strongly in whole brain extracts (C, D) from both control and *Ndn*-null mouse embryos. EID-1 protein abundance (relative to gamma-Tubulin) increases from E12.5 to E18.5 in control embryo brains (C), while EID-1 protein levels are significantly lower in *Ndn*-null embryo brains at E12.5 and E16.5 (t-test; $p<0.05$) (D). Limited availability of the GN735/736 antibody prevented repetition of this experiment and precluded inclusion in the published manuscript.

3.2.3 Necdin and EID-1 have altered localization in co-transfected cells

Since protein stability can be partly regulated by subcellular localization (Baker 2007; McKinsey et al. 2000), we investigated whether necdin or EID-1 were re-localized within the cell when co-expressed. Endogenous necdin is present mainly in the cytoplasmic compartment of maturing neurons (Lee et al. 2005), and localizes to both the nucleus and the cytoplasm in SAOS-2 osteosarcoma cells (Taniura, Kobayashi, and Yoshikawa 2005), while EID-1 is primarily cytoplasmic in transfected COS-7 cells (Bavner et al. 2002), but is proposed to be initially localized to the nucleus and then exported to the cytoplasm (Miyake, Yanagisawa, and Yuasa 2003; Bavner et al. 2002). We first transfected undifferentiated C2C12 myoblast cells with either HA-EID-1 or Xpress-Ndn then detected the proteins by immunofluorescence before or after differentiation in culture. The recombinant proteins were present in both the cytoplasm and the nucleus of transfected cells in the both states, but EID-1 protein accumulated in the cytoplasm of cells co-transfected with necdin versus cells singly transfected with HA-EID-1. However, the transfection efficiencies in C2C12 cells were too low to reliably quantify the extent of cytoplasmic enrichment in these cells.

We therefore transfected HEK293 cells with either HA-EID-1, Xpress-Ndn, or both plasmids, then performed cell fractionation to examine the intracellular distribution of the recombinant proteins (Figure 3.4). Xpress-Ndn protein was detected in both the nuclear and cytoplasmic fractions, and was 11-fold enriched in the cytoplasm in the presence of co-transfected HA-EID-1

compared to its relative abundance in the cytoplasm without co-expression of EID-1 (ratio of Xpress-Ndn protein detected in the cytoplasmic fraction to that detected in the nuclear fraction for lane 2 compared to the comparable ratio for lane 1, mean of three separate fractionation experiments).

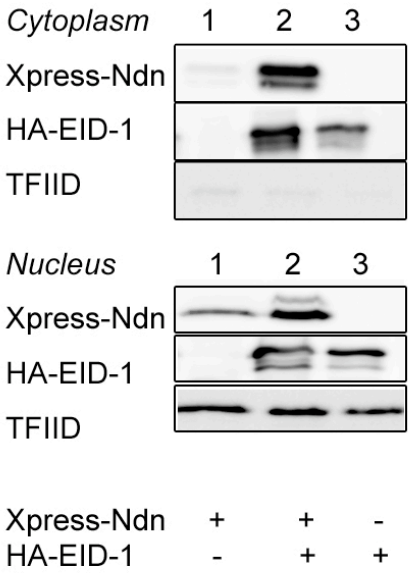
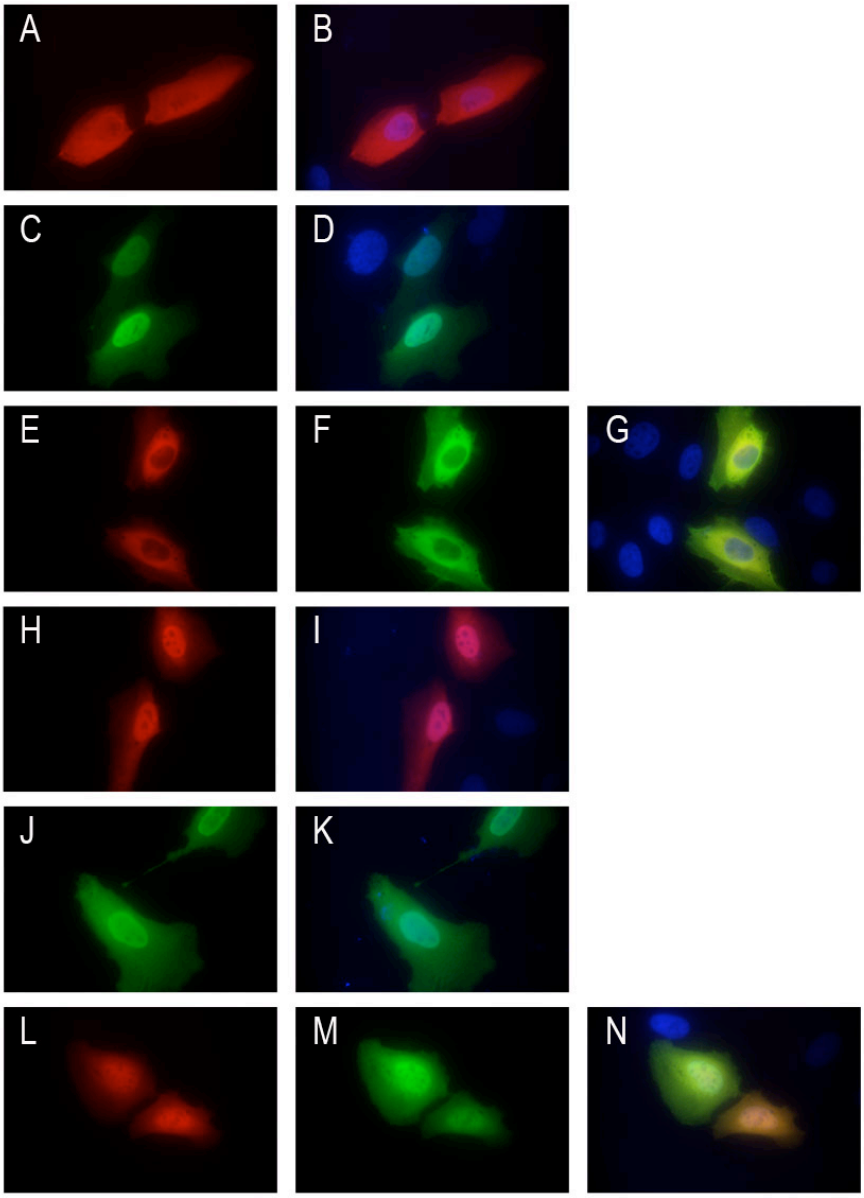


Figure 3.4. Co-expression of necdin and EID-1 enriched both proteins in the cytoplasm of transfected cells. Protein band doublets may reflect differential phosphorylation of necdin and EID-1 in cytoplasmic or nuclear pools. Lysates prepared from subcellular fractionation of HEK293 cells transfected with the expression plasmids indicated at the bottom of the figure and subjected to immunoblotting with antibodies against the Xpress and HA epitope tags, or against endogenous TFIID.

Likewise, HA-EID-1 was detected in both the nuclear and cytoplasmic fractions, but was preferentially enriched 7-fold in the cytoplasmic fraction compared to the abundance in the nuclear fraction in the presence of recombinant Xpress-Ndn (lane 2 compared to lane 3). To quantify this relocalization on a cell-by cell basis, we transfected HEK293 cells with HA-EID-1, Xpress-Ndn, or both plasmids, detected the proteins by immunofluorescence, and measured the proportion of cells displaying only nuclear staining, only cytoplasmic staining, or staining in both compartments for each of the two proteins (n=80 to 110 transfected cells counted per transfection experiment) (Figure 3.5). In cells singly transfected with HA-EID-1, all cells had staining in the cytoplasm, and 26% of cells had additional staining in the nucleus (Figure 3.5A,B; Table 4). Singly transfected cells expressing Xpress-Ndn had diffuse staining throughout the cell, with 98% of cells having some Xpress-Ndn in the nucleus (Figure 3.5C,D; Table 5). We then co-expressed both proteins and re-examined their subcellular localization. In contrast to the singly-transfected cells, only 5% of transfected cells retained any HA-EID-1 in the nucleus, and only 12% of transfected cells had detectable Xpress-Ndn in the nucleus (Figure 3.5E-G; Tables 4 and 5).

We then treated singly-transfected cells with leptomycin B, an inhibitor of Crm-1-mediated nuclear export. This led to the accumulation of HA-EID-1 (Figure 3.5H,I; Table 4) or Xpress-Ndn (Figure 3.5J,K; Table 5) in the nucleus, although many cells retained some cytoplasmic staining. These results indicate that both EID-1 and necdin are subject to Crm1-mediated nuclear export, and are

consistent with a previous finding for EID-1 (Miyake, Yanagisawa, and Yuasa 2003; Bavner et al. 2002).


A
B
C
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F
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I
J
K
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Figure 3.5. Immunofluorescent detection of HA-EID-1 and Xpress-necdin in transfected U2OS cells. Singly transfected HA-EID-1 (red) (A, B) and singly transfected Xpress-necdin (green) (C, D) are present in both the cytoplasm and nuclear compartments (blue is Hoechst stain). Co-transfection of HA-EID-1 and Xpress-necdin results in cytoplasmic accumulation of both proteins (E-G). Leptomycin B treatment increases nuclear pools of singly transfected HA-EID-1 (H, I), Xpress-necdin (J, K), and co-transfected proteins (L-N).

TABLE 4. Subcellular distribution of EID-1 in cells transfected with HA-EID-1 alone or with HA-EID-1 and Xpress-Ndn.

	EID-1	EID-1 and necdin
- Leptomycin B		
Nuclear	0%	0%
Nuclear and Cytoplasmic	26%	5%
Cytoplasmic	74%	95%
+ Leptomycin B		
Nuclear	76%	11%
Nuclear and Cytoplasmic	24%	89%
Cytoplasmic	0%	0%

The proportion of cells (n=50 to 100) with staining for EID-1 was assessed for the indicated compartments by immunofluorescence.

TABLE 5. Subcellular distribution of necdin in cells transfected with Xpress-Ndn alone or Xpress-Ndn and HA-EID-1.

	necdin	necdin and EID-1
- Leptomycin B		
Nuclear	10%	0%
Nuclear and Cytoplasmic	88%	12%
Cytoplasmic	2%	88%
+ Leptomycin B		
Nuclear	37%	10%
Nuclear and Cytoplasmic	60%	84%
Cytoplasmic	3%	6%

The proportion of cells (n=50 to 100) with staining for necdin was assessed for the indicated compartments by immunofluorescence.

Leptomycin B-treated cells co-expressing Xpress-Ndn and HA-EID-1 also had increased nuclear retention of both HA-EID-1 (Table 4) and Xpress-Ndn (Table 5) compared to untreated, co-transfected cells (Figure 3.5L-N). However, even when Crm-1-mediated nuclear export is blocked, the percentage of cells with some EID-1 staining in the cytoplasm increased from 24% to 89% on co-expression of necdin, and the percentage of cells having some cytoplasmic staining of necdin increased from 63% to 90% on co-expression with EID-1. These results indicate that co-expression of both proteins increases the relative amount of both proteins in the cytoplasm, in part through Crm1-mediated export, but also in part in a manner independent of Crm1.

3.2.4 Necdin relieves EID-1-dependent inhibition at myoD-dependent promoters

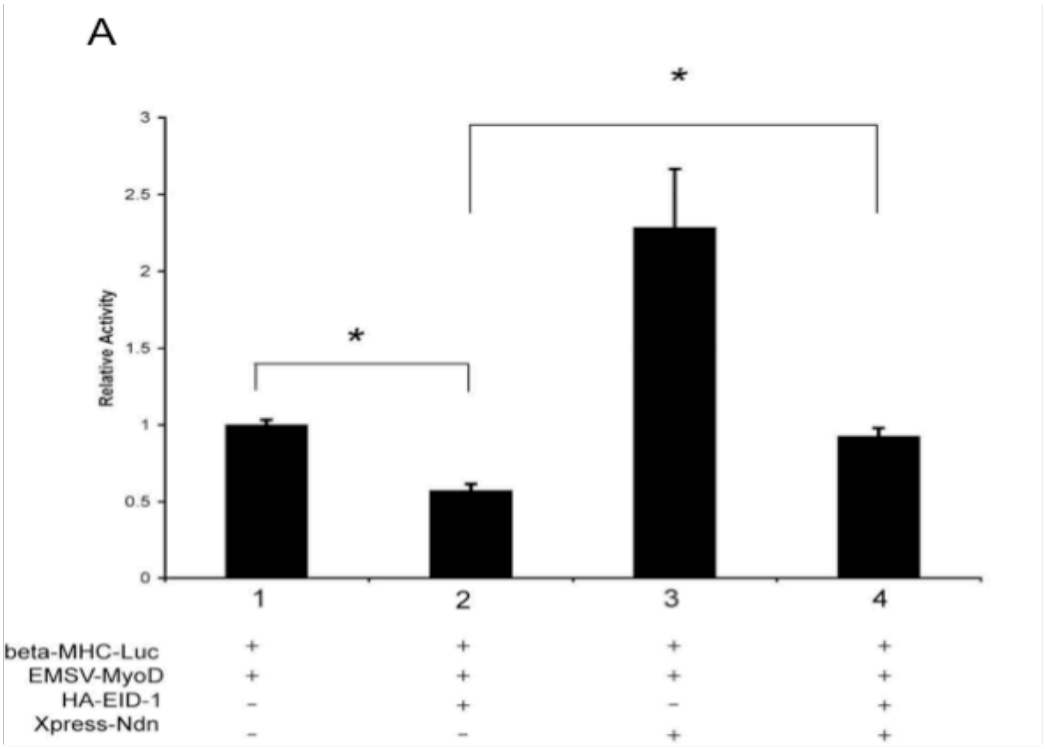
In dual-luciferase reporter assays in U2OS cells, EID-1 inhibits activity of the myogenic regulatory factor MyoD at the *beta-myosin heavy chain* (MHC) promoter (MacLellan et al. 2000). We assessed whether necdin alters EID-1 activity in the same assay. U2OS cells were transfected with necdin, EID-1, or both, along with EMSV-MyoD, a beta-MHC promoter-driven Luciferase reporter construct, and a control Renilla Luciferase reporter construct. In the absence of MyoD, no change in luciferase activity above baseline was detected on transfection with necdin, EID-1, or with both plasmids, while transfection with MyoD increased luciferase activity 5-fold over baseline (Figure 3.6A). However, transfection with EID-1 inhibited transactivation in the presence of MyoD, consistent with its previously reported activity at this promoter (MacLellan et al. 2000) (Figure 3.6A, assay 2 versus assay 1). Co-transfection with necdin and

EID-1 returned promoter activation to a level similar to that achieved with MyoD alone (assay 4 versus assay 2). Expression of necdin did not have a statistically significant effect on MyoD-dependent transactivation in the absence of EID-1, in any of three repeated experiments (assay 3). We performed a similar experiment using the 4RTK reporter construct containing a promoter with 4 E-box binding sites, previously shown to be activated by MyoD (MacLellan et al. 2000), and obtained similar results (Figure 3.6B). Thus, necdin relieves EID-1 induced inhibition at two MyoD dependent promoters, in the presence of MyoD. EID-1 does not bind DNA, but rather binds CBP/p300 and interferes with its activity, so it is not feasible to detect an EID-1/necdin complex at endogenous promoters. In summary, these experiments suggest that necdin is well placed to promote muscle differentiation by modulating the stability, subcellular localization, and activity of EID-1, thereby facilitating the release of EID-1 repression of MyoD-dependent transcription.

3.2.5 Ndn-null myoblasts are impaired in their differentiation into myotubes

Necdin is normally expressed in the myotome at E10.5 (Brunelli et al. 2004), in the fore and hind limb buds at E11.5 (Lee et al. 2000), and continues to be expressed in skeletal muscle during embryogenesis (Lee, Walker, and Wevrick 2003), while expression of EID-1 in developing limbs has not been examined. Considering that necdin facilitates myosin heavy chain gene expression through de-repression of EID-1, we predicted that the transition from MyoD expressing myoblasts to MHC-expressing muscle cells would be deficient in mice with a gene targeted loss of necdin. To test this hypothesis, we examined the subset of

the myogenic precursor cells that migrate from the dermomyotome into the nascent limb buds, subsequently express MyoD, become myoblasts, and differentiate into myotubes expressing MHC. We previously showed that motor neuron pools are reduced in *Ndn*-null mice (Pagliardini et al. 2005). To avoid the possible confounding effect of reduced innervation of skeletal muscles, we examined the differentiation of myoblasts present in cultures of isolated murine embryonic limb buds. Forelimb buds from E10.5 *Ndn*-null and wild-type littermate embryos were pooled by genotype, dissociated, plated, and cultured *in vitro*.


A
*
*
Relative Activity
3
2.5
2
1.5
1
0.5
0
1
2
3
4
beta-MHC-Luc + + + +
EMSV-MyoD + + + +
HA-EID-1 – + – +
Xpress-Ndn – – + +

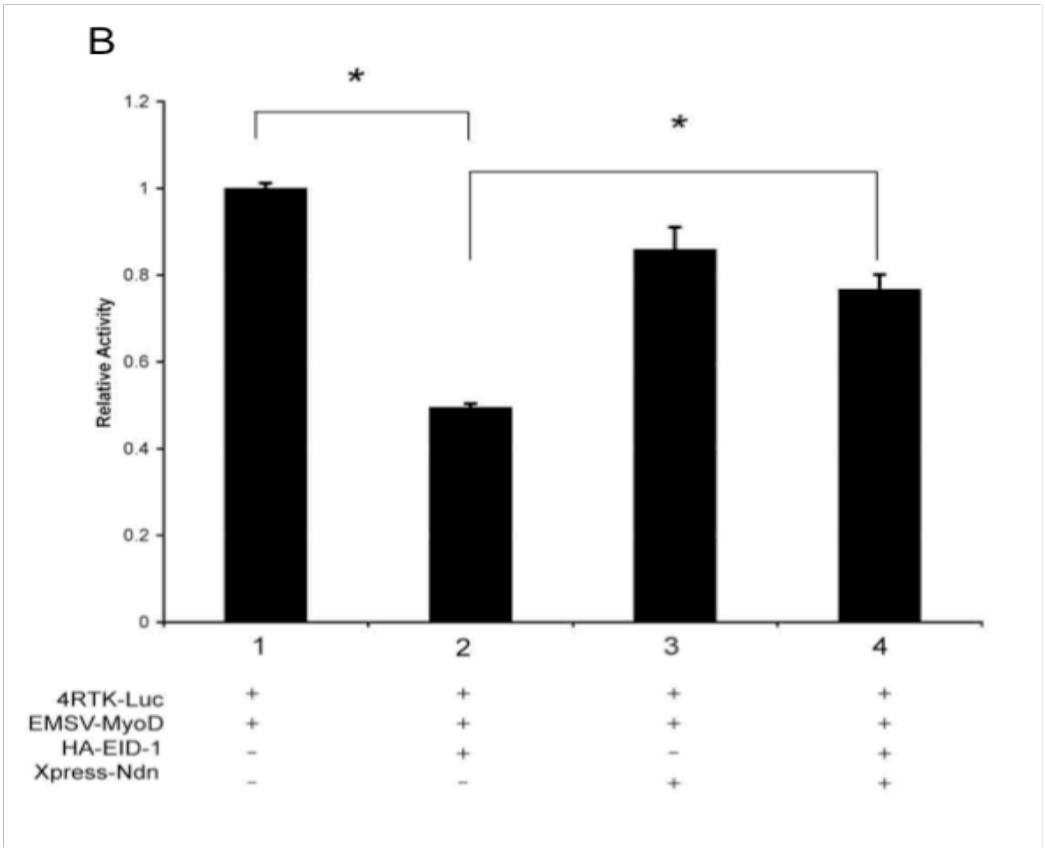

B
*
*
Relative Activity
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
4RTK-Luc + + + +
EMSV-MyoD + + + +
HA-EID-1 – + – +
Xpress-Ndn – – + +

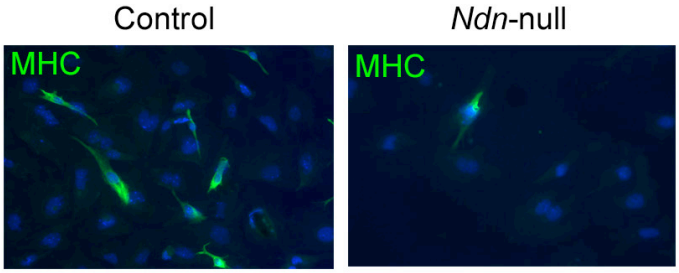
Figure 3.6. Necdin relieves EID-1-dependent transcriptional repression at MyoD-dependent promoters. Dual luciferase transcription assays following transfection of U2OS cells with an expression plasmid encoding EMSV-MyoD and (A) a beta-MHC luciferase reporter plasmid or (B) a 4RTK luciferase reporter plasmid, and combinations of HA-EID-1 and Xpress-Ndn. Data are normalized to the baseline activity of MyoD at the respective promoters. Differences that are significant at $p<0.05$ are labeled with an asterisk. These results represent one of three experiments completed in triplicate. Error bars represent the standard error of the mean.

