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Cellular localization and expression of Ski family proteins in rhabdomyosarcoma

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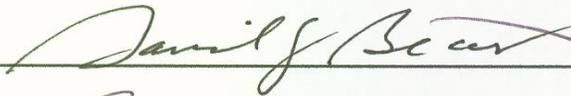
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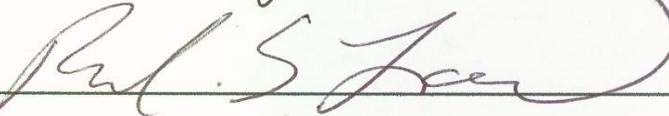
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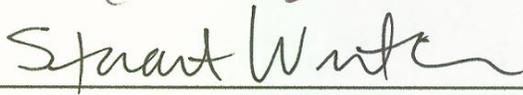
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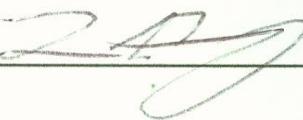
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Approved by the Dissertation Committee:

 _____, Chairperson

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**CELLULAR LOCALIZATION AND EXPRESSION OF
SKI FAMILY PROTEINS IN RHABDOMYOSARCOMA**

BY

BRIAN LEE REINERT

B.S., Biology, University of New Mexico, 1999
B.S., Psychology, University of New Mexico, 1999

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Doctor of Philosophy
Biomedical Sciences**

The University of New Mexico
Albuquerque, New Mexico

May 2010

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Acknowledgments

Ten years ago I was an undergraduate student preparing to finish my studies and apply to medical school. I had very little experience with biomedical research and had only recently started to work as a technician in a lab. My years in the lab as a student and then as a technician have had a profound effect on my life. I can never thank my mentor Dr. David Bear enough for what you have given me. If not for your influence and guidance, I would not be on my way to becoming a physician scientist. In your lab I, perhaps foolishly, chose to take on a project that was not in your area of expertise. At times this proved to be very challenging and we both second guessed this decision. But we struggled through, have learned a lot, and made some interesting discoveries along the way. The independence that you have sought from me was not always easy to return. However, in retrospect, it has made me learn more and given me a better understanding and appreciation of what I have accomplished.

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ABSTRACT OF DISSERTATION

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Abstract

Ski and SnoN are two highly related transcription factors that are transcribed from two separate genes named *SKI* and *SKIL*, respectively. They are both co-repressors of the Smad-mediated transforming growth factor beta (TGF β) signaling pathway. Originally they were classified as oncogenes as they have the ability to transform quail fibroblasts. However, they have also been found to have anti-proliferative properties so they are also considered tumor suppressors. They are involved in normal growth and development and thought to be essential as there is evidence that a homozygous mouse knockout of either *SKI* or *SKIL* is embryonic lethal. Both Ski and SnoN have been found to be overexpressed in some cancers. This overexpression has been found to be prognostic in some cases. Another prognostic factor in certain cancers is the cytoplasmic localization of these normally nuclear proteins. Other than with the TGF β signaling pathway, Ski and SnoN are known to have interactions with retinoic acid receptor signaling and the retinoblastoma protein. In order to obtain a better understanding of Ski and SnoN we have studied several aspects of the proteins. First, we identified the nuclear localization signal (NLS) of Ski and proved that this sequence was both necessary and sufficient for nuclear localization. Next, we looked into the correlation of Ski subcellular location and serine phosphorylation status. Here we found that serine phosphorylated Ski is found predominately in the cytoplasm. Finally, we looked at possible involvement of Ski and SnoN in the pediatric cancer rhabdomyosarcoma (RMS). We found there was expression of both of these proteins in cell lines derived from the cancer and in tumor tissue samples. We also found that Ski protein levels in RMS tumor

tissue are negatively correlated with RMS tumor group. The data from the Ski phosphorylation studies suggest that this modification may work with the NLS to regulate the subcellular location of Ski. Misregulation of this process may be responsible for the cytoplasmic localization of Ski that we found in RMS.

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Chapter 1: Ski and SnoN

1.1 Proteins and Post-translational Modifications

The Ski protein family is named for the founding member, v-Ski, and in humans the family consists of Ski, SnoN, SnoA, and Snol (Nomura et al., 1989; Pearson-White, 1993). These proteins were found when looking for the human homolog to v-ski (Nomura et al., 1989). v-ski and human Ski show 91% protein homology. Ski was named after the Sloan Kettering Institute where it was discovered. Sno is short for Ski-related novel, and is also called Ski-like protein or Skil. SnoN (Ski-related novel non-Alu containing) does not have an ALU containing motif while SnoA (Ski-related novel Alu containing) has the Alu motif. Snol contains an insertion (Ski-related novel with insertion) with an in-frame termination codon resulting in a truncated protein. These proteins are transcription factors that act as co-repressors of the Smad mediated TGF β signaling pathway (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999). The Ski protein is the largest of the family and consists of 728 amino acids. SnoN contains 684 amino acids while SnoA and Snol have 415 and 399 amino acids respectively. There are splicing variants of the Ski and SnoN proteins in other species that are not found in humans. Both Ski and SnoN contain nuclear localization signals (NLS) and are found in the nucleus of normal cells (Barkas et al., 1986; Krakowski et al., 2005; Nagata et al., 2006). Despite the conservation between the two proteins, the NLS is not conserved. A pair-wise protein BLAST between the human Ski and SnoN revealed that 37% of the amino acids are identical while 55% of the amino acids were identical or had

conservative changes (Tatusova and Madden, 1999). And a BLAST between the chicken homologs reported 42% identical amino acids and 60% that were identical or had conservative changes.

There are several functional domains in Ski and SnoN including the familial Ski domain also called the Dachshund homology domain (DHD or DACH) (see Figure 1) (Medrano, 2007). The Ski domain, which is essential for transformation, is roughly 100 amino acids long, contains a conserved CLPQ motif, and is found toward the N-terminus of the protein. There is a SAND-like (Sp100, Aire-1, NucP41/75, DEAF-1) domain in the region containing amino acids 219-312 that was discovered by 3-dimensional homology when examining the crystallized structure of this peptide (Wu et al., 2002). Also found in the Ski protein are a proline rich domain, three leucine zipper-like domains, two tandem helix-loop-helix motifs, a basic amino acid region, a serine/threonine domain, three tandem 25 amino acid repeats, and two alpha helical domains. The Ski domain is responsible for protein interaction with Smad4, PML, MeCP2, SKIP, pRb, HIPK-2, N-CoR, FHL2, and mSin3. Ski has multiple post-translational modifications including phosphorylation and sumoylation (Sutrave et al., 1990a).

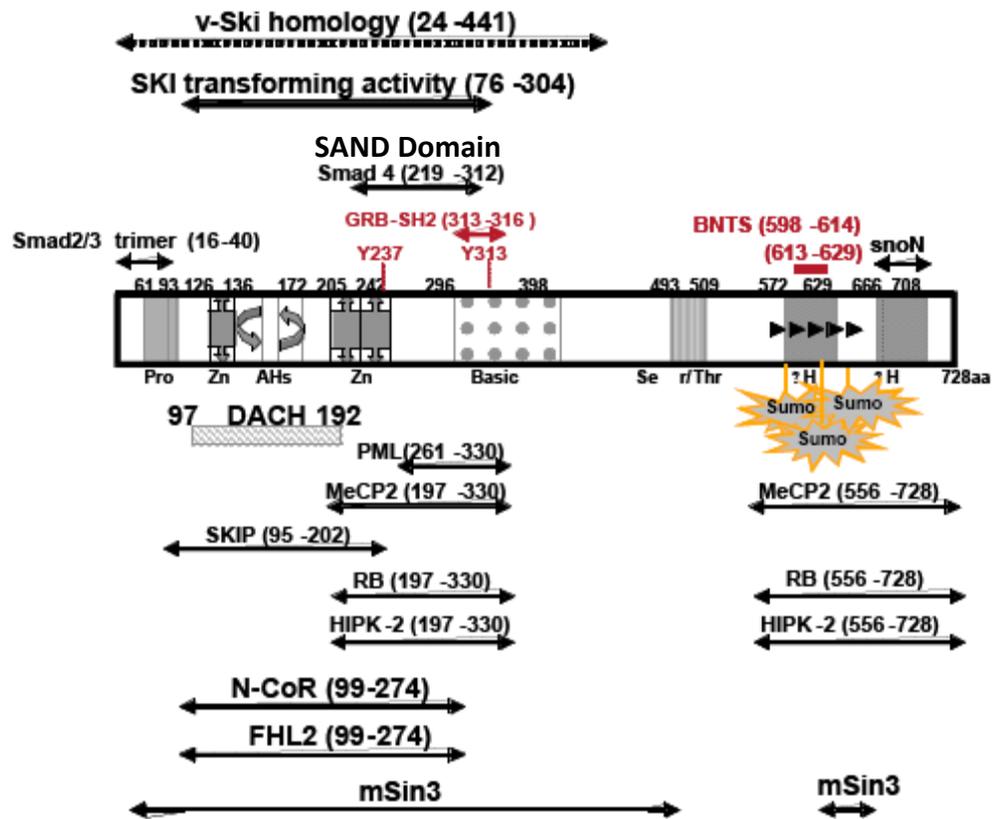


Figure 1: Ski domains and interactions [adapted from (Medrano, 2007)].

The overlapping 25 amino acid repeats and two alpha helical domains are located in the C-terminal domain of the protein and are thought to be responsible for the dimerization of Ski (Heyman and Stavnezer, 1994). In order to investigate this interaction a series of eight GST tagged deletion constructs were generated and expressed in cell lines. When chemically cross-linked proteins were immunoprecipitated, only the constructs that contained both the repeats and the alpha helical domains exhibited strong dimerization capability. Further biochemical studies including hydrodynamic, cross-linking, and circular dichroism support this model (Zheng et al., 1997a). Ski can also form heterodimers with SnoN, and SnoN can form homodimers, although this requires an upstream element in addition to the C-terminal domain (Cohen et al., 1999). This work, however, suggests that the Ski:SnoN heterodimer is more stable than either of the homodimers. They also demonstrate that the heterodimer will not only bind a GTCT element, but will also have a stronger transforming ability in chicken embryo fibroblasts than either of the Ski or SnoN homodimers. It should be noted that functions of Ski and SnoN other than DNA binding and transformation were not examined in these studies.