These cultures contain a mixture of cell types, a small fraction of which are myoblasts (expressing MyoD) that differentiate into myotubes (expressing myosin heavy chain (MHC)) (Cusella-De Angelis et al. 1994). One, three, and five days after plating, the cultures were immunostained with either MF20 antibody directed against MHC or an anti-MyoD antibody, and the percentage of cells that were stained for each protein was determined (Figure 3.7A). As expected, the percentage of control cells that appeared to be myotubes and expressed MHC increased from Day 1 to Day 5 (Figure 3.7B), consistent with the number of myotubes obtained in similarly prepared embryonic limb bud cultures (Cusella-De Angelis et al. 1994).

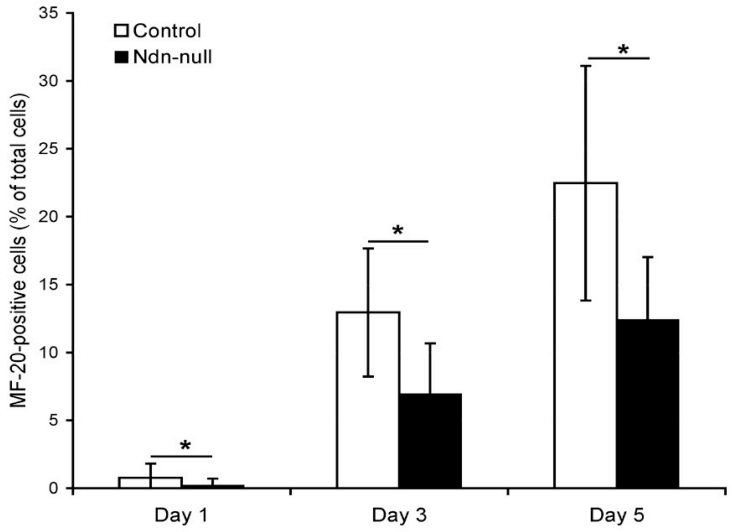
However, there were significantly fewer MHC-positive cells in the Ndn-null forelimb bud cultures at all time points. On Day 1, 1% of wild-type control cells were MHC-positive, while only the occasional MHC-positive cell was detected in the Ndn-null cultures. On Day 3, 13% of wild-type and 7% of Ndn-null cells were MHC-positive, and on Day 5, 22% of wild-type and 12% of Ndn-null cells expressed MHC. There was no difference in the number of MyoD positive cells in cultures collected at any time point (Figure 3.7C), suggesting that the formation of myoblasts is not dependent on necdin or that the limb bud culture assay is not sensitive enough to detect differences in the number of myoblasts due to the loss of necdin. We could not determine whether the intracellular localization of EID-1 was altered in these cells because cross-reactivity of the anti-EID-1 antibody with other proteins precluded its use in immunofluorescence.

Our results suggest that despite normal expression of MyoD, Ndn-null myogenic precursor cells have an impaired capacity to differentiate into muscle.

A


Control
Ndn-null
MHC
MHC

B


Control
Ndn-null
MF-20-positive cells (% of total cells)
35
30
25
20
15
10
5
0
*
*
*
Day 1
Day 3
Day 5

C

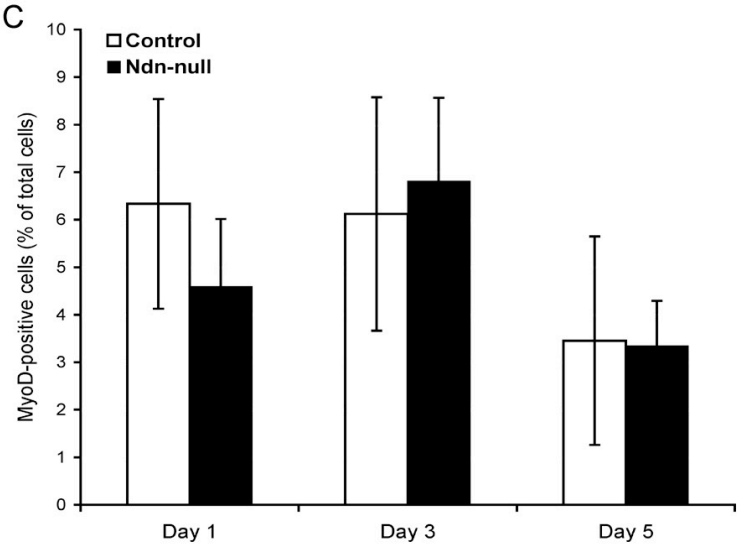

Control
Ndn-null
MyoD-positive cells (% of total cells)
10
9
8
7
6
5
4
3
2
1
0
Day 1
Day 3
Day 5

Figure 3.7. Conversion of MyoD expressing cells to MHC-expressing cells is decreased in limb bud cultures from *Ndn*-null mouse embryos. Limb buds from E10.5 embryos were dissociated and cultured, then immunostained for either MHC or MyoD. (A) MHC (green) was detected in differentiated myotubes by immunofluorescence in wild type (left) and *Ndn*-null (right) cells held in culture for 5 days. Cell nuclei were counterstained with DAPI (blue). (B) The number of MF20-positive cells (expressing MHC) in each culture was counted on Days 1 (the day after plating), 3 and 5 and compared to the total number of cells. (C) The number of immunostained MyoD-positive cells was compared to the total number of cells, also on Days 1, 3, and 5. *, $p < 0.001$.

3.3 Discussion

Differentiation of muscle precursor cells requires recruitment of chromatin remodeling proteins to gene promoters where they modulate the function of myogenic regulatory factors. The E1A-like inhibitor of differentiation EID-1 forms a complex with MyoD at the promoters of MyoD-dependent genes, where it inhibits differentiation by interfering with the histone acetyltransferase function of the p300 and CBP co-activator proteins. EID-1 interacts with the retinoblastoma tumor suppressor protein pRb, which itself promotes differentiation by modulating the activity of transcription factors, including MyoD. The MAGE family protein, necdin, promotes differentiation in cells of mesodermal, neural crest, and neural origin, as previously demonstrated in gain and loss of function experiments *in vivo* and in cultured cells (Hayashi et al. 1995; Brunelli and Cossu 2005; Brunelli et al. 2004; Kurita et al. 2006; Kuwajima et al. 2004; Takazaki, Nishimura, and Yoshikawa 2002; Taniura et al. 1998; Yoshikawa 2000; Lee et al. 2005; Deponti et al. 2007). We now demonstrate that necdin, like pRb, binds to EID-1 and suppresses its activity, consistent with other functional similarities between these two pro-differentiation proteins. We propose that like pRb, necdin promotes muscle differentiation in part by modulating the activity of EID-1, thereby facilitating the release of EID-1 repression of MyoD-dependent transcription (Bavner et al. 2002; MacLellan et al. 2000; Miyake et al. 2000) (Figure 3.8).

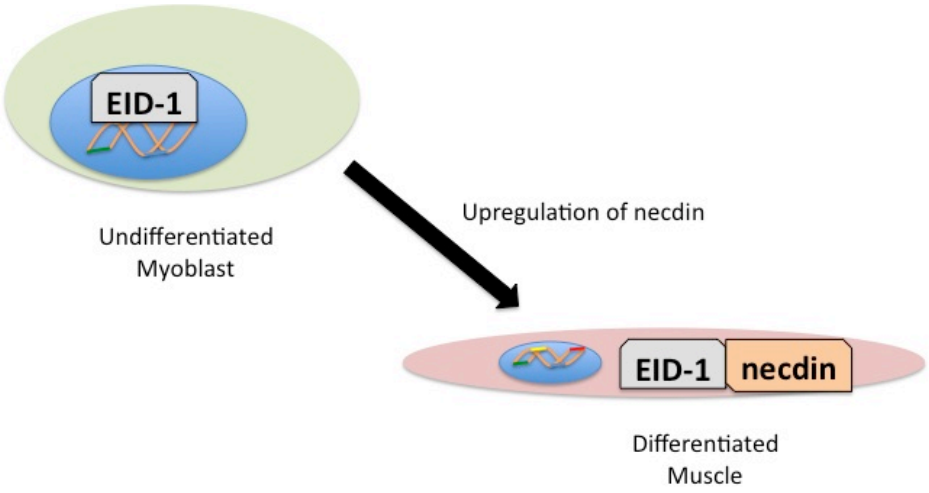


Figure 3.8. Proposed model for the necdin-EID-1 interaction during myogenesis. Accumulation of necdin during myogenesis results in sequestration of EID-1 in the cytoplasm. In the absence of EID-1, robust MyoD-dependent transcription leads to myotube formation.

In some circumstances, EID-1 level is modulated by proteasomal degradation. For example, the terminal differentiation and cell-cycle exit of U937 leukemia cells or WI38 fibroblasts correlates with reduced phosphorylation of pRB, ubiquitination of EID-1 by Mdm2, and proteasomal degradation of EID-1 (Krutzfeldt et al. 2005; MacLellan et al. 2000; Miyake et al. 2000). Additionally, inhibiting proteasomal degradation stabilizes EID-1, as does the over-expression of pRb mutant proteins that can bind to EID-1 but not recruit Mdm2 (Miyake et al. 2000). Given the functional similarities between necdin and pRb (Taniura et al. 1998), and a previous observation that necdin inhibits HIF-1α activity and promotes HIF-1α proteasomal degradation (Moon et al. 2005), it was *a priori* possible that necdin would de-repress EID-1-inhibition by promoting EID-1 proteasomal degradation. Alternatively, it was possible that necdin shares functional similarity with the pRb-interacting RET finger protein RFP, which stabilizes EID-1 by preventing pRb-dependent degradation (Krutzfeldt et al. 2005). RFP associates with pRb on DNA and is thought to prevent the physical association of EID-1 with pRb and Mdm2, both of which are necessary for EID-1 proteasomal degradation (Krutzfeldt et al. 2005; Taniura, Matsumoto, and Yoshikawa 1999). Consistent with the second hypothesis, co-transfection with necdin in pRb-proficient U2OS cells increased in the steady-state levels of EID-1. While it is possible that altered amounts of EID-1 are from an effect of necdin on translation initiation, we favor a model where necdin normally stabilizes EID-1 protein, but that EID-1 becomes less active in transcriptional repression during this process because of re-localization to the cytoplasm. Unfortunately, the

intracellular localization of endogenous EID-1 and interaction of endogenous EID-1 with necdin could not be assessed because cross-reactivity of the anti-EID-1 antibodies with other proteins precluded their use in immunofluorescence and immunoprecipitation experiments. Experiments that investigate the stability of exogenous EID-1 with a suitable antibody after silencing of endogenous necdin via RNA knock-down in a cell line could address this question.

In the presence of MyoD, EID-1 inhibits transcription from MyoD dependent promoters, but co-expression of necdin and EID-1 neutralizes this activity in transient transfection experiments. Notably, the expression of necdin alone in this system has no effect on either of the two MyoD-dependent promoters. Our subcellular localization experiments suggest that the neutralization of EID-1 activity by necdin occurs in part through the accumulation of both proteins in the cytoplasm, while both proteins occupy both the nuclear and cytoplasmic compartments in each other's absence. Nucleocytoplasmic shuttling of other proteins is important in muscle differentiation. For example, the repressive chromatin remodeling protein HDAC5 is exported from the nucleus to the cytoplasm during muscle differentiation, and this relocalization is required for MyoD-dependent gene activation (McKinsey et al. 2000; Verdin, Dequiedt, and Kasler 2003), and Crm1-independent nucleocytoplasmic shuttling and accumulation of the RNA-binding protein HuR in the cytoplasm also plays a critical role in muscle differentiation (van der Giessen et al. 2003). The effect of necdin on the activity, stability, and subcellular localization of EID-1 suggests

that necdin could also modulate co-repressor function in transcription in other cell types, including in neurons where both *Ndn* and *EID-1* are more highly expressed.

Necdin interacts with DNA-binding transcription factors, affecting their activity and stability. For example, necdin suppresses the activity of the repressive homeodomain transcription factor Msx2 thereby facilitating the differentiation of smooth muscle cells from mesoangioblast stem cells (Kuwajima et al. 2004; Brunelli and Cossu 2005; Brunelli et al. 2004). Necdin suppresses transcriptional activation by the hypoxia-inducible transcription factor HIF-1α and destabilizes HIF-1α in a proteasome-dependent manner (Moon et al. 2005), suppresses p53-dependent and E2F1-dependent promoter activation (Taniura, Matsumoto, and Yoshikawa 1999; Taniura et al. 1998; Kurita et al. 2006), but enhances the activity of the homeodomain protein Dlx2 thereby promoting GABAergic neuron differentiation (Kuwajima et al. 2004). The transcriptional activities of HIF-1α, p53, and E2F proteins are regulated by nucleocytoplasmic shuttling. While these previous studies implicate necdin in the modulation of DNA-binding transcriptional factor activity, our study expands the repertoire of necdin-mediated transcriptional regulation to include the co-repressor EID-1, which is not known to directly bind DNA. Furthermore, our results are consistent with experiments that show that necdin promotes skeletal muscle differentiation without directly interacting with MyoD, myogenin, or Mef2A (Deponti et al. 2007). Chromatin immunoprecipitation of necdin at MyoD-dependent promoters followed by immunoblot analysis to detect other myogenic transcription factors,

co-activators, or co-repressors could further elucidate the mechanisms by which necdin normally promotes muscle differentiation.

Necdin and other MAGE family proteins mediate the transduction of growth and survival signals in neurons, through associations with neurotrophin receptors (Kuwako et al. 2005; Kuwako, Taniura, and Yoshikawa 2004; Salehi et al. 2000; Tcherpakov et al. 2002; Williams et al. 2003). Although necdin was originally described as a nuclear protein (Maruyama et al. 1991), recent studies with more reliable antibodies demonstrate that in fact endogenous necdin is present mainly in the cytoplasmic compartment of maturing neurons (Yoshikawa 2000). While EID-1 was originally shown to inhibit p300-dependent transcriptional co-activation in the nucleus of undifferentiated cells, EID-1 continues to be expressed in adult mouse tissue (MacLellan et al. 2000; Miyake et al. 2000) and is normally found in the cytoplasm (Bavner et al. 2002). Taken together, these observations suggest that a second role of EID-1 may be to promote the relocalization of necdin to the cytoplasm, where it facilitates the transduction of growth and differentiation signals.

It is important to consider the embryonic time interval during which the interaction between necdin and EID-1 could affect myogenic differentiation. One of the earliest events in limb muscle formation is the expression of the transcription factor Pax3, which is crucial for the specification of myogenic cells in the limb. Pax3-expressing cells migrate from the dermomyotome into the limbs, express myogenic regulatory genes, and finally differentiate into myotubes. In previous studies, ectopic production of EID-1 in differentiating C2C12

myoblasts was shown to inhibit MHC expression and myotube formation (MacLellan et al. 2000; Miyake et al. 2000) but did not inhibit the expression of early myogenic markers such as MyoD (MacLellan et al. 2000). This places EID-1 temporally downstream of the initial specification and commitment of myogenic precursor cells. We observed that the number of MyoD-expressing myogenic progenitor cells was unaffected by loss of necdin, also placing necdin downstream of myogenic commitment. Thus, the necdin-EID-1 interaction is unlikely to be important in myoblast commitment or proliferation. In contrast, the proportion of MHC-expressing cells was significantly reduced in the *Ndn*-null cultures. This suggests a delay in MyoD-dependent differentiation and is consistent with a model whereby necdin is required for the timely de-repression of EID-1 at MyoD-dependent promoters. While indirect, these studies suggest that the interaction between EID-1 and necdin could be functionally important during normal skeletal myogenesis. Immunohistochemical analysis of necdin and EID-1 in the myoblasts of a developing mouse would reveal how these proteins respond temporally to differentiation cues during myogenesis.

The Structural Maintenance of Chromosomes complex (SMC5-6) is important in chromosome dynamics and transcription, and contains four essential non-SMC proteins (Nse1-4). The Nse3 protein of *Schizosaccharomyces pombe* is homologous to the MAGE family, while Nse4, a member of the kleisen superfamily, is most similar to EID-3 (Bavner et al. 2005) and to an EID-related protein encoded on human chromosome 10 (C10orf86) (Taylor et al. 2008). A recent study identified the MAGE family protein MAGE-G1 (Chibuk, Bischof,

and Wevrick 2001) and the C10orf86 encoded EID protein (named hNSE4a) as endogenous components of the human SMC5-6 complex (Taylor et al. 2008). Both the single zebrafish and chicken MAGE related genes encode proteins that are most similar to human MAGE-G1 (Bischof, Ekker, and Wevrick 2003; Lopez-Sanchez et al. 2007) which, like necdin, contains little coding sequence other than the MAGE homology domain. Like necdin, MAGE-G1 induces growth arrest in cells, interacts with the transcription factor E2F1 via its transactivation domain, represses E2F1-dependent transcription, antagonizes E2F1-induced apoptosis, and interacts with the intracellular domain of the p75 neurotrophin receptor (Kuwako, Taniura, and Yoshikawa 2004). The identification of a functional interaction between MAGE-G1 and an EID-family protein is consistent with our hypothesis that the MAGE protein necdin functionally interacts with EID-1 in mammalian cells.