The *SKI* and *SKIL* genes are thought to be paralogs and therefore they share many of the same protein domains. There is only one *SKI/SKIL* gene in *Drosophila* and it has more homology with the human *SKIL* than the human *SKI* gene, suggesting that *SKIL* is the founding member of the family (Takaesu et al., 2006). The protein interacting domains of SnoN have not been mapped as extensively as these, but as can be seen

from the protein sequence comparison, much of the general homology is conserved (see Figure 2). This is also apparent in the similarities in function between the two proteins. However, one notable difference is a region at the N-terminus of SnoN that contains an NLS is missing in Ski (Krakowski et al., 2005). There are also gaps in SnoN compared to Ski in the alignment that probably affect interaction with other proteins leading to the slight difference in function seen between the proteins.



Figure 2: Graphical representation of Ski/SnoN protein alignment from NCBI BLAST 2 sequences. The blue sections are regions that are similar while the red regions are gaps in the similarities. (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>)

The function of Ski and SnoN can be modified by post-translation modifications including phosphorylation and sumoylation. Phosphorylation is a very common modification, yet is very diverse in the types of functions that it can regulate including enzyme activity, protein-protein interactions, and protein degradation (Lodish, 2008). Ski phosphorylation was first examined in the chicken homolog of Ski. Chicken embryo fibroblasts were transfected with plasmids containing chicken *ski* cDNA (Sutrave et al., 1990a). The cells were labeled with $^{32}\text{P}_i$ in order to identify phosphorylated proteins. Extracts from the transfected cells were immunoprecipitated (IP) with antisera generated against Ski peptides and the IP eluates were electrophoresed on an SDS-polyacrylamide gel. Proteins were extracted from the gel, hydrolyzed with hydrochloric acid, and analyzed on cellulose plates. The results indicated that chicken Ski is phosphorylated only on serine residues and not on threonine or tyrosine. The other

study of Ski phosphorylation was in the context of the cell cycle (Marcelain and Hayman, 2005). In this study the authors overexpressed a T7-tagged human Ski in the MG63 osteosarcoma cell line and then synchronized the cells with nocodazole. They collected protein extracts at different phases of the cell cycle and performed a Western blot showing that there are two different molecular weights of Ski. The larger band disappeared when the extracts were treated with a phosphatase, suggesting that this form is phosphorylated. When phosphatase inhibitors were added to the extracts and phosphatase, the larger band did not disappear. The prevalence of the larger band throughout the cell cycle differed in the two cell lines they examined. Further examination suggested that Ski was phosphorylated by either the Cdk1-cyclin B kinase complex, or by another kinase which itself is regulated by Cdk1. Neither of these studies examined the effect of phosphorylation on subcellular localization.

SnoN has also been shown to be modified by the small ubiquitin-like modifier (SUMO) (Hsu et al., 2006). This post-translational modification has been associated with the regulation of many cell processes including nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and progression through the cell cycle (Hay, 2005). Studies have shown that SnoN is sumoylated at two separate lysines and that the modification helps to regulate SnoN's function in transcription repression (Hsu et al., 2006; Wrighton et al., 2007). This has been shown to be promoter-specific and to strongly inhibit myogenin transcription (Hsu et al., 2006). Sumoylation of Ski has not been reported in the literature, but there is

evidence that it may occur. There are three putative sumoylation sites located within the tandem repeats near the carboxy terminus of Ski (Medrano, 2007). Sumoylation is reportedly required at one of these sites for protein stability (Medrano, 2007).

Smad-mediated TGF β signaling has been shown to induce Ski and SnoN protein degradation (Sun et al., 1999). Ski is stabilized during mitosis by Cdc34 but degraded during interphase, leading to a cell-cycle dependent regulatory process (Macdonald et al., 2004; Marcelain and Hayman, 2005). Degradation of SnoN by the proteasome is preceded by ubiquitination of SnoN by one of two different E3 ubiquitin ligases. Smurf2 was the first E3 shown to mediate the ubiquitination of SnoN through an interaction with Smad2 (Bonni et al., 2001). It was also shown that the anaphase promoting complex (APC) can ubiquitinate SnoN when recruited by Smad3 (Stroschein et al., 2001; Wan et al., 2001).

1.2 Genes/Gene Expression/Transcripts

The *SKI* gene was initially mapped to 1q22-q24 using Southern blot analysis to probe human spermatocyte pachytene and lymphocyte metaphase chromosomes with a *v-SKI* probe (Chaganti et al., 1986). It was later remapped to 1p36.3 using the radiation hybrid (RH) method (Shinagawa et al., 2001). Only one functional protein has been reported from the *SKI* gene in humans while the chicken version has been shown to have several splicing variants (Sutrave and Hughes, 1989). According to the Celera build, the *SKI* gene extends from 351748 to 432073 on the positive strand, effectively spanning 80,325 bases (NCBI Accession AC_000044.1). Intron 1 is unusually large consisting of

73,242 bases. The mature mRNA is 2187 bases long and contains eight exons. The 3'untranslated region (UTR) has several A-rich regions that can cause false-priming when using oligo(dT) to prime the reverse-transcriptase (RT) reaction. It has also been reported that the GC-rich region in the 5'UTR may cause processivity problems with the RT enzyme (Nomura et al., 1989). The mature mRNA may be much larger than has been reported. One report states that the 3'UTR is much longer, with a total mRNA length of more than 4000 bases (Grimes et al., 1992). The gene is highly conserved through different species, as can be seen in Table 1. Although over 3000 single nucleotide polymorphisms (SNPs) for *SKI* have been submitted to NCBI, there has been only one report examining SNPs in *SKI* which was in the context of the risk of orofacial clefting (Lu et al., 2005). They found one SNP in the first exon that was associated with a decreased risk of orofacial clefting.

Table 1: *SKI* conservation from HomoloGene at NCBI (<http://www.genecards.org/cgi-bin/carddisp.pl?gene=SKI>)

Gene		Identity (%)	
Species	Symbol	Protein	DNA
Homo sapiens	SKI		
vs. Canis lupus familiaris	SKI	93.4	89.8
vs. Bos taurus	SKI	90.2	89.3
vs. Mus musculus	Ski	93.4	88.8
vs. Gallus gallus	SKI	83.6	76
vs. Danio rerio	skia	73	72

According to Build 36.3 of the Human Genome Project, the *SKIL* gene is located from 9,907.9 to 9,909.43 on chromosome 3q26 (<http://www.ncbi.nlm.nih.gov/genome/guide/>). There is disagreement as to the size and exon content of the mRNAs from the *SKIL* gene. Like the *SKI* gene, the *SKIL* gene has

a GC-rich region in the 5'UTR that appears to complicate RT reactions. According to NCBI, the mature mRNA coding for SnoN is 3111 bases long, has seven exons, and the coding sequence extends from 672 to 2726. This version of the mRNA also has an intron within the 5'UTR. The mRNA coding for SnoI is alternatively spliced and contains a premature stop codon (Pearson-White, 1993). The mRNA for SnoA is also alternatively spliced. The *SKIL* gene is highly conserved throughout different species as can be seen in Table 2 (NCBI, 2008).

Table 2: *SKIL* conservation from HomoloGene at NCBI (<http://www.genecards.org/cgi-bin/carddisp.pl?gene=SKIL>)

Gene		Identity (%)	
Species	Symbol	Protein	DNA
Homo sapiens	SKIL		
vs. Pan troglodytes	SKIL	99.4	99.4
vs. Canis lupus familiaris	SKIL	91.5	90.5
vs. Bos taurus	SKIL	90.9	91.2
vs. Mus musculus	Skil	89	85.4
vs. Rattus norvegicus	Skil	89.7	85.5
vs. Gallus gallus	SKIL	76.2	75.3
vs. Danio rerio	LOC556403	48.3	52.2

SKIL has been found to be an immediate early serum response gene in quiescent fibroblasts (Pearson-White and Crittenden, 1997). *SKI* does not exhibit this response. *Ski* expression has been shown to be induced by phorbol 12-myristate 13-acetate (PMA) in myeloid cell lines (Namciu et al., 1994). *Ski* expression is also up-regulated in muscle differentiation (Ambrose et al., 1995; Namciu et al., 1995). *SnoN* expression is up-regulated upon serum deprivation in myoblasts, and upon serum stimulation in fibroblasts (Mimura et al., 1996; Pearson-White and Crittenden, 1997). *Ski* and *SnoN* are both up-regulated during liver regeneration, while they are down-regulated in the

fibrotic kidney (Macias-Silva et al., 2002; Yang et al., 2003). The up-regulation of Ski was correlated with the progression of esophageal squamous cell carcinoma while the up-regulation of SnoN was correlated with a negative prognosis in the same cancer (Akagi et al., 2008; Fukuchi et al., 2004). *SKI* gene amplification is a negative prognostic marker in early colorectal cancer (Buess et al., 2004). Both Ski and SnoN have been found to be up-regulated in melanoma (Boone et al., 2008; Chen et al., 2003; Fumagalli et al., 1993; Heider et al., 2007; Poser et al., 2005; Reed et al., 2001; Reed et al., 2005).

Ski and SnoN were found to be involved in various aspects of development and their gene expression patterns throughout development have been examined in lower animals. Data from studies of *Xenopus laevis* indicate that Ski is maternally regulated during early development and then widely expressed in adult tissues (Sleeman and Laskey, 1993). In mouse embryos Ski is expressed in all tissues with varying expression levels at different developmental stages (Lyons et al., 1994). Gene expression has also been performed in tissues using microarrays as can be seen in Figures 3 and 4 (Shmueli et al., 2003). These charts were created using the GCRMA method of gene expression quantification and normalization. The median expression of the samples examined is arbitrarily set at 7 and the rest of the transcripts are graphed relative to this value. This illustrates the relative expression of mRNA transcripts for both Ski and SnoN in normal and tumor tissue and that there is mRNA for both Ski and SnoN in all tissues examined. An interesting observation of these charts is that the relative mRNA levels of Ski and SnoN are very similar in the different tissues with the notable exception of skeletal

muscle where Ski has a higher than average mRNA level and SnoN has a lower than average mRNA level.

There are three other genes that have been found that are related to *SKI* and contain the Ski domain. They are *Dach1*, *Fusnel-15*, and *Fusnel-18* (Zhang and Stavnezer, 2008). The *Dach1* gene has been mapped to 13q22 and the mRNA is expressed predominately in adult kidney, heart and placenta with weaker expression in brain, lung, skeletal muscle, and pancreas (Kozmik et al., 1999). Expression studies in mice suggest that it may be involved in eye and limb development (Hammond et al., 1998). The protein consists of 706-amino acids and has a predicted molecular weight of 73 kDa (Kozmik et al., 1999). The *Fusnel-18* gene was mapped to 18q22.1 and encodes a 297-amino acid protein (Arndt et al., 2005). The mRNA is expressed only in neural tissue and the protein has been found to also inhibit TGF β signaling. The *Fusnel-15* gene was mapped to 15q23 and shares a high homology with the *Fusnel-18* gene, although it encodes a much larger, 921-amino acid, protein (Arndt et al., 2007). mRNA expression of *Fusnel-15* is detected in adult Purkinje cells of the cerebellum where the protein has been found to interact with Smad1, Smad2, and Smad3 protein, inhibiting bone morphogenic protein (BMP) signaling.