In summary, we propose that one way in which necdin promotes muscle differentiation is by relieving EID-1-dependent transcriptional repression, likely in concert with its other activities related to transcription. While central hypotonia rather than abnormal muscle development is a cardinal feature of Prader-Willi syndrome in infants, histochemical studies of muscle biopsies from children with PWS have documented small type 2 fibers, with a decrease in the number of type 2B fibers and an increase in the number of type 2C (immature) fibers (Miike et al. 1988; Sone 1994; Argov et al. 1984). PWS infants typically have normal electromyography, suggesting that if there is any myopathy it is not detectable in the presence of profound central hypotonia (Richer, Shevell, and

Miller 2001). Regardless of the relative importance of the necdin-EID-1 interaction in muscle differentiation, the observation that necdin deficiency compromises myotube formation in the limb buds provides new insights into the origins of body composition abnormalities and reduced muscle mass in PWS. We speculate that the loss of necdin in people with PWS contributes to delayed or deficient skeletal muscle development, contributing to reduced muscle mass and exacerbating the low muscle tone that is present at birth.

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Chapter 4. Impaired myosin activation and cellular polarization contribute to defective neuronal and muscle development in a necdin-deficient mouse model of Prader-Willi syndrome

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Jason Bush completed all of the experiments presented in this chapter.

4.1 Summary

NDN is one of several genes inactivated in Prader-Willi syndrome (PWS), a developmental disorder characterized by hyperphagic obesity, hypotonia, and developmental delay. The encoded protein necdin modulates transcription and has less well understood cytoplasmic functions. We now demonstrate that loss of necdin in mouse and PWS fibroblasts impairs polarity initiation through a Cdc42-myosin-dependent pathway, thereby reducing cell migration. Given that cellular migration and polarity are integral processes in both the developing muscle and nervous systems, we investigated these tissues in *Ndn*-null mice and found polarization defects in both primary neuron cultures and in the developing limb. *Ndn*-null neurons fail to activate myosin light chain and display defective polarization with respect to a brain derived neurotrophic factor gradient. Pax3-positive muscle progenitors in *Ndn*-null developing forelimbs display defective polarization, do not adequately migrate into the dorsal limb bud, and the extensor muscles are consequently smaller. These results provide strong evidence that necdin is a key protein regulating polarization of the cytoskeleton during development. Further, this report is the first to demonstrate a cellular defect in PWS, and suggests a novel molecular mechanism to explain neurological and muscular pathophysiologies in PWS.

4.2 Introduction

Five protein-coding genes, including *NDN* encoding necdin, and a set of small nucleolar RNAs are typically inactivated in people with PWS, complicating

our understanding of the underlying etiology. Murine *Ndn* was originally identified as a gene whose expression is upregulated in retinoic-acid treated P19 embryonal carcinoma cells (Maruyama et al. 1991), and is strongly expressed during development, including nervous (Aizawa et al. 1992), muscle (Gerard et al. 1999; Kuwajima et al. 2004), and somitic tissues (Andrieu et al. 2003; Brunelli et al. 2004). In mice, loss of necdin causes respiratory defects, pain insensitivity, and hypotonia (Andrieu et al. 2006; Bush and Wevrick 2008; Kuwako et al. 2005; Muscatelli et al. 2000; Pagliardini et al. 2005; Ren et al. 2003; Deponti et al. 2007; Gerard et al. 1999). Necdin is a transcriptional modulator that inhibits apoptosis and promotes differentiation in both neurons and muscle (Barker and Salehi 2002; Bush and Wevrick 2008; Deponti et al. 2007), but can also act as a cytoplasmic modulator of signaling events through a poorly understood mode of action (Bronfman et al. 2003; Liu et al. 2009; Tcherpakov et al. 2002). Loss of function of other PWS candidate genes also produces phenotypes in the mouse, primarily affecting post-natal growth, metabolism, and behavior (Ding et al. 2008; Doe et al. 2009; Skryabin et al. 2007; Mercer et al. 2009; Mercer and Wevrick 2009).

Proper development of the nervous and skeletal muscle systems requires the coordination of cellular differentiation with migration and, in neurons, axonal extension. Consequently, ablation of gene function necessary for either cellular specification or migration can produce similar phenotypes at later stages of development. For example, either failure to specify muscle progenitors in *Splotch* mutant mice or inactivation of the hepatocyte growth factor receptor *c-met* results

in the absence of forelimb musculature (Bladt et al. 1995; Goulding, Lumsden, and Paquette 1994). While cell migration is critical for normal development, the molecular basis of cell motility is best described by studies *ex vivo*. In fibroblasts and muscle precursors, initiation of migration involves asymmetrical partitioning of intracellular components to establish polarity with respect to the intended direction of movement (Gotlieb and Spector 1981; Kupfer, Louvard, and Singer 1982; Ueda et al. 1997). Developing neurons have additional cytoskeletal requirements during migration and axonal projection. Positioning of the centrosome predicts which neurite will become the axon in hippocampal neurons (de Anda et al. 2005), and the bundling of microtubules (Bielas et al. 2007) via activated myosin (Burnette et al. 2008) at the wrist of axonal growth cones facilitates proper axonal extension and guidance. Axonal defects consistent with cytoskeletal disorganization and migration defects are observed in *Ndn*-null mice (Kuwajima, Hasegawa, and Yoshikawa 2010; Kuwako et al. 2005; Lee et al. 2005; Miller, Wevrick, and Mellon 2009; Pagliardini et al. 2005; Tennese, Gee, and Wevrick 2008; Zanella et al. 2008). We now report that loss of necdin impairs Cdc42-dependent myosin activation and the subsequent polarization of fibroblasts, neurons, and muscle, both *in vitro* and *in vivo*.

4.3 Results

4.3.1 Loss of necdin delays polarization in both mouse embryonic fibroblasts and human fibroblasts

We and others previously observed deficient migration of *Ndn*-null embryonic GnRH, sympathetic neuron, and GABAergic interneuron populations

(Kuwajima, Hasegawa, and Yoshikawa 2010; Miller, Wevrick, and Mellon 2009; Tennese, Gee, and Wevrick 2008). While investigating the myogenic potential of embryonic limb bud cultures, we noticed that those from *Ndn*-null mice contained fewer Pax3-positive muscle precursors (Figure 4.1). This reduction may be due to increased apoptosis; however, the reduced number of Pax3-positive cells may also reflect a migration defect into the limb bud due to loss of necdin. To resolve this ambiguity, we examined whether loss of necdin impairs migration of other cell types and to investigate the mechanism for this cellular phenotype.

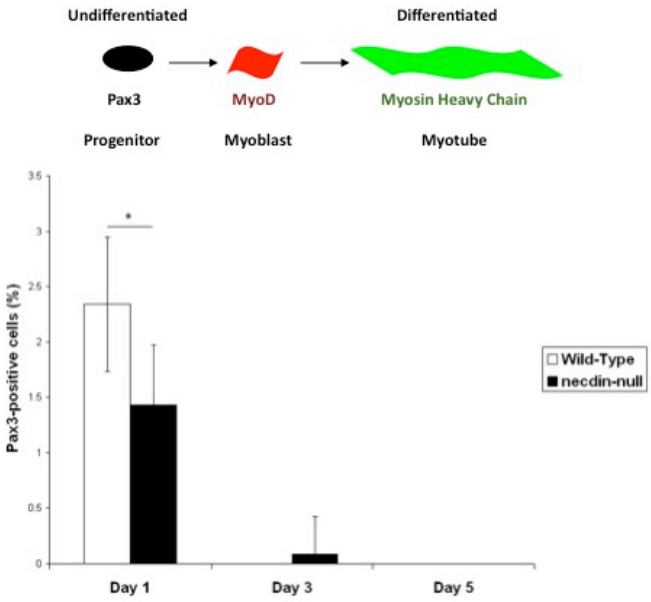


Figure 4.1. *Ndn*-null limb buds contain fewer Pax3-positive muscle precursors. Pooled E10.5 limb bud cultures were prepared from control or *Ndn*-null embryos and cultured for 1, 3, or 5 days and the proportion of Pax3-positive cells was determined by dividing the number of Pax3-expressing nuclei in a field by the total number of nuclei counted (t-test, mean±SD, * $p<0.05$).

We first performed wound-healing assays using *Ndn*-null and control mouse embryonic fibroblasts (MEFs) because fibroblasts are highly motile cells that express necdin. MEF monolayers of each genotype were wounded and migration distances measured over 96 hours. Notably, *Ndn*-null MEFs were motile, but migrated shorter distances at all time points (Figure 4.2A). *Ndn*-null fibroblasts migrated at a reduced rate in the first 12 hours (Control = 33 μm/h vs. *Ndn*-null = 21 μm/h), but at a comparable rate after 24 hours (Control = 14 μm/h vs. *Ndn*-null = 11 μm/h).

The reduced initial velocity suggested that loss of necdin compromises the initiation of fibroblast migration. Cells on the edge of a wound typically initiate migration by re-orienting their centrosome to a location between the leading edge of the cell and the nucleus, polarizing the cell with respect to the direction of migration (Kupfer, Louvard, and Singer 1982). We wounded monolayers of MEFs, then processed them at time intervals, staining with Hoechst to label nuclei, and with antibodies to α-tubulin and γ-tubulin, defining the cell periphery and labeling the centrosome. As previously shown, 4 hours after wounding approximately 67% of wound-edge control fibroblasts orient with the centrosome between the leading edge and the nucleus (Gomes, Jani, and Gundersen 2005; Palazzo et al. 2001). In contrast, *Ndn*-null cells were not oriented toward the wound (Figure 4.2B), suggesting that the delay in *Ndn*-null MEF migration is due to reduced ability of cells to polarize towards the wound edge. We performed similar experiments using PWS (therefore necdin-null) and control human fibroblasts. The proportion of re-oriented wound-edge fibroblasts was not

different from expected in the seven control cell lines examined 4 hours after wounding (Figure 4.2C). However, analogous to the *Ndn*-null MEFS, neither PWS fibroblast line was significantly oriented.

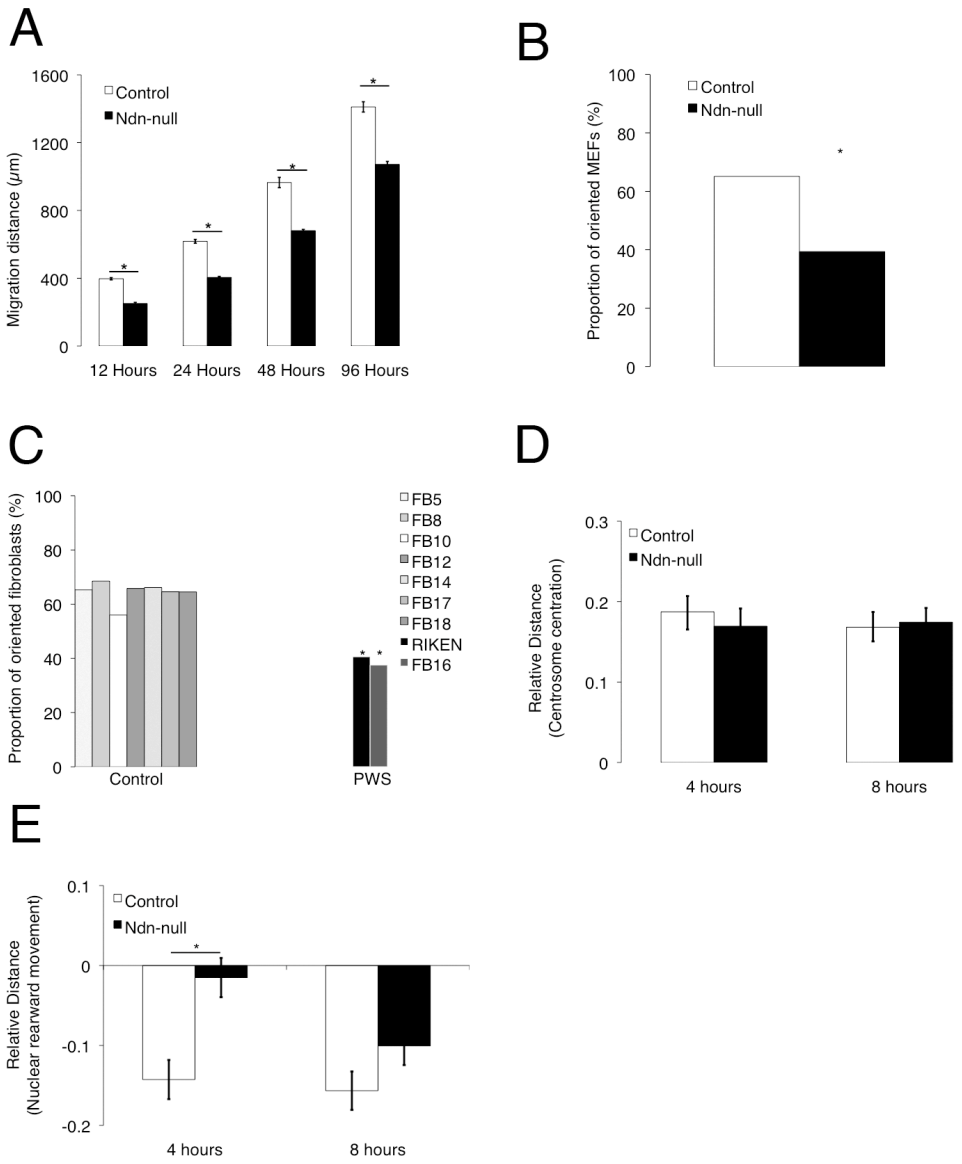

A
Migration distance (μm)
1600
1200
800
400
0
Control
Ndn-null
12 Hours
24 Hours
48 Hours
96 Hours
B
Proportion of oriented MEFs (%)
100
80
60
40
20
0
Control
Ndn-null
C
Proportion of oriented fibroblasts (%)
100
80
60
40
20
0
FB5
FB8
FB10
FB12
FB14
FB17
FB18
RIKEN
FB16
Control
PWS
D
Relative Distance
(Centrosome centration)
0.3
0.2
0.1
0
Control
Ndn-null
4 hours
8 hours
E
Relative Distance
(Nuclear rearward movement)
0.1
0
-0.1
-0.2
Control
Ndn-null
4 hours
8 hours

Figure 4.2. Impaired myosin-dependent activities in *Ndn*-null migrating fibroblasts. (A) MEF monolayers were grown to confluence, wounded, and the distance and rate of migration into the wound were measured (t-test, $p<0.05$). (B) Centrosome orientation was measured in wound-edge MEFs 4 hours after wounding (chi-square, $p<0.05$). (C) Centrosome orientation was measured in wound-edge human fibroblasts 4 hours after wounding (left, control lines: FB5, 8, 10, 12, 14, 17, 18; right PWS lines: FB16 and RIKEN, chi-square, $p<0.05$). The position of the centrosome (D) and the nucleus (E) was measured in wound-edge MEFs. *Ndn*-null MEFs have normal centrosome centration but do not experience nuclear rearward movement at 4 hours (Fisher's exact, $p<0.05$).

Centrosome re-orientation in fibroblasts is facilitated by both microtubule-dependent activities that maintain the centrosome at the center of the cell and actin retrograde flow that forces the nucleus away from the leading edge (Gomes, Jani, and Gundersen 2005). We measured the position of the centrosome and the center of the cell in wound-edge MEFs at 4 or 8 hours after wounding, but found no inter-genotype differences in the distance between the centrosome and the cell center at either time point (Figure 4.2D). We then measured the distance between the center of the cell and the center of the nucleus along the axis of migration, such that negative values indicated that the nucleus had undergone retrograde movement. At both time points, control cells had undergone measureable nuclear rearward movement, but rearward movement was not apparent in *Ndn*-null cells after 4 hours with some recovery at 8 hours (Figure 4.2E). These results suggest that lack of centrosome re-orientation in *Ndn*-null MEFs is caused by defective actin-dependent nuclear rearward movement, while microtubule-dependent cell centration is unperturbed.

Given the importance of actin-myosin regulation during nuclear rearward movement (Gomes, Jani, and Gundersen 2005), we asked whether other actin-associated cellular responses are sensitive to the loss of necdin. MEFs were plated at low density, maintained in serum-free media for 16 hours, and then re-fed with serum-replete media. Cells were then labeled with rhodamine-conjugated phalloidin to visualize F-actin and with antibodies to vinculin to label mature focal adhesions. We found that *Ndn*-null MEFs had significantly fewer lamellipodial ruffles compared to controls (control = 6.3±0.2 ruffles/cell, *Ndn*-null

= 5.6±0.2 ruffles/cell; $p<0.05$) and significantly smaller focal adhesions compared to control cells (control = 3.5±0.1 relative units, *Ndn*-null = 2.8±0.08 relative units; $p<0.05$). These results demonstrate that necdin is required for prompt cell polarization and other actin-associated cellular responses.

4.3.2 Loss of necdin impairs phosphorylation of myosin light chain through reduced Cdc42 activation

We next sought to determine the mechanism by which loss of necdin impairs in actin-dependent nuclear rearward movement. Phosphorylation of the regulatory protein myosin light chain (MLC) positively regulates the generation of force through the actin-myosin cytoskeleton, and impairment of this modification disrupts nuclear rearward movement during centrosome reorientation (Gomes, Jani, and Gundersen 2005). We serum-deprived *Ndn*-null and control MEFs for 16 hours, then stimulated them with serum, collected cell lysates, and measured the activation of serum response pathways. Control MEFs responded as expected through a robust increase in the relative amount of pMLC to total MLC or γ-tubulin (Figure 4.3A, C). In contrast, *Ndn*-null MEFs failed to increase pMLC in response to serum (Figure 4.3B, C). There was no genotype-dependent difference in the activation of the MAP kinase pathway (pERK to total ERK) measured in the same cell lysates (Figure 4.3A, B). Together, these data suggest that loss of necdin in MEFs results in defects in a signaling pathway upstream of MLC and that *Ndn*-null MEFs do not have generalized defects in intracellular signaling following serum stimulation. We performed similar experiments to test whether PWS fibroblasts also show an impaired serum

response. Control fibroblasts had variable responses to serum. We observed a significant increase in pMLC in two of four control lines, with a trend towards activation in the other two cell lines, while none of the three PWS cell lines achieved significant MLC activation (Figure 4.3D). Since absence of necdin is the only trait shared among *Ndn*-null MEFs and PWS cell lines, we conclude that necdin is required for timely centrosome re-orientation and serum-induced myosin activation in both mice and humans.