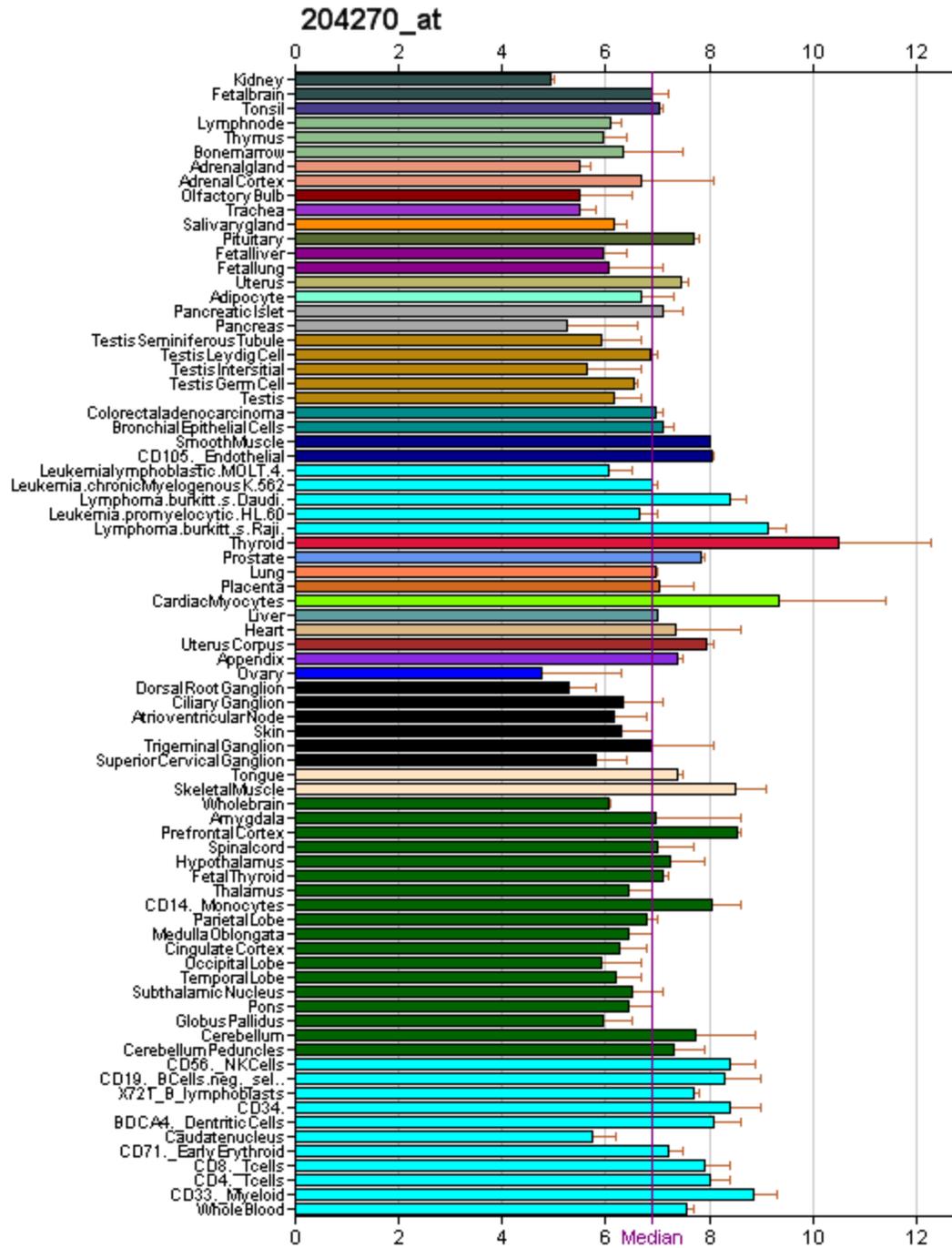


Figure 3: Ski gene expression as determined by Affymetrix gene chip 204270_at (<http://biogps.gnf.org/#goto=genereport&id=6497>). The horizontal axis indicates fluorescence intensity.