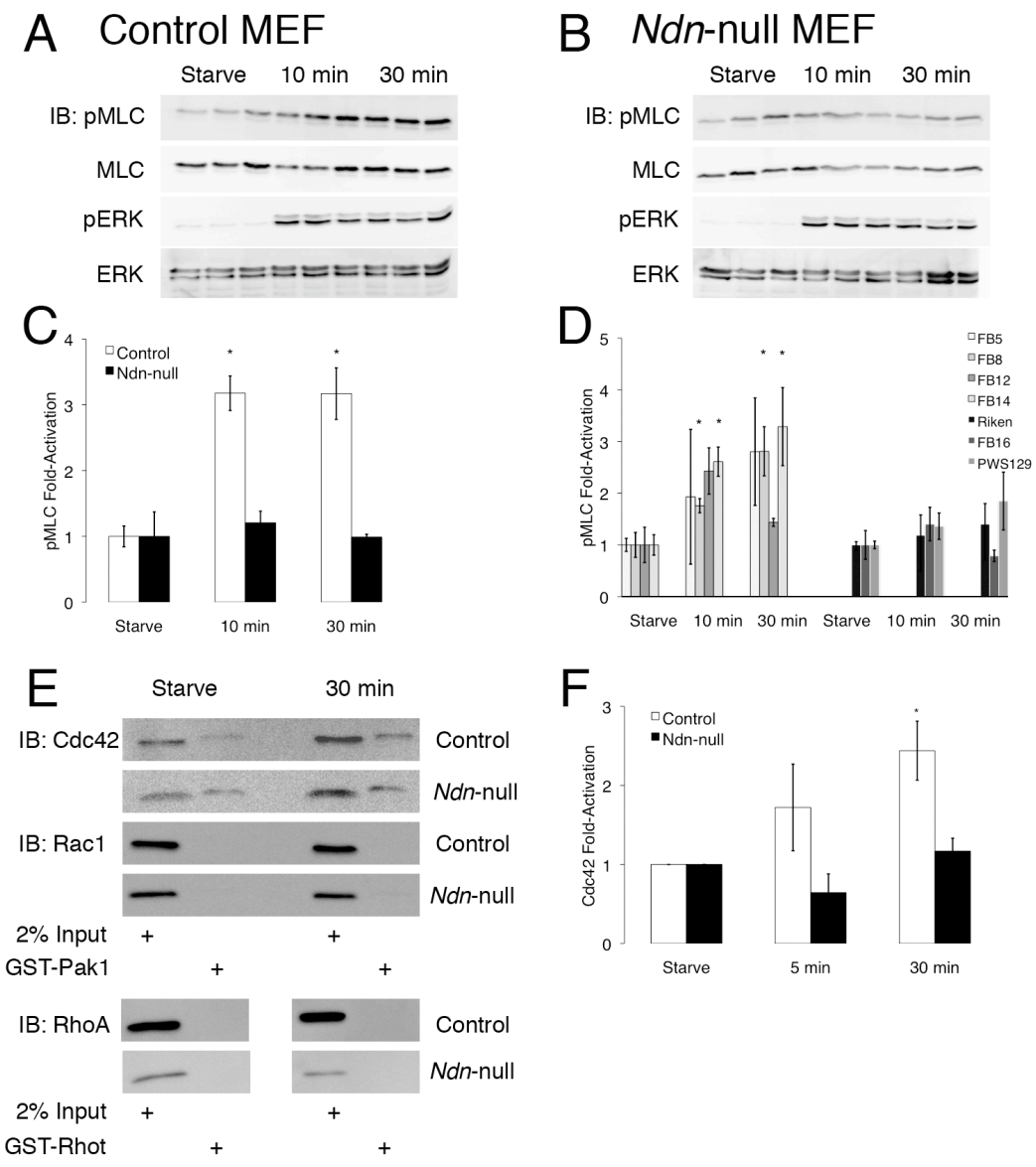

A Control MEF
Starve
10 min
30 min
IB: pMLC
MLC
pERK
ERK
B Ndn-null MEF
Starve
10 min
30 min
IB: pMLC
MLC
pERK
ERK
C
Control
Ndn-null
pMLC Fold-Activation
Starve
10 min
30 min
D
FB5
FB8
FB12
FB14
Riken
FB16
PWS129
pMLC Fold-Activation
Starve
10 min
30 min
Starve
10 min
30 min
E
Starve
30 min
IB: Cdc42
Control
Ndn-null
IB: Rac1
Control
Ndn-null
2% Input
GST-Pak1
IB: RhoA
Control
Ndn-null
2% Input
GST-Rhot
F
Control
Ndn-null
Cdc42 Fold-Activation
Starve
5 min
30 min

Figure 4.3. Defective activation of myosin and Cdc42 in *Ndn*-null fibroblasts. Phosphorylation of myosin light chain (pMLC) and ERK (pERK) in control (A) and *Ndn*-null (B) MEFs in response to serum. (C) Serum induces significant pMLC activation above basal levels (starve) in control but not *Ndn*-null MEFs (t-test, $p<0.05$). (D) Serum induces significant pMLC activation above starved levels in some control cell lines while no PWS cell line shows significant activation (left, control lines: FB5, 8, 10, 12; right, PWS lines: FB16, RIKEN, PWS129, t-test, $p<0.05$). (E) Representative immunoblots measuring serum-induced Cdc42, Rac1, and RhoA activation. Rac1 and RhoA are not activated in control or *Ndn*-null MEFs. (F) Quantification of Cdc42 fold-activation over unstimulated levels via GST-Pak1 precipitation from lysates of MEFs treated with serum for 5 and 30 minutes demonstrates that Cdc42 is not activated in *Ndn*-null MEFs (one-tailed t-test, $p<0.05$).

Cdc42 activation at the leading edge of wounded cells is important in transducing signals that promote centrosome re-orientation through both microtubule- and actin-dependent pathways, and Cdc42 is both necessary and sufficient for centrosome reorientation in MEFs (Gomes, Jani, and Gundersen 2005). We asked whether serum-induced Cdc42 activation was compromised, potentially explaining the morphological and biochemical defects observed in *Ndn*-null MEFs and PWS fibroblasts. Lysates from serum-deprived then re-fed MEFs were subjected to a Cdc42 pull-down assay using GST-bound Pak1 (Benard, Bohl, and Bokoch 1999). Thirty minutes of serum stimulation strongly activated Cdc42 in control cells but not in *Ndn*-null cells (Figure 4.3E, F). The related GTPases Rac1 and RhoA also regulate cytoskeletal dynamics (Gomes, Jani, and Gundersen 2005). However, 5 or 30 minutes of serum stimulation did not lead to significant Rac1 or RhoA activation in MEFs of either genotype in parallel pull-down assays (Figure 4.3E, and data not shown). While myosin or Cdc42 may eventually become activated at later time points in *Ndn*-null cells, these results suggest that the failure of *Ndn*-null MEFs to activate MLC in response to serum is due, at least in part, to an inability to activate Cdc42.

4.3.3 Myosin activation is impaired in Ndn-null neurons

We postulated that loss of necdin may also compromise Cdc42 activation in neurons, the cell type in which necdin is most abundant. BDNF activates TrkB, and subsequently Cdc42 (Cheung et al. 2007; Shen et al. 2006; Chen et al. 2006) to promote downstream changes to the cytoskeleton. We collected cortical neurons from E16.5 *Ndn*-null and control littermates and cultured them for 4 or 5

days *in vitro* (DIV), a time when axonal and dendritic processes were readily identifiable. We then treated the cultures with 50 ng/ml BDNF or vehicle for 30 minutes, prepared lysates, and probed for activated Cdc42 or Rac1. However, we did not detect significant Cdc42 or Rac1 activation in either control or *Ndn*-null cultures (data not shown).

We then examined cortical neurons for BDNF-induced pMLC response. Both control and *Ndn*-null cultures contain similar levels of TrkB, the cognate receptor for BDNF in cortical neurons, and each genotype displayed significant activation of TrkB, Akt, and ERK (Figure 4.3A, B, data not shown). Control cultures displayed a robust activation of MLC in response to BDNF, but *Ndn*-null cultures did not significantly activate pMLC (Figure 4.4A-C). These results suggest that loss of necdin impairs myosin activation independent of Cdc42 in neurons, although it remains possible that BDNF-induced activation of Cdc42 occurred at a level below the detection ability of our assay.

Regulation of the actin-myosin cytoskeleton is important during neuritogenesis (Bradke and Dotti 1999) and in the consolidation of microtubules at the base of the growth cone during axonal extension (Burnette et al. 2008; Schaefer et al. 2008). Polarity establishment and axonal extension are easily examined in hippocampal neurons, which project a single axon *in vitro* (Dotti, Sullivan, and Banker 1988). Hippocampal neurons (E18.5) were prepared, allowed to adhere to poly-L-lysine-coated coverslips, then transferred to wells in which a BDNF-containing agarose drop had been placed on one end. Media was then added to release BDNF in a graded manner. After 8 hours, we found that

46% of control neurons projected their axon in the direction of the agarose drop, while the axons of *Ndn*-null neurons projected in random directions (Figure 4.4D).

In addition to the defective polarization observed in response to a BDNF gradient, we noted that *Ndn*-null hippocampal neurons more had more frequent axonal branches (14.6% of axons were forked in control cultures vs. 54.3% in *Ndn*-null cultures, Figure 4.4E, F). These defects were present in hippocampal neurons cultured in both Neurobasal media and in neurons exposed to BDNF.

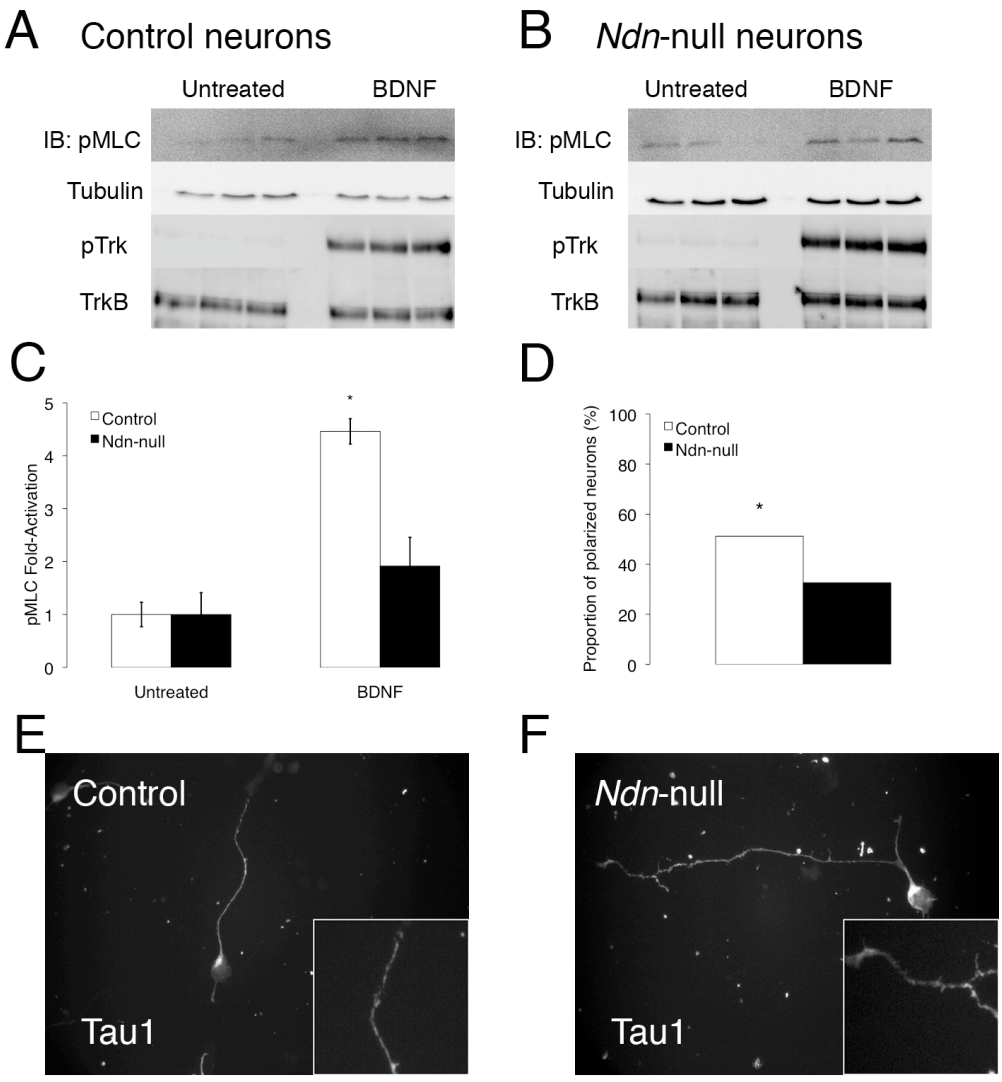

A
Control neurons
Untreated
BDNF
IB: pMLC
Tubulin
pTrk
TrkB
B
Ndn-null neurons
Untreated
BDNF
IB: pMLC
Tubulin
pTrk
TrkB
C
pMLC Fold-Activation
Control
Ndn-null
Untreated
BDNF
D
Proportion of polarized neurons (%)
Control
Ndn-null
E
Control
Tau1
F
Ndn-null
Tau1

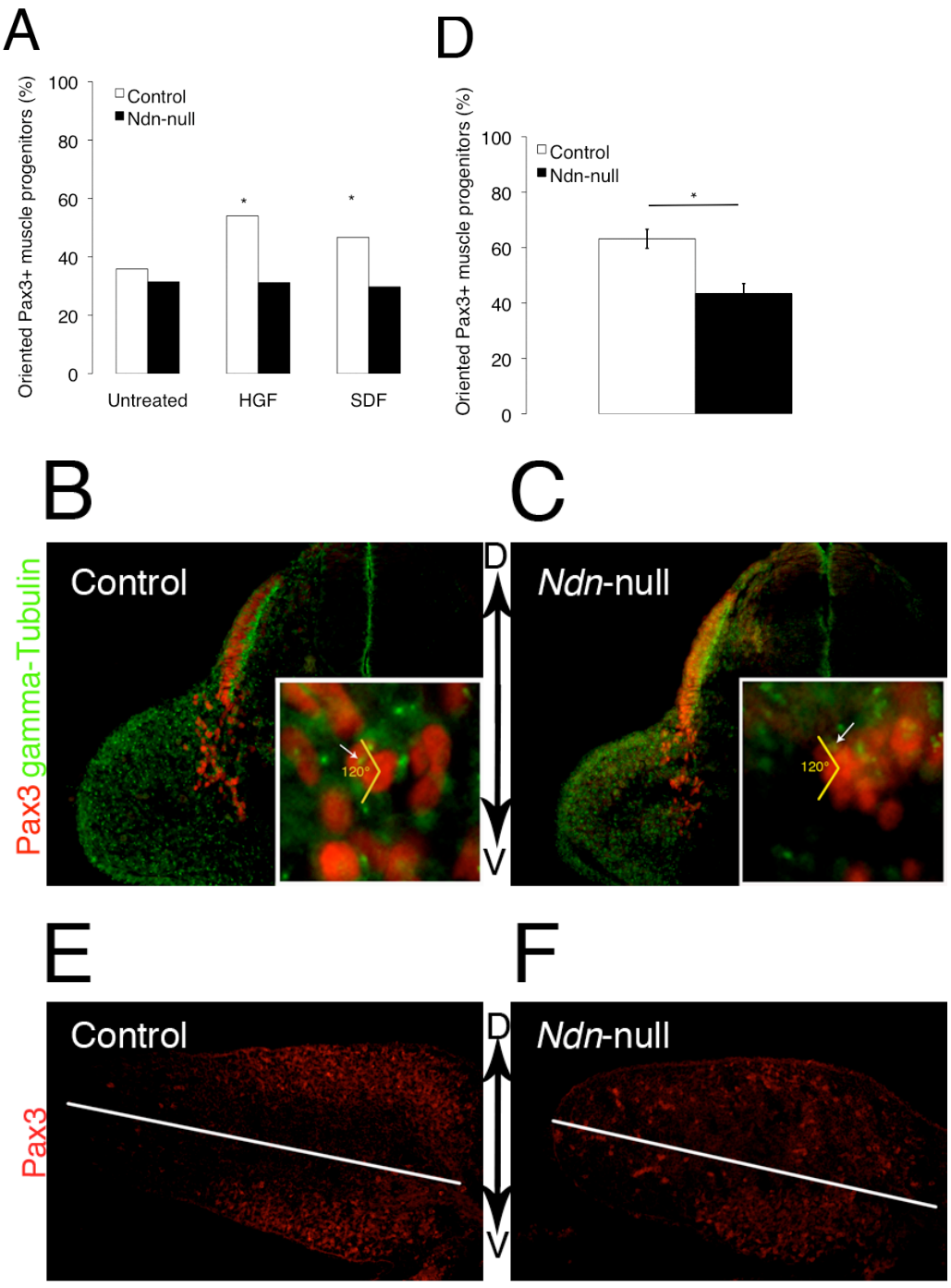
Figure 4.4. Cytoskeletal disorganization and defective response to BDNF in *Ndn*-null primary neuronal cultures. Immunoblot analysis of cortical neuron cultures from E16.5 control (A) and *Ndn*-null (B) embryos treated with BDNF for 30 minutes. (C) Control cultures show a significant increase in pMLC activation (normalized to γ-tubulin), while *Ndn*-null neurons do not activate pMLC (t-test, $p<0.05$). (D) The proportion of hippocampal neurons projecting an axon from the third of the cell oriented towards a BDNF-containing agarose bead was compared to random (33%). Control, but not *Ndn*-null cultures showed significant polarization (chi-square, $p<0.05$). Untreated hippocampal neurons from control (E) and *Ndn*-null (F) E18.5 embryos labeled with antibodies to Tau1 revealed increased axonal branching in *Ndn*-null cultures (14.6% in control vs. 54.3% in *Ndn*-null; $p<0.05$).

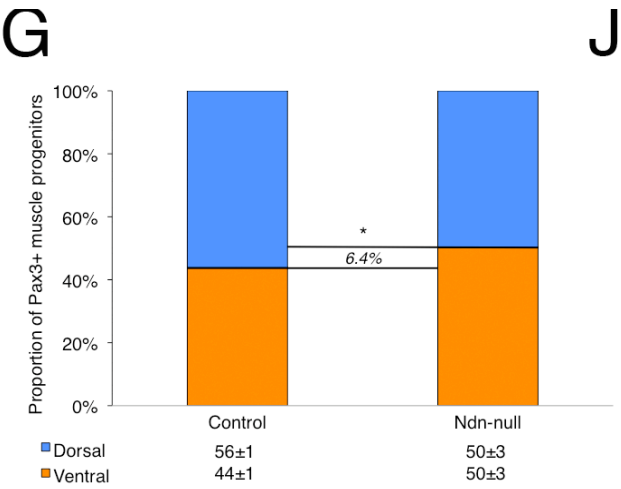
Similar defects were noted in *Ndn*-null neurons of the sympathetic nervous system (Lee et al. 2005; Tennese, Gee, and Wevrick 2008) and neurons regulating respiratory control (Pagliardini et al. 2005; Zanella et al. 2008), and are strikingly similar to those observed in cultured neurons from mice carrying mutations causing defects in microtubule bundling (Bielas et al. 2007; Kappeler et al. 2006; Koizumi et al. 2006). These results suggest that necdin promotes myosin activation during axonal projections to facilitate cytoskeletal bundling and establish efficient connectivity during development of the nervous system.

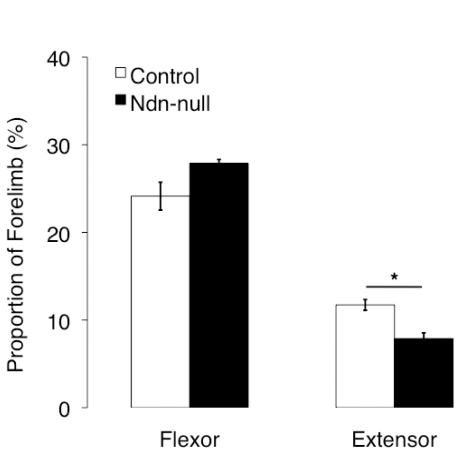
4.3.4 Defective migration of progenitors results in muscle defects in Ndn-null mice

Muscle progenitor cells form another major migratory cell population in the developing embryo. At the axial level of the limb buds, muscle progenitor cells expressing the transcription factor Pax3 (Pax3-positive) delaminate from the dermomyotome, in which necdin is normally expressed (Brunelli et al. 2004). These progenitors migrate into the limb bud in response to factors produced in the distal limb, including the stromal-derived factor-1α (SDF-1α). We asked whether muscle progenitors from *Ndn*-null or control littermate embryo limb buds are competent to polarize in response to growth factors secreted by the limb mesenchyme. We prepared limb bud micromass cultures from E10.5 embryos and plated them on coverslips between an agarose bead containing hepatocyte growth factor (HGF) or SDF-1α, and a bead with no growth factor. After 4 or 8 hours, Pax3-positive cells were scored as 'oriented' or 'not' depending on whether the centrosome was located in the third of the cell facing the source of the

growth factor. While most Pax3-positive muscle progenitor cells from the limb buds of control embryos were oriented towards the source of the growth factor, the centrosomes of Pax3-positive cells derived from the limb buds of *Ndn*-null embryos were not oriented towards either growth factor (Figure 4.5A).


A
Oriented Pax3+ muscle progenitors (%)
100
80
60
40
20
0
Control
Ndn-null
*
*
Untreated
HGF
SDF
D
Oriented Pax3+ muscle progenitors (%)
100
80
60
40
20
0
Control
Ndn-null
*
B
C
Pax3 gamma-Tubulin
Control
120°
D
V
Ndn-null
120°
E
F
Pax3
Control
D
V
Ndn-null


G
Proportion of Pax3+ muscle progenitors
100%
80%
60%
40%
20%
0%
*
6.4%
Control
Ndn-null
Dorsal
Ventral
56±1
44±1
50±3
50±3


J
Proportion of Forelimb (%)
40
30
20
10
0
Control
Ndn-null
*
Flexor
Extensor

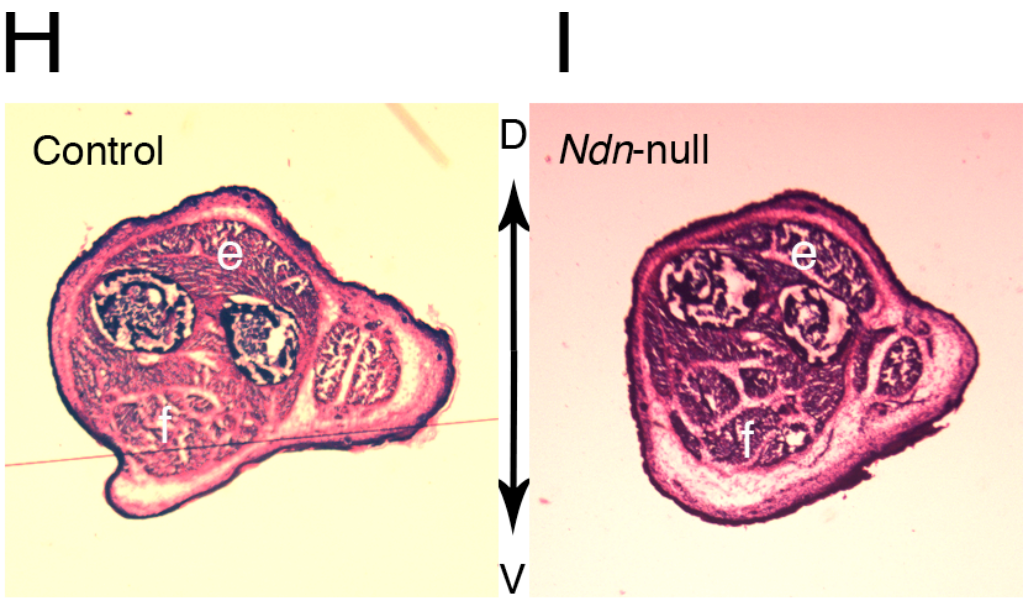

H
I
Control
e
f
D
V
Ndn-null
e
f

Figure 4.5. Polarity, migration, and dorsal patterning defects in *Ndn*-null mouse forelimbs. (A) Micromass limb bud cultures were plated between agarose beads containing HGF, SDF-1α, or neither growth factor, and examined after 24 hours. Pax3-positive cells were scored as either centrosome located in the one third of the cell oriented toward the growth factor, or not oriented. Control muscle progenitors polarize with respect to both HGF and SDF-1α, while *Ndn*-null cells do not (33%=not oriented, chi-square, $p<0.05$). Representative forelimb images from E9.5 control (B) and *Ndn*-null mice (C) co-labeled with antibodies to γ-tubulin and Pax3. Delaminated Pax3-positive cells were scored as either oriented toward the limb bud or not oriented. (D) A smaller proportion of *Ndn*-null Pax3-positive muscle progenitors in embryos polarize with respect to the developing limb compared to control (Fisher's exact, $p<0.05$). Representative forelimb images from E10.5 control (E) and *Ndn*-null (F) mice stained with antibodies to Pax3. (G) The pool of dorsal muscle progenitors is reduced in *Ndn*-null mice (t-test, $p<0.05$). Representative sections of E18.5 control (H) and *Ndn*-null (I) forelimbs show extensor (e) and flexor (f) muscles. (J) Extensor muscles constitute a smaller proportion of the average limb cross-section in *Ndn*-null forelimbs (t-test, $p<0.05$).

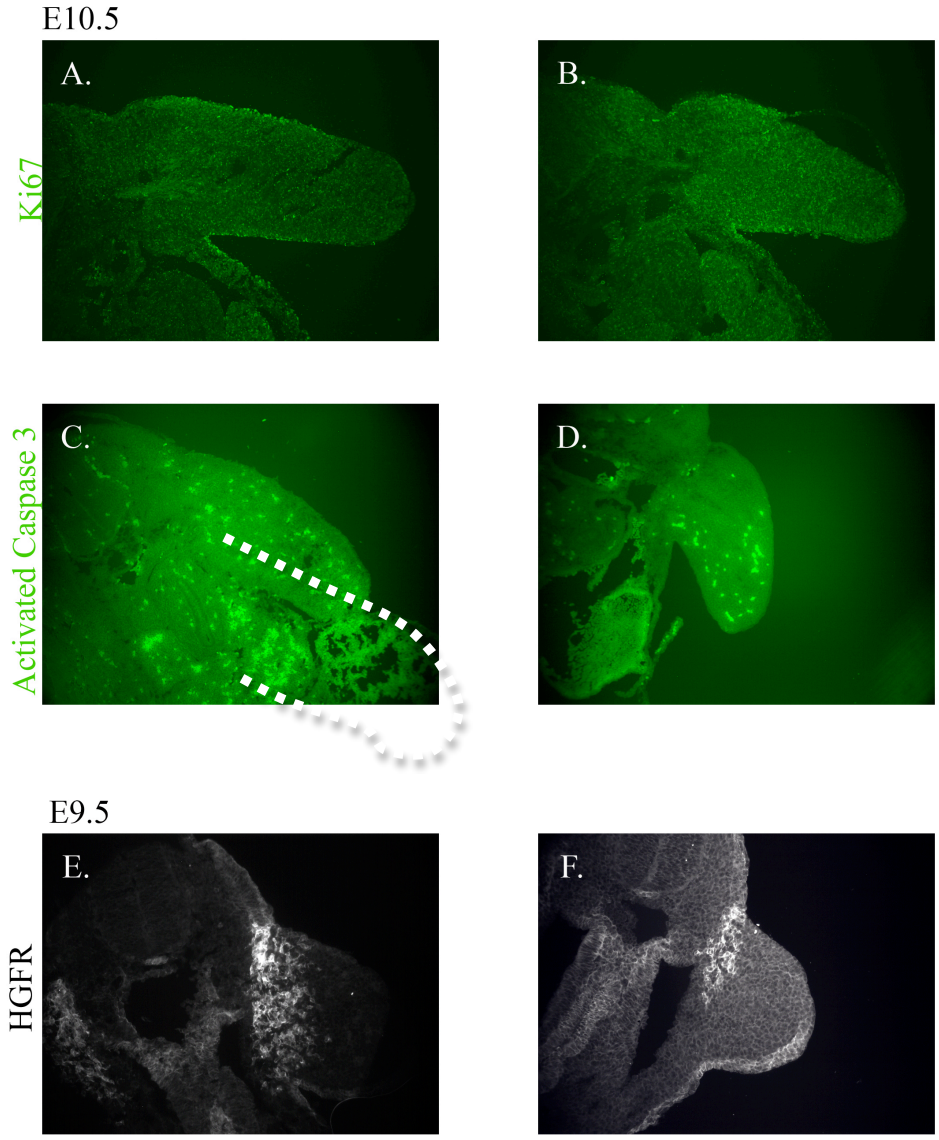

Control
Ndn-null
E10.5
A.
B.
Ki67
C.
D.
Activated Caspase 3
E9.5
E.
F.
HGFR

Figure 4.6. Normal cell proliferation, cell death, and HGFR expression in *Ndn*-null mice. There is widespread cellular proliferation, as determined by immunostaining with an antibody to the cell proliferation nuclear antigen Ki67 (A, B), and very limited cell death, as detected with an antibody to activated Caspase 3 (C, D), in the limb bud of both genotypes at E10.5. HGFR is detected in migrating muscle progenitors of both genotypes (E, F).

At the axial level of the limb, a subset of ventrally-migrating muscle progenitors respond to growth factors secreted by the limb mesenchyme and begin to move laterally out into the limb. To our knowledge, the polarity of these cells has not previously been reported. We asked if Pax3-positive muscle progenitor cells polarize *in vivo* with respect to the developing limb bud and, if so, whether polarity is affected in *Ndn*-null mouse embryos. Transverse sections of the limb bud from E9.5 mouse embryos were labeled with antibodies to Pax3 and γ-tubulin, and scored as 'oriented' or 'not' with respect to the developing limb (Figure 4.5B, C). Since not all Pax3-positive cells are migratory, we examined only those that had delaminated from the somite adjacent to the limb bud. Significantly more Pax3-positive cells had oriented their centrosome with respect to the limb bud in control embryos (63±3% of cells polarized, n=5 embryos) than would be expected by random chance alone (33%)(Figure 4.5D). A significant proportion of Pax3-positive cells were also oriented towards the limb in *Ndn*-null embryos (44±3% of cells polarized, n=3 embryos). However, the proportion of polarized muscle progenitors in *Ndn*-null embryos was significantly reduced compared to control embryos (63% oriented in control versus 44% oriented in *Ndn*-null; Fisher's exact test p=0.0002) (Figure 4.5D). These results are consistent with the polarity defects observed in *Ndn*-null MEFs, PWS fibroblasts, and *Ndn*-null muscle progenitor cells in culture.

We next asked if the failure of muscle progenitors to polarize in *Ndn*-null embryos results in a migration defect. Limb muscle defects in *Lbx1*-/- and *Gab1*-

/- embryos result from the specific loss of extensor groups derived from muscle progenitor cells that normally migrate into the dorsal half of the limb bud (Brohmann, Jagla, and Birchmeier 2000; Gross et al. 2000; Sachs et al. 2000). The distribution of Pax3-positive muscle progenitor cells was examined in E10.5 *Ndn*-null and control littermate embryos. We quantified the number of Pax3-positive cells in dorsal and ventral halves of the limb buds (Figure 4.5E, F), and found significantly fewer dorsally located Pax3-positive cells in the *Ndn*-null embryos (Figure 4.5G). These results are consistent with the hypothesis that delayed polarization results in defective migration of *Ndn*-null cells *in vivo*.

Failure of muscle progenitors to migrate into the limb bud results in subsequent defects in limb muscle development in other gene-targeted or spontaneous mutant mice (Bladt et al. 1995; Bober et al. 1994; Dietrich et al. 1999; Goulding, Lumsden, and Paquette 1994; Maina et al. 1996; Maina et al. 2001; Sachs et al. 2000; Schaeper et al. 2000; Vasyutina et al. 2005). We asked whether the reduction in dorsally located limb muscle progenitors would result in decreased limb muscle size. Extensor (e) and flexor (f) muscles were examined in cross-sections of E18.5 *Ndn*-null and control mouse forelimbs (Figure 4.5H, I). Consistent with a reduction in the dorsal muscle progenitor pool, the average proportional cross-sectional area of the extensor muscles in the forelimbs of *Ndn*-null mouse embryos was smaller than the equivalent muscle area in control embryos, whereas the average proportional cross-sectional area of the flexor muscles was not different between genotypes (Figure 4.5J). Taken together, these results suggest that loss of necdin impairs the polarity of muscle progenitors

migrating into the limb bud, and that the reduced dorsal pool of muscle progenitors consequently gives rise to smaller extensor muscles in the *Ndn*-null forelimb.

We detected ubiquitous cell proliferation throughout the limb buds of both genotypes (Figure 4.6A,B) and the number of apoptotic cells in the dorsal region of the *Ndn*-null embryos did not appear different from controls (Figure 4.6C,D), suggesting that differences in proliferation or cell death are not responsible for the different limb bud muscle progenitor distributions between genotypes. Furthermore, HGFR was detected in the migrating muscle progenitors entering the limb bud at E9.5 in both embryos (Figure 4.6E,F), indicating that cells of both genotypes should be competent to respond to chemotactic stimuli.

4.4 Discussion

Understanding how organisms regulate growth, differentiation, and patterning during embryogenesis is one of the most fascinating problems in biology. *Ndn*-null mice are one model for studying the developmental aspects of PWS, and understanding their phenotypes has clarified how loss of necdin contributes to the disease pathology, including neonatal respiratory compromise and autonomic dysfunction. We now present evidence from studies of PWS patient fibroblasts and *Ndn*-null mouse cells that together suggest a novel mechanism whereby loss of necdin impairs cellular polarization during migration initiation through a Cdc42-myosin-dependent pathway. Notably, this is the first evidence of a cellular defect in Prader-Willi syndrome. The polarization and axon branching defects observed in *Ndn*-null neurons are consistent with a failure to

activate myosin in response to cues necessary for normal neuronal development. Finally, the polarity of *Ndn*-null muscle progenitors is altered and forelimb musculature is subsequently affected, consistent with a role for necdin during muscle development. Taken together, our results identify a signaling pathway defective in PWS patient-derived cells and provide evidence that polarization defects likely contribute to the neurological defects and hypotonia observed in people with PWS.

Ndn was originally identified as a gene whose transcript is markedly induced during neuronal differentiation (Maruyama et al. 1991). *Ndn* is highly expressed in developing embryos, in nervous (Aizawa et al. 1992), muscle (Gerard et al. 1999; Kuwajima et al. 2004), and somitic tissues (Andrieu et al. 2003; Brunelli et al. 2004). Necdin shares functional similarity with Rb, interacting with E2F1 (Taniura et al. 1998) and E2F4 (Kobayashi, Taniura, and Yoshikawa 2002) to promote cell-cycle exit. In muscle, necdin co-operates with MAGE-D1 to relieve Msx2-mediated transcriptional repression (Kuwajima et al. 2004) and antagonizes EID-1-dependent repression of MyoD (Bush and Wevrick 2008), while necdin interacts with Dlx proteins in neurons to promote GABAergic neuronal differentiation (Kuwajima, Nishimura, and Yoshikawa 2006). *Ndn*-null mice have increased levels of apoptosis in both nervous tissue (Andrieu et al. 2006; Hasegawa and Yoshikawa 2008; Kurita et al. 2006; Kuwako et al. 2005; Takazaki, Nishimura, and Yoshikawa 2002) and muscle (Deponti et al. 2007; Sciorati et al. 2009), and necdin modulates both the activity of p53 (Hasegawa and Yoshikawa 2008; Taniura, Matsumoto, and Yoshikawa 1999) and the

expression of *Cdc2* (Kurita et al. 2006). However, necdin is also present in the cytoplasm and interacts with TrkA and p75NTR receptors to enhance TrkA activation in dorsal root ganglion neurons (Bronfman et al. 2003; Kuwako et al. 2005). We now show that Cdc42 and myosin activation are defective in stimulated *Ndn*-null cells (Figure 4.3E), demonstrating that necdin is either directly or indirectly required for the coupling of receptor activation to downstream signaling molecules.

We previously demonstrated that some neuronal cell populations failed to complete migration in developing *Ndn*-null mice. Our current study shows that *Ndn*-null embryonic fibroblasts are motile but have delayed migration initiation rather than defective steady state migration (Figure 4.2A). *Ndn*-null MEFs and fibroblasts from individuals with PWS are delayed in reorienting their centrosome to establish polarity with respect to the direction of migration (Figure 4.2). Centrosome re-orientation involves microtubule-dependent centrosome centration and myosin-dependent actin retrograde flow leading to nuclear rearward movement, with both processes dependent on Cdc42 activation (Gomes, Jani, and Gundersen 2005). We found that while centrosome centration was normal, nuclear rearward movement was delayed, suggesting that loss of necdin selectively impairs activation of the actin-myosin cytoskeleton. Immunoblot analysis confirmed that loss of necdin impairs myosin activation in mouse and human cells in response to extracellular stimulation (Figure 4.3C, D, Figure 4.4C). Importantly, *Ndn*-null cells maintain normal stimulation responses in other pathways, including activation of TrkB, AKT, and MAP kinase, and microtubule-

dependent centrosome centration in response to wounding (Figure 4.2D, Figure 4.3A, B, Figure 4.4A, B, data not shown). Further experiments are required to address whether necdin normally promotes cellular polarization through transcriptional mechanisms, by coupling receptor activation to down-stream myosin activation as an adaptor protein, or through a combination of mechanisms.

Polarization during the initiation of cellular migration involves reorganizing the cytoskeleton, which depends on receptor activation and modulation of intracellular signaling by regulatory proteins. Receptors important for polarization and migration typically signal through the Rho family GTPases, including RhoA, Rac1, and Cdc42. These proteins cycle between active GTP-bound states and inactive GDP-bound states, and interact with various downstream effectors depending on their state of activation. We did not detect significant activation of either RhoA or Rac1 in serum-stimulation of MEFs (Figure 4.3E) and did not detect activation of Cdc42 or Rac1 in response to BDNF in neurons of either genotype. We expected BDNF to activate Cdc42 in cortical neurons (Cheung et al. 2007); however, shorter culture duration (4-5 DIV vs. 7 DIV in (Cheung et al. 2007)) and differences in cortical culture preparation might explain this disparity. Despite delayed centrosome reorientation and significant impairment in Cdc42 and pMLC activation, *Ndn*-null MEFs do eventually migrate into a wound and maintain a normal migration velocity. It remains possible that weak Cdc42 activation occurs in *Ndn*-null MEFs and this activation eventually drives polarization. Alternatively, *Ndn*-null MEFs might require prolonged receptor stimulation in order to achieve sufficient Cdc42 activation, causing a

delayed response. Finally, compensatory activation of GTPases other than Cdc42 could function in a necdin-independent manner to eventually promote migration in *Ndn*-null MEFs. It is likely that necdin is not absolutely required for Cdc42 activation, since Cdc42 regulates both the microtubule and actin-myosin cytoskeleton during fibroblast polarization (Gomes, Jani, and Gundersen 2005), and we did not observe any defect in the microtubule-dependent process of centrosome centration in *Ndn*-null MEFs during wound healing assays (Figure 4.2D). However, we have not ruled out the possibility that necdin is also important in regulating microtubule dynamics or in regulating interactions between the actin and microtubule cytoskeletons.