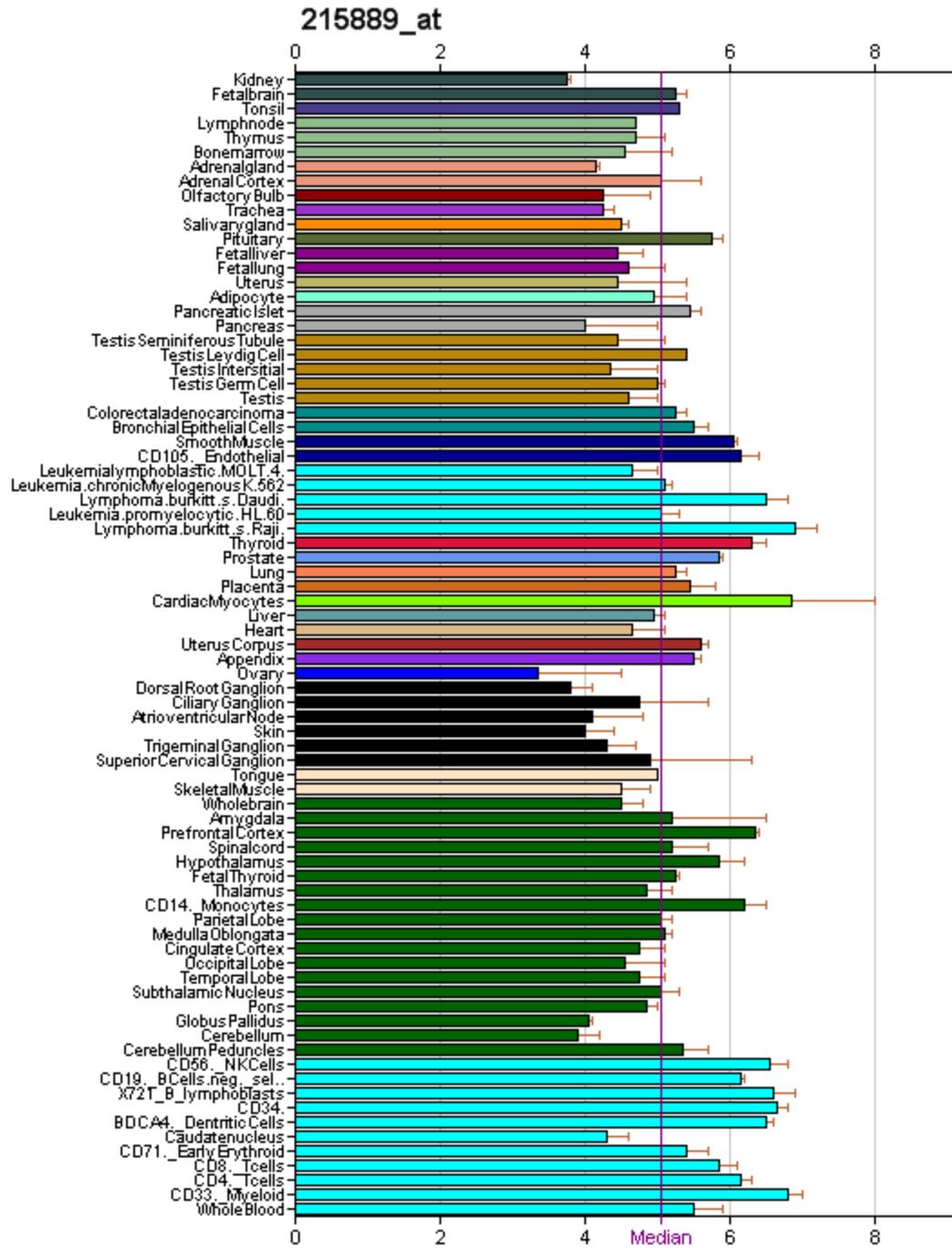


Figure 4: Sno gene expression as determined by Affymetrix gene chip 215889_at (<http://biogps.gnf.org/#goto=genereport&id=6498>). The horizontal axis indicates fluorescence intensity.