Axonal defects and disorganization of the nervous tissue have previously been reported in *Ndn*-null mice (Lee et al. 2005; Pagliardini et al. 2005; Tennese, Gee, and Wevrick 2008; Zanella et al. 2008). We observed increased branching in hippocampal neurons cultured from *Ndn*-null embryos, consistent with cytoskeletal disorganization (Figure 4.4E, F). Failure to activate myosin at the wrist of the growth cone during axonal extension results in unconsolidated microtubules along the length of the axon (Burnette et al. 2008). Microtubule bundles are maintained by microtubule binding proteins, including doublecortin, the product of *DCX* mutated in some individuals with Type 1 lissencephaly (des Portes et al. 1998; Gleeson et al. 1998; Sossey-Alaoui et al. 1998). Neurons cultured from mice in which the doublecortin pathway has been disrupted display increased branching (Bielas et al. 2007; Kappeler et al. 2006; Koizumi et al.

2006), so further investigation of biologically relevant interactions between necdin and the pathways implicated in lissencephaly are warranted.

Brain-derived neurotrophic factor (BDNF) is important during brain development and activates Cdc42 to promote neurite extension in cultured neurons (Cheung et al. 2007). While BDNF is not known as a polarity cue *per se*, it does promote neuronal migration (Polleux et al. 2002). We therefore predicted that a spatial gradient of BDNF would result in polarized neurite extension towards the growth factor and increase the probability that an axon would project in that direction. Indeed, BDNF induced a small but significant increase in the proportion of polarized neurons in control cultures (Figure 4.4D). Hippocampal neurons from *Ndn*-null mouse embryos did not project axons towards the BDNF gradient, and treatment of *Ndn*-null cortical neurons with BDNF failed to activate myosin (Figure 4.4B). Necdin interacts with TrkA receptors in sympathetic neurons treated with nerve growth factor to promote cell survival (Kuwako et al. 2005), suggesting that necdin may similarly promote receptor activation by binding to TrkB in cortical neurons. Notably, missense mutations in the human gene encoding TrkB, *NTRK2*, cause severe obesity with developmental delay, a phenotype highly reminiscent of the PWS (Gray et al. 2007). However, TrkB was activated in both cultures, as were AKT and MAPK, suggesting a signaling defect downstream of TrkB but upstream of MLC activation. This further supports a role for necdin in promoting the neuronal migration, as inhibition of the MAP kinase pathway had no effect on TrkB-mediated migration (Polleux et al. 2002). The impairment of MLC activation in multiple cell types suggests that necdin

normally participates with, or regulates the expression of, proteins that promote signal transduction. Future work will clarify the molecular mechanism through which necdin acts to potentiate receptor signaling during cellular polarization and in mature neurons.

Limb muscle development requires both correct specification and subsequent migration of progenitors into the limb anlagen. Necdin is expressed in the somite and in both differentiating and regenerating skeletal muscle (Andrieu et al. 2003; Brunelli et al. 2004; Deponti et al. 2007; Sciorati et al. 2009). Loss of necdin does not affect the specification of limb muscle progenitors, as we can detect Pax3-positive cells (and hepatocyte growth factor receptor-positive (HGFR-positive) cells, data not shown) migrating from the somite at the axial level of the limb in *Ndn*-null mouse embryos. Homozygous *Splotch* mice, which have a null mutation affecting *Pax3*, and *Hgfr*-/- mice have no limb musculature due to a lack of migratory muscle progenitors (Bladt et al. 1995; Bober et al. 1994; Daston et al. 1996; Dietrich et al. 1999; Franz et al. 1993; Goulding, Lumsden, and Paquette 1994). *Lbx1*-/- and *Gab1*-/- mice form only flexor muscles in the forelimb, as the dorsal muscle progenitors are absent (Brohmann, Jagla, and Birchmeier 2000; Gross et al. 2000; Sachs et al. 2000). A recent study examined muscle progenitor migration in a *Cdc42flox/flo;Lbx1Cre* mouse in which *Cdc42* expression is deleted only in Lbx1-positive myoblasts and surprisingly found no difference in the distribution of limb muscle progenitors (Vasyutina et al. 2009). However, *Lbx1* expression does not appear in the somite until E8.5 (Jagla et al. 1995) and the protein half-life of Cdc42 is 15 hours

(Backlund 1997), so residual Cdc42 protein in the *Cdc42flox/flo;Lbx1Cre* mouse may be sufficient to promote muscle precursor polarity and normal migration.

Muscle dysgenesis in *Ndn*-null mice most closely resembles that found in mice deficient for the SDF-1α chemokine receptor CXCR4, where dorsal muscle progenitors are specified but fewer progenitors migrate into the dorsal limb field and the extensor muscles are consequently smaller (Figure 4.4) (Vasyutina et al. 2005). *In vitro* polarization assays with SDF-1α or HGF (Fig 4.5A) and examination of muscle progenitors *in vivo* (Figure 4.5D) demonstrate that *Ndn*-null muscle progenitors respond poorly to migratory cues, resulting in a delay in dorsal muscle progenitor migration, and a subsequent reduction in dorsal-derived extensor muscles (Figure 4.5G). While hypotonia is a cardinal feature of PWS, there are limited examples of other human genetic diseases in which limb musculature is affected. Anomalies of the upper limb are observed in patients with Klein-Waardenburg syndrome (Goodman et al. 1982), a disorder caused by mutations in *PAX3* (Hoth et al. 1993), the same gene mutated in the *Splotch* mouse model of limb muscle development (Franz et al. 1993). The murine homolog of the *Drosophila* single-minded gene, *Sim1*, is expressed in the developing somite and migrating muscle precursors (Coumailleau and Duprez 2009; Fan et al. 1996), and deletions or haploinsufficiency of *SIM1* are found in people with a PWS-like phenotype of syndromic obesity and hypotonia (Bonaglia et al. 2008; Faivre et al. 2002; Varela et al. 2006). Defects in specific limb muscle groups have not been reported in PWS individuals, but overlying hypotonia may impair the detection of weakness in specific muscle groups.

Specificity of Cdc42 signal transduction is achieved via a diverse group of regulatory proteins including GTPase activating proteins (GAPs) and guanine-nucleotide exchange factors (GEFs) (Pertz et al. 2008). Intriguingly, genetic alterations of GAPs and GEFs have been identified in individuals with cognitive impairment, including mutations in *oligophrenin-1* (Billuart et al. 1998), mental disorder-associated GAP protein (*MEGAP*) (Endris et al. 2002), a Rho guanine exchange factor 6 (*ARHGEF6*) (Kutsche et al. 2000), Neurofibromatosis Type 1 (*NF1*) (Martin et al. 1990; Xu, Lin et al. 1990; Xu, O'Connell et al. 1990; Ballester et al. 1990), and the cytoplasmic Fragile X syndrome (FXS) protein interacting protein 1 (*CYFIP1*) (Schenck et al. 2001). In addition to developmental delay, these patients typically have hypotonia or motor delay, suggesting that these proteins may also be involved in normal muscle development. About 75% of PWS is caused by deletion of the paternal chromosome 15 and a lack of compensatory expression due to genomic imprinting that silences the expression of PWS genes from the maternal allele. The paternal deletion includes a number of genes not imprinted on the maternal allele, and while maternal expression is thought to compensate for loss of paternal expression, there is some evidence to suggest that haploinsufficiency of these genes contributes to the PWS phenotype. *CYFIP1* is missing from the paternal chromosome in PWS patients with the larger (type 1) deletions that are associated with more severe cognitive impairment (Butler et al. 2004). Additionally, *CYFIP1* mRNA levels are reduced in a subset of FXS patients with PWS phenotype (PWP) (Nowicki et al. 2007), suggesting a genetic interaction between

NDN and *CYFIP1* that is disrupted in individuals with PWS and in cases of FXS with PWP. Our results demonstrate that necdin, like the GEFs and GAPs described above, modulates Cdc42 and related protein activities, and that failure to regulate cytoskeletal reorganization during development of the nervous and muscle tissues contributes to cognitive impairment and hypotonia, phenotypes shared between PWS and other developmental disorders, including X-linked mental retardation and Fragile X syndrome.

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Chapter 5. Conclusion

5.1 The molecular basis of Prader-Willi syndrome

Several PWS mouse models have been generated, in which a single gene or combination of genes from the region of mouse chromosome 7c syntenic to the PWS region of chromosome 15 in humans has been deleted or replaced. Characterization of these models has succeeded in dissecting some aspects of the PWS phenotype and attributing those defects to the loss of a particular gene. For example, incomplete puberty is a typical characteristic of PWS (Prader 1956), and some PWS individuals experience excessive daytime sleepiness (Helbing-Zwanenburg, Kamphuisen, and Mourtazaev 1993). MAGEL2 is expressed almost exclusively in the hypothalamus and *MAGEL2*-null mice display circadian and reproductive defects (Bischof, Stewart, and Wevrick 2007; Kozlov et al. 2007; Mercer and Wevrick 2009), become obese as adults following neonatal growth retardation (Bischof, Stewart, and Wevrick 2007), and have altered serotonin metabolism (Mercer et al. 2009). MAGEL2 interacts with centrosomal proteins (Lee et al. 2005); however, the molecular mechanism by which MAGEL2 normally functions to regulate metabolism, circadian rhythm, and fertility are not known. One intriguing possibility is that MAGEL2 functions with Bardet-Biedl syndrome (BBS) proteins to regulate metabolism, since both PWS and BBS are characterized by severe obesity. Leptin receptor signaling, which is critical for energy homeostasis, is impaired in cells from various BBS-null mouse models and BBS1 interacts with the leptin receptor (Seo et al. 2009). Therefore, if MAGEL2 normally interacts with BBS proteins to facilitate leptin signaling, loss of either MAGEL2 or BBS proteins could lead to remarkably similar obesity

phenotypes. Furthermore, this hypothesis is consistent with my observation that necdin is important for coupling receptor signaling, such as BDNF-induced TrkB activation in cortical neurons (Figure 4.4), to downstream pathways. Further studies are necessary to determine if the related MAGE proteins necdin and MAGEL2 share similar functions in different tissues.

Short stature and growth retardation are characteristics of PWS (Holm et al. 1993; Prader 1956) and both *MAGEL2*-null (Bischof, Stewart, and Wevrick 2007) and the *MBII-85*-null mice (Ding et al. 2008; Skryabin et al. 2007) display postnatal growth retardation. While a mechanism by which MBII-52, the human orthologue HBII-52, or MAGEL2 regulate growth has not been described, MBII-52 and HBII-52 transcripts do regulate the editing of serotonin receptor mRNA (Kishore and Stamm 2006; Vitali et al. 2005). Defective editing of the mRNA encoding the serotonin receptor is found in a number of patients with psychiatric disorders, including schizophrenia (Dracheva et al. 2003; Sodhi et al. 2001), and similar neurological disturbances in both PWS and schizophrenia could result from shared defects in serotonin processing. Although the targets of MBII-85/HBII-85 are not known, these snoRNAs are suspected to regulate RNA editing in a manner similar to MBII-52/HBII-52.

Necdin remains the best-studied of the PWS candidate proteins. Most *Ndn*^{tm2stw}-mutant mice die of respiratory distress shortly after birth (Gerard et al. 1999), and both rhythm generation by and organization of the pre-Bötzinger complex are defective in this mouse (Pagliardini et al. 2005; Ren et al. 2003). PWS individuals often have breathing difficulties reminiscent of Congenital

Central Hypoventilation syndrome (CCHS), where patients present with cyanosis at birth, have life-long night-time hypoventilation, and occasionally have concomitant Hirschsprung's disease or neuroblastomas of neural crest origin (Weese-Mayer, Berry-Kravis, and Marazita 2005). CCHS appears to be caused by mutations in a number of genes, including *END3* (Bolk et al. 1996), *RET* and *GDNF* (Amiel et al. 1998), *BDNF* (Weese-Mayer et al. 2002), *HASH-1* (de Pontual et al. 2003), and mutations or expansions in *PHOX2B* (Amiel et al. 2003; Sasaki et al. 2003; Weese-Mayer et al. 2003; Matera et al. 2004). The affected genes encode proteins important for the specification, survival, or migration of autonomic nervous system neural crest derivatives. Ventral migration of neural crest cells does not appear to be affected in Ndn^{tm2stw}-mutant mice, but rostral migration of the neural crest-derived superior cervical ganglia is impaired (Tennese, Gee, and Wevrick 2008). Given that loss of necdin impairs BDNF-TrkB signaling and polarity (Figure 4.4), it is possible that necdin promotes the normal migration of respiratory and other neural crest-derived neurons through direct or indirect interactions with the Ret and/or Endothelin-3 receptors.

Developmental delay of varying severity is a major characteristic of PWS. With the exception of enlarged ventricles, polymicrogyria, incomplete closure of the cortical insula (Miller et al. 2007), and an unusual ability to solve jigsaw puzzles (Dykens 2002), neurological abnormalities in PWS are unremarkable. *MAGEL2*-null mice have behavioral defects and changes in both brain volume and neurochemistry (Mercer et al. 2009), although these defects are not mechanistically understood. Some *Ndn*-null mice also display behavioral defects

(Muscatelli et al. 2000) and others demonstrate pain insensitivity consistent with observations in PWS patients (Kuwako et al. 2005). Neurons cultured from *Ndn*-null mice have axonal outgrowth and fasciculation defects (Figure 4.4) (Lee et al. 2005), suggesting that impaired neuronal pathfinding due to loss of necdin could lead to faulty connectivity during brain patterning and learning.

Genetic lesions affecting GAPs and GEFs have been identified in individuals with cognitive impairment, including mutations in *oligophrenin-1* (Billuart et al. 1998), *MEGAP* (Endris et al. 2002), *ARHGEF6* (Kutsche et al. 2000), and *CYFIP1* (Schenck et al. 2001). These proteins modulate the function of Rho family GTPases, which are critical for cytoskeletal regulation during axonal and dendritic growth. We have not identified the mechanism through which necdin modulates TrkB-dependent cytoskeletal changes in neurons, but it is reasonable to include necdin among the proteins described above based on functional similarity. However, it remains to be determined whether necdin functions as a GAP, GEF, or scaffolding molecule, or if necdin regulates the transcription of genes encoding proteins of this nature. Regardless of the mechanism, loss of necdin affects cellular polarity and migration in a number of cells types (Figure 4.2, 4.5) (Kuwajima, Hasegawa, and Yoshikawa 2010; Lee et al. 2005; Miller, Wevrick, and Mellon 2009; Tennese, Gee, and Wevrick 2008), including PWS patient fibroblasts, suggesting that migration defects result in altered distribution of cells that would normally express *NDN* in people with PWS.

5.2 The Ndn-null mouse is a model of hypotonia in Prader-Willi syndrome

Mammalian skeletal development involves the coordination of complex events to correctly form properly functioning skeletal muscle. Hypotonia in people with PWS is believed to be of complex origin, not arising from a single defective process, although there is likely a neural basis. The work presented in this thesis complements studies from others supporting the notion that individuals with PWS suffer from defects in multiple developmental steps during muscle development, and that these defects synergistically combine with neural deficits to produce the severe hypotonia observed in PWS. Specifically, I provide evidence that loss of necdin in mice impairs limb muscle progenitor polarity, subsequent migration into the dorsal domain, and reduces the proportional area of forelimb extensor muscles. Muscle differentiation is compromised by the failure to de-repress transcriptional inhibition by EID-1 in primary myoblasts derived from *Ndn*-null embryo limb buds (Bush and Wevrick 2008), further exacerbating defects due to progenitor migration. Discoveries by other investigators have demonstrated defective satellite muscle cell differentiation (Deponti et al. 2007) and motor neuron defects (Pagliardini et al. 2005) in *Ndn*-null mice, and the detrimental contributions of these impairments to skeletal muscle development are likely magnified in the context of the migration and primary differentiation defects. Thus, loss of necdin impairs four temporally sequential events critical for skeletal muscle development: progenitor migration, primary differentiation, motor neuron development, and satellite cell differentiation. Finally, the centrosome re-orientation and myosin activation defects shared between *Ndn*-null primary cells

and PWS fibroblasts provide compelling evidence that loss of necdin contributes to the pathophysiology of PWS and validates the *Ndn*-null mouse as a model for this disease. See Figure 5.1 for a current summary of pathways involving necdin.

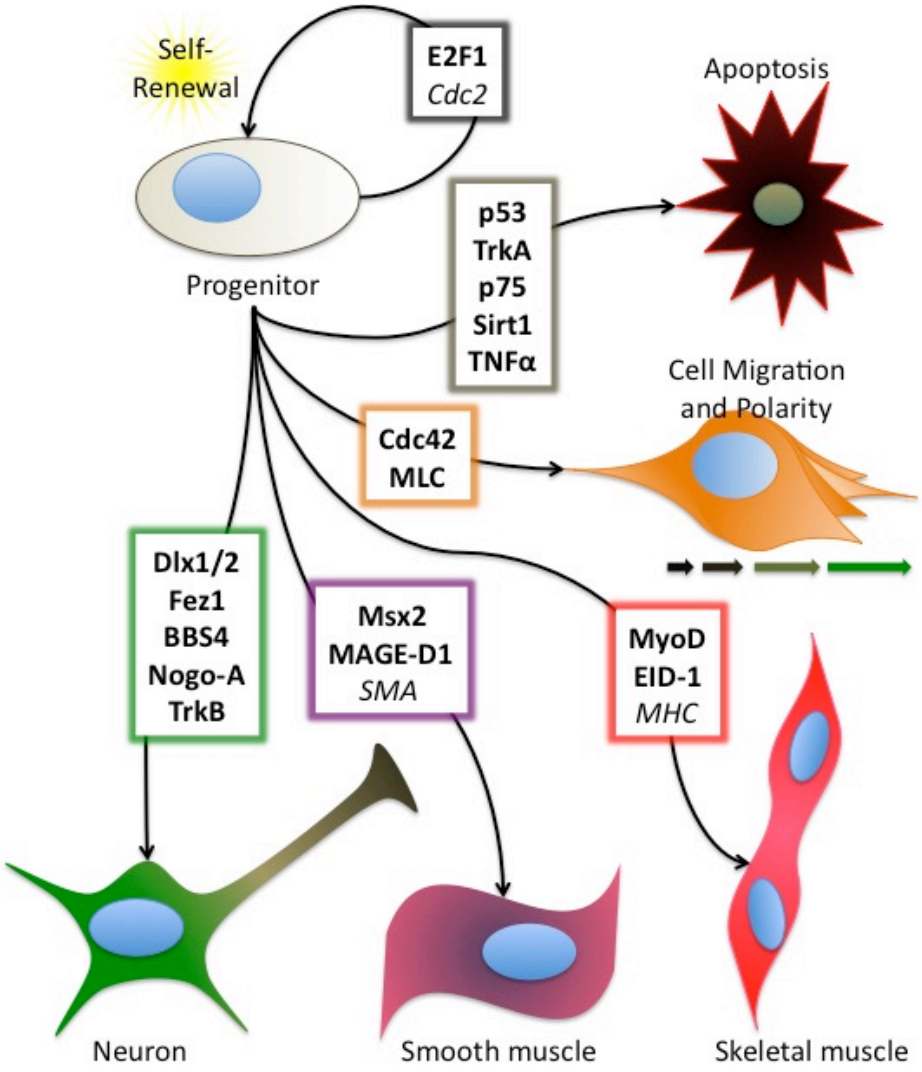

Figure 5.1. Current summary of pathways involving necdin. Necdin promotes cell cycle exit by inhibiting E2F1-dependent *Cdc2* expression, regulates apoptosis by inhibiting Sirt1-dependent p53 acetylation and TNFα signaling, promotes myosin-dependent cell polarity via Cdc42, relieves EID-1-dependent inhibition of MyoD-dependent *myosin heavy chain* (*MHC*) expression and muscle differentiation, and dynamically regulates neurite extension by differentially interacting with Nogo-A or modulating TrkB signaling.

5.3 Necdin promotes cell polarity and limb muscle progenitor migration

The hypothesis that the loss of necdin would impair cellular migration, thus impairing muscle progenitor migration and contributing to hypotonia in PWS, was plausible for several reasons. First, the superior cervical ganglia (SCG) in *Ndn*-null mice do not complete rostral migration during embryonic development (Tennese, Gee, and Wevrick 2008). Second, gonadotropin hormone-releasing hormone (GnRH) neurons in *Ndn*-null mice have reduced migration from the olfactory bulb towards the hypothalamus (Miller, Wevrick, and Mellon 2009). Both GnRH neurons and muscle progenitors require HGF for normal migration *in vivo* (Dietrich et al. 1999; Giacobini et al. 2007; Takayama et al. 1996). Third, necdin is expressed in the developing dermomyotome of mice (Brunelli et al. 2004), and micromass cultures from *Ndn*-null mouse limb buds contain fewer Pax3-expressing cells than those cultured from control embryos (Figure 4.1). The reduced number of Pax3-expressing cells in *Ndn*-null limb bud cultures could be explained by increased apoptosis or precocious differentiation of these cells. However, there was no difference between genotypes in the number of MyoD-expressing myoblasts present in cultures (Figure 3.7), suggesting that cell death or premature differentiation to myoblasts cannot explain the reduced number of Pax3-expressing cells in *Ndn*-null cultures.

Ndn-null mouse embryonic fibroblasts (MEFs) were studied to test the hypothesis that loss of necdin impairs cellular migration because these primary cells are more easily prepared than muscle precursors and because fibroblasts are the most common cell type used for migration assays. Results from experiments

using *Ndn*-null MEFs revealed a delay in cellular migration due to delayed polarization with respect to the direction of migration. Specifically, loss of necdin impaired Cdc42-dependent myosin activation and nuclear rearward movement in response to serum activation, which is a critical component of centrosome re-orientation and the establishment of polarity in a wound healing assay (Gomes, Jani, and Gundersen 2005) (Figure 4.3). This impairment did not globally alter signal transduction in *Ndn*-null MEFs, as Extracellular Regulated Kinase (ERK) was significantly activated in MEFs of both genotypes (Figure 4.3).

Brain-derived neurotrophic factor (BDNF) is not a polarity cue *per se*, but this growth factor induces neurite extension (Lindsay, Thoenen, and Barde 1985) which requires myosin (Wylie et al. 1998), so it was reasonable to hypothesize that BDNF activates myosin. BDNF-TrkB signaling is important for the tangential migration of GABAergic interneurons (Polleux et al. 2002), a population whose migration was recently demonstrated to be impaired in *Ndn*-null mice (Kuwajima, Hasegawa, and Yoshikawa 2010). Mutations in *BDNF* are associated with obesity and cognitive impairment (Gray et al. 2006), and *BDNF*-null animals display breathing difficulties (Erickson et al. 1996) similar to *Ndn*-null mice. BDNF should also activate Cdc42 in cortical neurons (Cheung et al. 2007); however, BDNF treatment did not significantly activate Cdc42 in cortical neuron cultures of either genotype although identical BDNF treatments lead to significant myosin activation in control but not *Ndn*-null cultures (Figure 4.4). I cultured cortical neurons for 5 days *in vitro* (DIV) before completing the GTPase assay, while Cheung and others cultured their cells for 7 DIV. From the methods

described, it is difficult to determine exactly how the investigators prepared the cortical neurons, since they provide a description for experiments using rat cortical neurons but not for the mouse cortical neurons used in the GTPase assay. Regardless, the protocol I used appears similar to the one they used for rat cortical cultures, and I am able to detect significant activation of TrkB in cultures of both genotype in response to BDNF (Figure 4.4), indicating that my cortical cultures are viable and that the BDNF treatment protocol works. It is possible that neurons respond to BDNF via Cdc42 at later stages but employ another mechanism at early stages. I could complete the assay at 7 DIV to address this question. Another possible explanation for the difference between my results and those of Cheung and others is that *Ndn*-null neurons could up-regulate components of an alternative signaling pathway in order to compensate for Cdc42 activation. However, because BDNF does not significantly activate myosin in *Ndn*-null neurons, such an alternative pathway would also appear to require necdin. RNA knock-down of necdin could be done to test whether neurons respond differently to BDNF under chronic or acute loss of necdin. Finally, it is also possible that a different Cdc42 antibody, Pak1-GST fusion protein, or other conditions might affect the sensitivity of this assay thereby producing apparently different results. This question might be best addressed by sending *Ndn*-null mice to Cheung and others so they could test for Cdc42 activation in neurons with their reagents under conditions in their laboratory.

Most studies of cell polarization during migration initiation involve *in vitro* or *ex vivo* analysis. While limb muscle progenitors delaminating from the

dermomyotome should, in theory, establish polarity with respect to the developing limb, this question has not been specifically addressed. I tested this hypothesis *in vitro* using HGF or SDF-1α in agarose plated adjacent to micromass limb bud cultures from *Ndn*-null or control embryos. The technique, where a growth factor soaked into an agarose creates a chemokine gradient *in vitro*, has been demonstrated using BDNF to induce neuronal migration (Zhou et al. 2007). Analogous to scoring centrosome re-orientation in the wound healing assay, I used the position of the centrosome with respect to the nucleus and the source of the growth factor to score polarity. Consistent with the hypothesis that pro-migratory chemokines would induce cellular polarization in limb muscle progenitors, I found that muscle progenitors from the limbs of control embryos, but not those from *Ndn*-null embryos, polarized with respect to both HGF and SDF-1α (Figure 4.5). The proportion of polarized cells after 8 hours of treatment did not increase in either genotype, presumably because the growth factor was then evenly distributed in the media and no longer acting as a gradient.

Parallel experiments using BDNF-stimulated hippocampal neurons produced similar results. Hippocampal neurons project a single Tau-positive axon in culture and therefore are suitable for studying cell polarity (Dotti, Sullivan, and Banker 1988). I reasoned that a spatial gradient of BDNF would increase the number of neurites on the side of the cell exposed to the growth factor and that this would increase the probability that one of those neurites would become the single axon projection. BDNF led to a small but significant increase in the number of polarized neurons in control but not *Ndn*-null cultures (Figure 4.4).

Thus, loss of necdin impairs growth factor-induced polarization in muscle and neurons *in vitro*. While I have not tested specifically whether *Ndn*-null muscle progenitors display impaired myosin activation in response to relevant growth factors, it is reasonable to conclude that the same mechanism impaired by loss of necdin in fibroblasts and neurons is adversely affected in migrating muscle precursors. Furthermore, failure to activate the actin-myosin cytoskeleton during the initiation of migration could underly both the skeletal muscle migration defects described here, as well as those of the tangentially migrating interneurons and GnRH neurons (Kuwajima, Hasegawa, and Yoshikawa 2010; Miller, Wevrick, and Mellon 2009).

The polarity of muscle progenitors has not been previously examined *in vivo*. Based on the results described above and the documented relationship between polarity and migration *in vitro*, I hypothesized that muscle progenitors delaminating from the dermomyotome should polarize with respect to the limb bud, and that this process would be defective in *Ndn*-null embryos. To address this question, I used the same re-orientation tool to score the position of the centrosome of muscle progenitors with respect to the developing limb bud in sections of embryonic day 9.5 (E9.5) control and *Ndn*-null embryos. Significantly more muscle progenitors polarized with respect to the limb bud than would be expected by chance alone in both control and *Ndn*-null embryos (Figure 4.5). However, significantly fewer muscle progenitors were polarized in *Ndn*-null embryos compared to controls. These results are consistent with those obtained from *Ndn*-null MEFs, and suggest that loss of necdin impairs polarity in multiple

cell types, including developing muscle, a tissue type affected in individuals with PWS. My experiments are the first to describe that muscle precursors polarize *in vivo* with respect to the limb bud and that necdin is important for this developmental process. Investigation of polarity in other cell types undergoing epithelial to mesenchymal transition in the *Ndn*-null mouse might yield similarly interesting results.

This assessment of muscle progenitor polarity *in vivo* is admittedly simple. This assay could be improved by mating the *Ndn*-null mouse line to the *Lbx1*-GFP line to visualize only those muscle progenitors destined to migrate into the limb bud (Vasyutina et al. 2005). Similarly, the use of an antibody to Lbx1 would identify migrating muscle progenitors. *In vitro* assays measure cell polarity in two dimensions, while polarization *in vivo* involves X, Y, and Z axes. However, muscle progenitors delaminating from the somite migrate ventrally and a proportion of those cells change trajectory to migrate laterally. My analysis is unlikely to be confounded because limb muscle precursors do not significantly migrate along the anterior-posterior axis in mice (Bober et al. 1994). More thorough analysis awaits the advent of new reagents, such as mice in which Lbx1 drives expression of GFP-γ-Tubulin to specifically identify the centrosome belonging to the migrating cell, or a construct encoding a receptor that only fluoresces when activated, thereby labeling the leading-edge of the migrating cell.

If the polarity defects in *Ndn*-null muscle progenitors produce a migration delay in a fashion similar to the delayed migration of polarity-impaired *Ndn*-null MEFs, the distribution of muscle progenitors in the developing forelimb of the

Ndn-null embryo should consequently be affected. As mentioned above, muscle progenitors in mouse embryos lacking the genes *Pax3*, *HGFR* (*c-met)*, or *HGF* fail to migrate from the dermomyotome and as a result these mice do not develop limb musculature. Some mouse mutants display an intermediate phenotype whereby the flexor muscles of the forelimb, which are derived from the ventral pool of migratory progenitors, form normally, while the dorsally-derived extensor muscles are absent or severely reduced. Mice missing the transcription factor *Lbx1*, *Gab1*, which encodes an *HGFR* adaptor protein, or *CXCR4* display absence or reduction of forelimb extensor muscles (Gross et al. 2000; Sachs et al. 2000; Schafer and Braun 1999; Vasyutina et al. 2005). Examination of these mouse embryos around E10.5 reveals that muscle progenitors delaminate from the dermomyotome but fail to migrate into the dorsal region of the front limb bud. Rather, they continue to migrate ventrally, with a proportion moving laterally into the ventral half of the limb bud. This type of migration defect is consistent with a model whereby delaminating muscle progenitors are slow or fail to polarize with respect to chemotactic cues secreted by the limb bud and the majority of muscle progenitors continue along the ventral migratory pathway. Migration of thoracic body wall progenitors was not examined but could contribute to hypoventilation in PWS.

Loss of necdin is unlikely to produce a severe muscle phenotype, as people with PWS and the *Ndn*-null mice have limb musculature, but a reduction in the number of limb muscle progenitors could impair subsequent muscle development and contribute to hypotonia in PWS. To address this possibility, I

examined the distribution of muscle progenitors in sections of *Ndn*-null and control embryos at E10.5, one day after assessing the polarity of these cells. Vasyutina and others compared the distribution of muscle progenitors in the forelimb by dividing the bud into four quadrants: proximal dorsal, distal dorsal, distal ventral, and proximal ventral (Vasyutina et al. 2005). Using a similar technique, the muscle progenitors in the dorsal and ventral pools of the limb bud were counted and the distribution of cells was compared between *Ndn*-null and control embryos. Consistent with the observation that muscle progenitors in *Ndn*-null embryos fail to polarize with respect to the developing limb, as well as the observed migration delay in *Ndn*-null MEFs, the dorsal pool of muscle progenitors is reduced in *Ndn*-null embryos (Figure 4.5). Clarification of the roles that cell proliferation and cell death play in shaping the distribution of muscle progenitors could be achieved by sorting dissociate limb buds using a flow cytometer.

Unlike *Lbx1*-/- or *Gab1*-/- mice, those lacking *CXCR4* develop extensor muscles despite reduced dorsal muscle progenitor pools. In fact, the forelimb musculature of *CXCR4*-/- mouse embryos is indistinguishable from controls at birth and the authors of the study suggest that the subsequent muscle developmental program is sufficient to compensate for the reduced pool of muscle progenitors observed earlier in development (Vasyutina et al. 2005). Given the difference in muscle progenitor distribution in the forelimb bud at E10.5, I hypothesized that loss of necdin would negatively affect the size of forelimb extensor muscles at E18.5. I measured the cross sectional area of flexor and

extensor muscles, as well as the total cross-sectional area, at a number of points along the length of the zeugopod of each forelimb. I then divided the area of the extensor or flexor muscle by the total cross-sectional area to obtain ratios that were then averaged for each embryo. Normalization of cross-sectional area and averaging of values along the forelimb length were necessary to account for size differences between genotypes and to effectively sample the size of the muscle along the entire length. Comparison of proportional muscle area revealed that extensor muscles were significantly smaller in *Ndn*-null mice, while the total proportional muscle area was not different (Figure 4.5). Dorsal limb bud-specific increases in apoptosis or similar region-specific decreases in myoblast differentiation were not detected in the *Ndn*-null mouse (Figure 4.6). Furthermore, region-specific changes in muscle precursor proliferation or death have not been described in the literature, while the defects observed in *Ndn*-null mice were consistent with the defective forelimb development described in *Lbx1*-/-, *Gab1*-/-, and *CXCR4*-/- mice (Gross et al. 2000; Sachs et al. 2000; Schafer and Braun 1999; Vasyutina et al. 2005).

In conclusion, these data provide compelling evidence that polarity defects and subsequent delay of muscle progenitor migration due to loss of necdin result in specific reduction of forelimb extensor muscles. No previous study has documented muscle development defects *in vivo* and impaired signal transduction and cytoskeletal regulation due to the loss of a single protein. Given the central role of Cdc42 during centrosome re-orientation and cell migration *in vitro*, and in the light of my results demonstrating that Cdc42 activation is impaired in *Ndn*-

null cells, it is reasonable to expect that *Cdc42*-null mice could have a similar muscle phenotype. Carmen Birchmeier and others recently addressed this question. These authors avoided the early embryonic lethality in *Cdc42*-null mice (Chen et al. 2000) by specifically eliminating Cdc42 in Lbx1-positive muscle precursors, therefore allowing the study of *Cdc42*-null cells in an otherwise normal mouse embryo (Vasyutina et al. 2009). Surprisingly, migration of muscle progenitors appeared unaffected in *Cdc42*flox/flox;*Lbx1*cre mice at E10.5 and the major muscle phenotype was attributed to impaired myoblast fusion. While it is possible that other GTPases compensate in the absence of Cdc42, as these authors suggest, the half-life of Cdc42 is 15 hours (Backlund 1997) and Lbx1 expression is not detected in the somites until E8.5 in mice (Jagla et al. 1995), so substantial Cdc42 protein levels (25-50% of normal levels) will be present in *Cdc42*flox/flox;*Lbx1*cre embryos at the stage when muscle progenitors must polarize with respect to the limb. Therefore, it cannot be concluded that Cdc42 is dispensable for muscle progenitor migration from the lack of limb muscle migration phenotype in the *Cdc42*flox/flox;*Lbx1*cre mouse. It is reasonable to hypothesize that both Cdc42 and necdin are important for polarization, but not necessarily migration, of muscle progenitors and potentially co-operate during this developmental process.

5.4 Myoblast differentiation

Necdin is the only PWS candidate gene strongly expressed in developing and skeletal muscle, suggesting that loss of necdin could impair muscle development and contribute to hypotonia in people with PWS. Necdin bears

functional similarity to Rb (Taniura et al. 1998), a protein that is critical for MyoD-dependent transcription during muscle differentiation (Gu et al. 1993). The E1A-like inhibitor of differentiation-1 (EID-1) inhibits MyoD-dependent transcription and is degraded by Rb in a cell-cycle-dependent manner (MacLellan et al. 2000; Miyake et al. 2000). EID-1 was identified as a possible necdin-interacting protein in a yeast two-hybrid screen of fetal brain cDNAs. I therefore hypothesized that necdin would antagonize EID-1-depenedent inhibition of muscle differentiation, similar to the function necdin performs to promote neuronal differentiation, and that prolonged EID-1 activity might contribute to hypotonia in PWS.

I confirmed that necdin and EID-1 interact in transiently transfected cells (Figure 3.1), but was surprised to see that EID-1 protein levels were higher and that the protein half-life of EID-1 was significantly extended (Figure 3.2). Necdin regulates the protein stability of other transcriptional regulators, including HIF-1α (Moon et al. 2005), which could contribute to muscle development by modulating local vasculature through HIF-1α-dependent expression of vascular-edothelial growth factor (*VEGF*) (Damert, Ikeda, and Risau 1997; Damert et al. 1997). EID-1 protein was more abundant in the presence of necdin than when proteasomal degradation was inhibited with the drug MG132. Furthermore, endogenous levels of EID-1 were significantly reduced in tissue from *Ndn*-null mice compared to littermate controls (Figure 3.3). These results were inconsistent with a model whereby necdin functioned similar to Rb by relieving EID-1-dependent transcriptional repression of MyoD by degrading EID-1 protein.

It therefore seemed plausible that necdin might actually co-operate with EID-1 to inhibit muscle differentiation, which was counter-intuitive given that necdin promotes cell cycle exit and differentiation in neurons (Kobayashi, Taniura, and Yoshikawa 2002; Maruyama et al. 1991; Takazaki, Nishimura, and Yoshikawa 2002), smooth muscle (Brunelli et al. 2004), and C2C12 murine satellite muscle cells (Kuwajima et al. 2004). However, I demonstrated that necdin promoted MyoD-dependent activation by relieving EID-1-dependent repression using a reporter construct containing the *Myosin Heavy Chain* (*MHC*) promoter and a reporter construct containing several E-box MyoD consensus binding sites (Figure 3.6). These results complement those of Deponti and others who demonstrated that necdin promotes transcriptional activation at the myogenin promoter and chromatin immunoprecipitation of C2C12 myoblasts identifies transfected necdin in a complex with the endogenous myogenin promoter (Deponti et al. 2007). Despite the multiple lines of evidence demonstrating that necdin is important for muscle differentiation, these authors were unable to identify an interaction between necdin and relevant transcription factors. My results provide a mechanism that potentially explains the results of Deponti and others, whereby necdin could promote MyoD activation by antagonizing EID-1. Although consistent with the concept of necdin as a pro-differentiation protein, the mechanism by which necdin stabilized EID-1 while antagonizing EID-1 function was not clear, but could be due to subcellular changes in protein location.

Immunofluorescent investigation of cells transiently transfected either necdin or EID-1 alone revealed that each protein is distributed throughout the cell,

in both nuclear and cytoplasmic compartments. However, transfection with both necdin and EID-1 demonstrated that co-expression of necdin and EID-1 depleted the nuclear pool of both proteins and resulted in their cytoplasmic accumulation (Figure 3.5). This observation was confirmed by immunoblot analysis of similarly transfected cells separated into nuclear and cytoplasmic fractions (Figure 3.4). EID-1 has a nuclear export signal that is mediated by CRM1 and EID-1 can be trapped in the nucleus upon treatment of cells with Leptomycin B (Bavner et al. 2002). Leptomycin B treatment increased nuclear accumulation of necdin alone, EID-1 alone, and both proteins together (Figure 3.5), suggesting that necdin-dependent nuclear export of EID-1 is mediated in a CRM1 dependent manner. Taken together, these data suggest a model whereby necdin relieves EID-1-dependent inhibition of MyoD-dependent transactivation by sequestering EID-1 in the cytoplasm (Figure 3.8).