1.3 Function

1.3.1 Overview

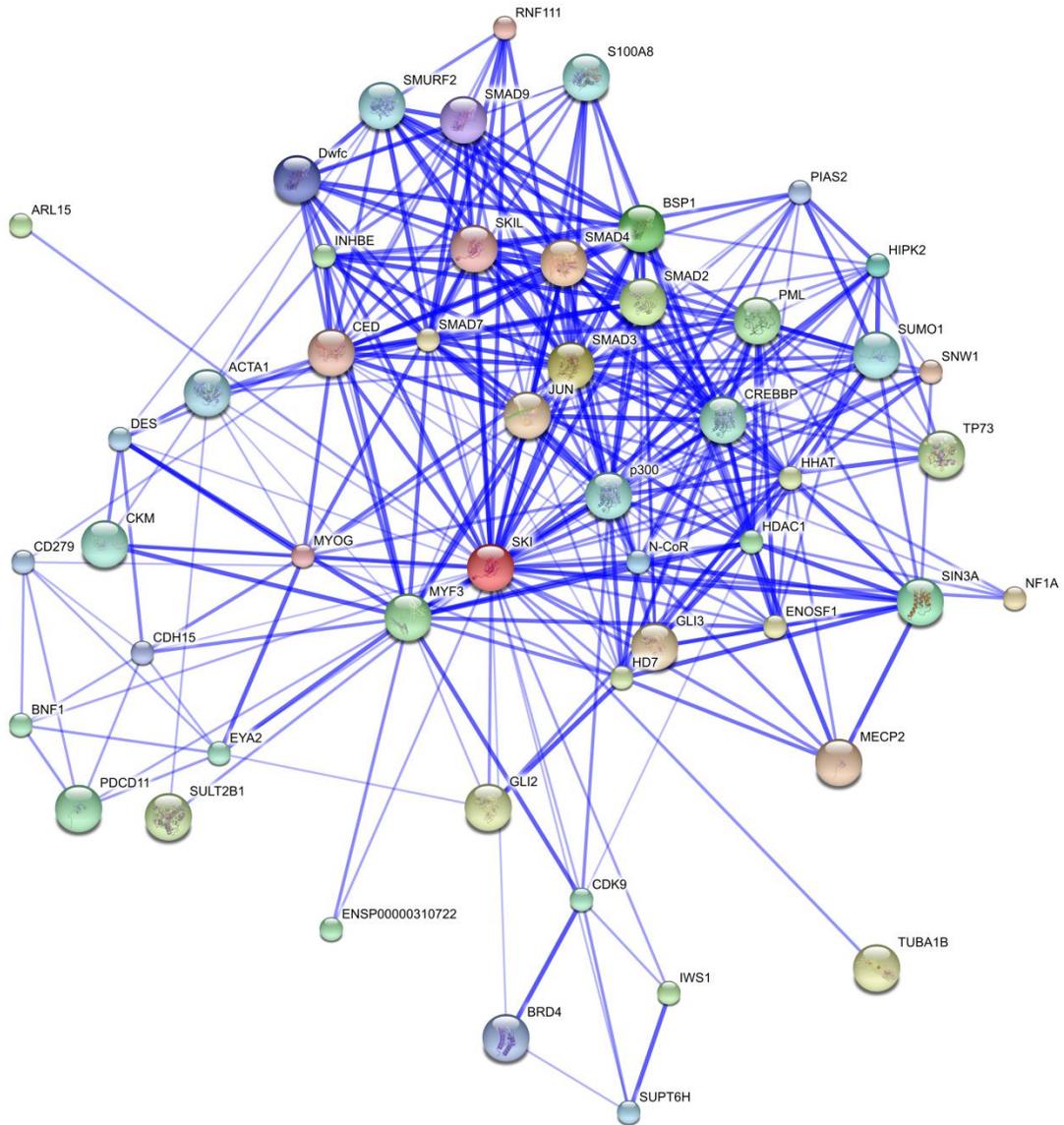


Figure 5: Protein interactions known and predicted between Ski and other proteins from the STRING 8.0 website (Jensen et al., 2008). Thicker connecting lines indicate a stronger probability of interaction.

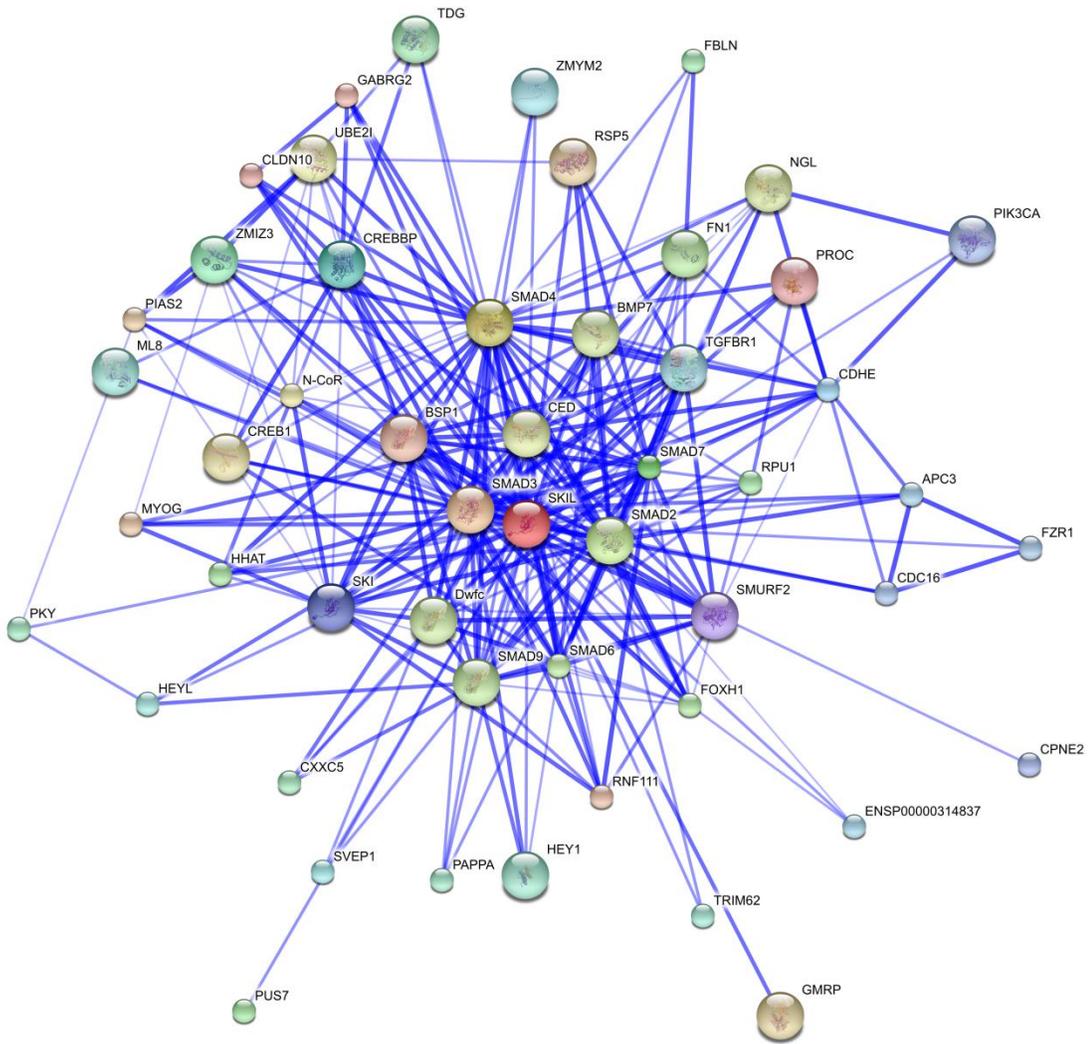


Figure 6: Protein interactions known and predicted between SnoN and other proteins from the STRING 8.0 website (Jensen et al., 2008). Thicker connecting lines indicate a stronger probability of interaction.