Necdin has not been thoroughly characterized as a modulator of nuclear-cytoplasmic shuttling, or as a regulator of protein stability. Cytoplasmic retention of NEFA, a calcium-binding protein, increases upon co-transfection with necdin in neuronal cell lines and promotes caffeine-evoked calcium retention (Taniguchi et al. 2000). As NEFA is normally secreted from the cell (Barnikol-Watanabe et al. 1994), and does not move between the nucleus and cytoplasm, necdin could function similarly to tether both EID-1 and NEFA in the cytoplasm. Calcium homeostasis is critical in differentiated neurons and muscle, two cell types that strongly express necdin, but a phenotype involving differences in cellular calcium levels has not been described in *Ndn*-null mice. However, NEFA is cleaved to

produce nesfatin-1, a satiety molecule that acts through the melanocortin pathway in the hypothalamus to regulate food intake (Maejima et al. 2009; Oh et al. 2006). It is possible that necdin, or the related protein MAGEL2, could modulate processing of NEFA into nesfatin-1 or the exocytic release of nesfatin-1. Dysregulation of this process could explain why people with PWS, who lack both necdin and MAGEL2, have insatiable appetites.

Necdin interacts with the RING finger ubiquitin ligase Praja-1, a protein that promotes proteasomal degradation of the Dlx-interacting protein Dlxin-1 (also known as MAGE-D1) and Msx2 to regulate transcription (Sasaki et al. 2002). Given that EID-1 is degraded in a proteasome-dependent manner upon cell cycle exit (MacLellan et al. 2000; Miyake et al. 2000), it is possible that necdin increases EID-1 stability, at least in part, by interfering with Praja-1-dependent degradation. However, the presence of necdin had a stronger stabilizing effect on EID-1 protein than proteasomal inhibition alone (Figure 3.2), indicating that factors other than the proteasomal pathway are involved.

I hypothesized that loss of necdin in the mouse would lead to protracted EID-1-dependent inhibition of MyoD-dependent transactivation and result in a reduction in the conversion of myoblasts to myotubes. I tested this hypothesis by culturing micromass limb bud cultures from E10.5 *Ndn*-null and control littermate embryos and monitoring the conversion of MyoD-positive myoblasts to MHC-positive myotubes at one, three or five days in culture. There was no difference between genotypes in the number of MyoD-positive cells, indicating that loss of necdin does not affect myoblast proliferation. However, *Ndn*-null cultures

contained significantly fewer MHC-positive myotubes, consistent with a model whereby loss of necdin leads to prolonged EID-1-dependent repression of MyoD-dependent transactivation and results in impaired myogenic differentiation (Figure 3.7).

Necdin is known to interact with Msx2 to promote differentiation of smooth muscle (Brunelli et al. 2004) and with both Msx1 and Msx2 in C2C12 cells (Kuwajima et al. 2004). While prolonged repression by Msx1 may contribute to impaired myoblast differentiation in *Ndn*-null mice, it is unlikely that Msx2 contributes to the defect because Msx2 is not expressed by migrating muscle progenitors (Bendall et al. 1999). Kuwajima and colleagues favor a model whereby necdin interacts with and removes Msx1 and Msx2 via the protein MAGED1 to promote binding of Dlx proteins to promote differentiation (Kuwajima et al. 2004). These authors show necdin protein accumulating in cytoplasmic regions in differentiated C2C12 myotubes, similar to the redistribution of co-expressed necdin and EID-1 in my experiments. It is also possible that regulation of Msx protein stability by a complex containing necdin, Dlxin-1/MAGE-D1, and Praja-1 contributes to relief of transcriptional repression. Brunelli and colleagues suggest that both necdin and Msx2 co-operate to promote smooth muscle cell differentiation by acting together as transcriptional repressors, potentially acting to relieve repression of an unknown repressor of smooth muscle cell differentiation or to switch from inhibitory to activating transcriptional regulators (Brunelli and Cossu 2005). According to unpublished data, these authors note that necdin and Msx2 both redistributed to the cytoplasm in

differentiating smooth muscle cells (Brunelli and Cossu 2005). It is interesting to note that Deponti and co-workers describe a skeletal muscle defect in a *Ndn*-null mouse, and while chromatin immunoprecipitation identified transfected necdin at the myogenin promoter, they were unable to detect an interaction between necdin and MyoD, myogenin, or Mef2A by co-immunoprecipitation in transfected C2C12 cells (Deponti et al. 2007). These results are consistent with the model I propose, in which necdin relieves EID-1-dependent repression of MyoD-dependent transactivation by excluding EID-1 from the nucleus. Thus, removal of repressive transcription factors and co-repressors from the nucleus may represent a common mechanism by which necdin promotes differentiation.

It is possible that EID-1 continues to function as an inhibitor of differentiation in the presence of necdin, sequestering necdin away from the nucleus where it normally promotes cell cycle exit (Hayashi et al. 1995; Taniura, Matsumoto, and Yoshikawa 1999), inhibits apoptosis (Hasegawa and Yoshikawa 2008; Kurita et al. 2006; Taniura, Matsumoto, and Yoshikawa 1999), promotes neuronal differentiation (Kuwajima, Nishimura, and Yoshikawa 2006), or promotes myogenesis (Deponti et al. 2007; Kuwajima et al. 2004). Indeed, co-expression of necdin and EID-1 in migrating myoblasts could maintain an intermediate state of differentiation, preventing terminal differentiation into myotubes until other pro-differentiation factors tip the balance. However, there was no difference in the number of myoblasts between *Ndn*-null and control mouse cultures (Figure 3.7), indicating either that necdin is dispensable for maintaining myoblast fate or that *in vivo* events promoting myoblast fate via

necdin are not recapitulated in the micromass limb bud culture assay. The micromass assay removes spatial cues normally found during limb bud development and simply follows the differentiation of cells previously specified. Finally, loss of necdin impairs terminal differentiation of myoblasts to MHC-positive myotubes (Bush and Wevrick 2008; Deponti et al. 2007), consistent with a model whereby up-regulation of necdin during muscle differentiation leads to relief of EID-1-dependent repression of MyoD-dependent transactivation.

The role of necdin outside of the nucleus is not well understood. In neurons, necdin interacts with the p75 neurotrophin receptor (Bronfman et al. 2003; Kuwako, Taniura, and Yoshikawa 2004; Tcherpakov et al. 2002) and TrkA (Kuwako et al. 2005) to promote prevent apoptosis. Necdin has been recently reported to interact with the Nogo-A receptor, a protein best described in oligodendrocytes as an inhibitor of axonal extension (Liu et al. 2009). These authors suggest that Nogo-A inhibits neurite extension by retaining necdin in the cytoplasm and inhibiting necdin-dependent transcriptional activity in the nucleus.

EID-1 is also poorly understood outside of the nucleus despite two independent observations of cytoplasmic accumulation (Bavner et al. 2002; Bush and Wevrick 2008) and the fact that EID-1 is ubiquitously expressed in neonatal and adult mouse tissue (MacLellan et al. 2000; Miyake et al. 2000), with EID-1 protein levels actually increasing in more differentiated muscle and neuronal tissue (Figure 3.3). Cytoplasmic accumulation of co-expressed necdin and EID-1 is unlikely to be an artifact of overexpression since co-expression of necdin with other proteins does not result in a similar re-localization (Lee et al. 2005).

Similarly, increased EID-1 protein in the presence of necdin is unlikely to be an artifact because neither over-expressed necdin nor control proteins accumulate (Lee et al. 2005), and endogenous EID-1 protein is less abundant in tissue from *Ndn*-null mice (Figure 3.3). Thus, the functional requirement for cytoplasmic retention of necdin and EID-1 remains elusive.

An intriguing possible role for EID-1 protein in the cytoplasm relates to the ability of EID-1 to inhibit HAT activity (MacLellan et al. 2000; Miyake et al. 2000). While histones, transcription factors, and other nuclear proteins are well-described targets of acetylation, the alpha-tubulin subunit of microtubule polymers is also dynamically acetylated (Greer et al. 1985; L'Hernault and Rosenbaum 1985, 1985). Acetylation stabilizes microtubules and can label a subset of microtubules to regulate sub-cellular trafficking of cargoes carried by microtubule motors in developing neurons (Reed et al. 2006). In differentiating L6 myoblasts, tubulin acetylation does not occur immediately after differentiation, but significantly increases after myoblast fusion, presumably establishing a stable microtubule array upon which the contractile actin-myosin network will be assembled (Gundersen, Khawaja, and Bulinski 1989). Elongator is the only the acetyltransferase known to promote cytoplasmic tubulin acetylation (Creppe et al. 2009), and both HDAC6 and Sirt2 possess tubulin deacetylase activity (Hubbert et al. 2002; North et al. 2003). Furthermore, mutations in genes encoding components of the Elongator complex are associated with Familial Dysautonomia (FD), in which patients suffer from numerous neurological defects, and motor neuron disease (Close et al. 2006; Simpson et al. 2009). Knock-down of

Elongator specifically reduces the expression of genes important for cell migration, and FD patient fibroblasts display impaired migration and reduced expression of similar genes (Close et al. 2006). The phenotypic similarities between FD and PWS patient fibroblasts suggest they could share similar dysregulation of chromatin acetylation at genes important for cell migration. Given the post-mitotic enrichment of both necdin and EID-1 in neurons and muscle, two cell types that regulate tubulin acetylation, it is possible that these proteins co-operate to modulate tubulin acetylation. Thus, loss of necdin likely impairs cell migration by compromising both transcriptional and cytoskeletal events.

Bardet-Biedl syndrome (BBS) is a genetic disorder that shares phenotypic overlap with PWS, with common features including obesity and developmental delay. BBS is caused by mutations in a number of heterologous genes (currently BBS1-BBS14, whose protein products interact in what has become known as the BBSome), many of which are important for development and function of the primary cilium (reviewed in (Zaghloul and Katsanis 2009)). Necdin interacts with BBS4 and is enriched around basal bodies in cells when both proteins are over-expressed (Lee et al. 2005), providing evidence that some aspects of PWS and BBS may have a common molecular basis. BBIP10 is a protein that tethers HDAC6 to the BBSome and depletion of BBIP10 expression results in reduced acetylated tubulin, which can be reversed by inhibiting HDAC6 in BBIP10 depleted cells (Loktev et al. 2008). These authors conclude that dysregulation of tubulin acetylation, a modification enriched in the primary cilium, contributes to

the pathogenesis of BBS. While EID-1 does not possess deacetylase activity, it is functionally similar to the HDACs because it is capable of reducing histone acetylation. Therefore, it is plausible that the expression of EID-1 in post-mitotic cells and increased EID-1 protein in the cytoplasm in the presence of necdin are important for the regulation of tubulin acetylation. The lack of an *EID-1*-/- mouse model or a reliable antibody to EID-1, necessary to validate RNA-mediated interference, make testing this hypothesis impractical at this time.

5.5 Future Directions

While there are reports suggesting that a microdeletion of the snoRNAs found in the PWS critical region is sufficient to cause this disease (de Smith et al. 2009; Sahoo et al. 2008), substantial evidence from animal models, cell biology, and biochemistry indicates that loss of necdin and MAGEL2 contributes to the PWS phenotype. Loss of necdin impairs development of nervous and muscle tissue development at multiple stages. Improved reagents, such as an antibody to necdin suitable for immunofluorescent detection of endogenous protein or the generation of a transgenic mouse expressing GFP-conjugated necdin, will permit high-resolution analysis of protein localization and interaction during various cellular responses. Although these approaches are challenging, they would advance our understanding of normal necdin function, allow the identification of defective molecular mechanisms in *Ndn*-null cells, and facilitate the investigation of potential therapeutic interventions.

The role of necdin in muscle development is complex, and targeting developmental pathways for treatment *in utero* is not a realistic goal at this time.

However, since necdin is important for satellite cell differentiation in adults (Deponti et al. 2007), pursuit of the molecular mechanism by which necdin promotes this process could lead to the development of therapies for other myopathies. Loss of Cdc42 impairs myoblast fusion in *Cdc42*flox/flox;*Lbx1*cre mice (Vasyutina et al. 2009). Failure to activate Cdc42 in *Ndn*-null muscles may contribute to fiber disorganization and impaired regeneration, in addition to reduced transcriptional activation (Deponti et al. 2007). To address this possibility, skeletal myoblasts from the limbs of E12.5 *Ndn*-null and control embryos could be prepared, plated in culture, and the fusion index measured. I predict that, like the *Cdc42*flox/flox;*Lbx1*cre myoblasts, those from *Ndn*-null embryos will have impaired fusion due to the loss of Cdc42 activation. Asymmetrical division and self-renewal of muscle satellite cells depends on the direction of division and the proteins that make contact with the dividing cells (Kuang et al. 2007). The extent to which Cdc42 and necdin participate in satellite cell regulation is not currently known, but given the muscle phenotype in the *Ndn*-null mouse and impaired Cdc42 activation due to the loss of necdin, investigation of this pathway is warranted.

Impaired myosin activation due to loss of necdin is an intriguing problem because myosin activation is important for a number of developmental and physiological processes perturbed in people with PWS. Phophorylation of MLC and activation of non-muscle myosin Va is important for insulin granule secretion from pancreatic beta cells and pituitary somatotrophs and growth hormone release (Iida et al. 1997; Ivarsson et al. 2005). Since people with PWS have low levels of

growth hormone (Lee et al. 1987), and loss of necdin impairs myosin activation in both mouse and human cells, it is reasonable to believe that mobilization of growth hormone, and potentially other hormones, is impaired in PWS due to the loss of necdin. Myosin activation in patient fibroblasts in response to serum can easily be measured by immunoblot analysis using the antibody to phosphorylated serine 19 of the regulatory myosin light chain and this assay could be adapted for use in a high-throughput screen of small molecule pharmaceuticals. Growth hormone therapy, while expensive, is of great benefit to individuals with PWS (Tauber et al. 2009) and the identification of a drug that might potentiate growth hormone release would be a remarkable advancement in the treatment of PWS. Therefore, it is critical to characterize the molecular mechanism by which necdin normally acts to promote Cdc42-dependent myosin activation and to verify that *Ndn*-null cells have impaired hormone release. This could be accomplished by monitoring hormone release following knock-down of necdin in a hormone releasing cell line, such as the hypothalamic gonadotropin hormone-releasing hormone neuron-derived GT1-7 cells. Validating that necdin indeed activates myosin to mobilize growth factors would be sufficient rationale for conducting a small molecule screen to identify a drug that potentiates serum-induced myosin activation in PWS fibroblasts.

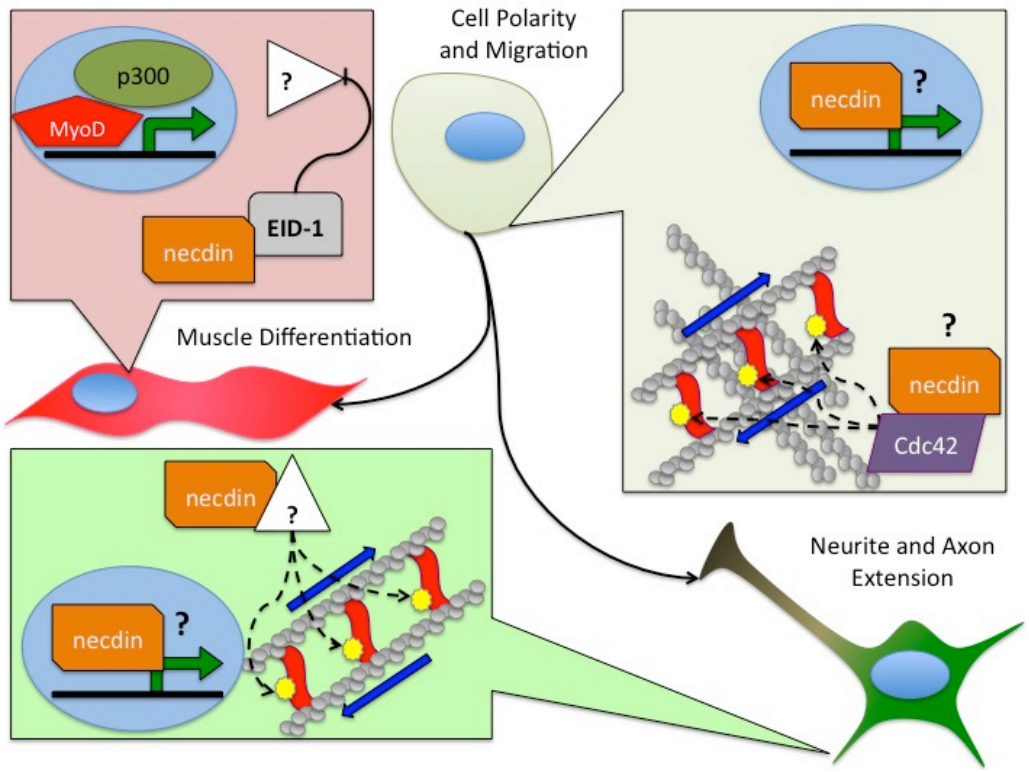

Figure 5.2. Proposed model of necdin function. Necdin promotes cell polarity in migrating cells by promoting Cdc42-dependent myosin activation (top right) either by coupling receptors to Cdc42 or by promoting the transcription of a receptor-Cdc42 scaffold. Necdin similarly promotes polarized neurite outgrowth and axonal extension in neurons (bottom left) through a Cdc42-independent pathway. Necdin promotes MyoD-dependent muscle differentiation (top left) by sequestering the inhibitor of acetyltransferase co-activator EID-1 in the cytoplasm, where EID-1 may further modulate acetylation of unknown proteins in various cell types.

5.6 Closing Remarks

The results of my thesis are summarized in Figure 5.2. Like many studies, my work raises many more questions than it has answered. Specifically, is there a cytoplasmic acetyltransferase modulated by the presence of post-mitotic necdin and EID-1? Does necdin promote Cdc42-dependent myosin activation in migrating cells by physically coupling receptors and downstream effectors, or does necdin regulate the transcription of such a scaffolding protein? Finally, how does necdin regulate BDNF-induced myosin activation in neurons in the absence of Cdc42 activation? Determining the answers to these questions will be the basis of subsequent studies in the Wevrick laboratory.

The multigenic nature of PWS confounds our understanding of this disorder, but targeted deletion of candidate genes and molecular examination of how the proteins encoded by these genes has vastly improved our understanding of PWS. With technical advances, our pursuit of this knowledge will accelerate and generate novel avenues for therapeutic intervention. For example, defective myosin activation in both *Ndn*-null and PWS fibroblasts is the first documentation of a cellular defect in PWS and was facilitated by the generation of a transgenic animal. While correcting myosin activation in people with PWS will not cure the disorder, it may significantly improve the quality of life for these individuals. Furthermore, it would provide a strong example of how basic science discoveries can lead to clinical therapies and why research in medical genetics is important.

5.7 Bibliography

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