Some of the functions of Ski and SnoN can be inferred from the protein interaction diagrams (see Figures 5 and 6). The width of the lines between the proteins signifies the confidence of the interaction, with the wider lines indicating stronger evidence of interaction. The main interaction partners are the Smad proteins that mediate signaling from transforming growth factor beta (TGF β) receptors. This family of transmembrane receptors consists of TGF β 1 and 2, activin 1 and 2, and bone morphogenic protein (BMP) 1 and 2 (Massague, 2008). The ligands for these receptors consist of more than 30 members and include activin, nodal, lefty, myostatin, TGF β , BMP, anti-muellerian hormone (AMH, also known as MIS), and other growth and differentiation factors (GDF). The receptors are classified as either type I or type II, with the latter having a binding site for the corresponding ligand. Binding of the type II receptor with a compatible ligand results in receptor dimerization and subsequent phosphorylation of a type I receptor. Upon phosphorylation, the receptor dimer is now considered activated, and the activated receptor then phosphorylates receptor Smads (R-Smads), which then bind to a co-Smad and translocate into the nucleus. There are also inhibitor Smads (I-Smads) that compete with Co-Smad binding and recruit Smad ubiquitination regulatory factors (Smurfs) to the TGF β receptors and mark them for degradation. In the nucleus, the Smad complex binds with co-factors that regulate DNA binding specificity and function. The end result of the signaling pathway is context and cell type specific. It is also dependent on the co-factors expressed in the cell, resulting in cell growth, cell differentiation, apoptosis, and/or general homeostasis regulation (Luo, 2004).

Ski's ability to regulate Smad-mediated TGF β signaling involves the recruitment of histone deacetylase (HDAC) to Ski/Smad complexes on DNA (Akiyoshi et al., 1999). Ski also inhibits the interaction between Smad3 and transcriptional activator p300, blocking transcription initiation. The Ski/HDAC complex has been shown to also contain N-CoR/SMRT and mSin3A (Nomura et al., 1999). The v-Ski viral protein lacks the domain that interacts with mSin3A and therefore acts in a dominant negative manner in regulating Ski activity. There is also evidence that retinoblastoma protein (pRb) is in an inhibitory complex with Ski, Sin3A, and HDAC that blocks pRb mediated transcription (Tokitou et al., 1999).

The Ski interacting protein (Skip), which is also known as SNW domain containing 1 (SNW1), is a transcription factor that appears to have opposing function to Ski and SnoN in the Smad mediated TGF β signaling pathway (Leong et al., 2001). Skip's function appears to be related to its interaction with not only Ski and SnoN but also the Smad proteins (Dahl et al., 1998b; Prathapam et al., 2001). Another important interaction appears to be a complex formed between pRb, Skip, and Ski that represses pRb's transcriptional activity (Prathapam et al., 2002). This complex can also bypass the G1 and flat cell phenotype mediated by pRb.

1.3.2 Transformation

The Ski containing avian virus was first discovered while examining Sloan-Kettering viruses (SKVs) (Li et al., 1986). These are a group of transforming retroviruses that were isolated from chicken embryo cells that had been infected with the avian

leukosis virus transformation-defective Bratislava 77 (tdB77). A conserved sequence was found in several of the isolated viruses that had a cellular homolog in the chicken genome. This sequence was not thought to be related to any of the known oncogenes at the time, although later it was said to have limited sequence and structural similarities to *v-myc* (Stavnezer et al., 1989). Infection of chicken embryo cells with the viruses induced focus formation in monolayer culture and colony formation in soft agar. These results led to the classification of *Ski* as an oncogene. *SnoN* was also found to have transforming abilities, but only at high expression levels (Boyer et al., 1993). This ability of the *Ski* gene family encoded proteins to transform cells was subsequently found to be the result of their capacity to inhibit Smad-mediated TGF β signaling (He et al., 2003). This pathway has many diverse functions, including roles in regulation of epithelial and neural tissues, wound repair, and the immune system (Massague, 2008). In the context of cancer, the TGF β pathway is important because it contributes to the regulation of cellular proliferation, differentiation, survival, adhesion, and the cellular microenvironment. Ligands in the TGF β family bind to cell surface receptors in the TGF β family. This interaction leads to the activation of the receptors via dimerization and subsequent cross-phosphorylation of the receptors. The activated receptors then phosphorylate Receptor-Smads (R-Smads) which are anchored in close proximity by a Smad anchor for receptor activation (SARA). The activated R-Smad binds to a Co-Smad and translocates to the nucleus. In the nucleus the Smads bind to Smad binding elements (SBE) on DNA in a complex with other cofactors. The cofactors in the complex determine the end result of the signal. *Ski* and *SnoN* are cofactors that interact with R-

Smads (Smad 2 and Smad 3) and the Co-Smad (Smad 4) (He et al., 2003). This interaction has been shown to be responsible for their transforming activities.

1.3.3 Myogenesis

In addition to having transformation properties, Ski was also found to have myogenic properties that were first demonstrated in quail embryo cells (Colmenares and Stavnezer, 1989). Although Ski has potent myogenic activities, SnoN is only weakly myogenic in chicken embryo fibroblasts or quail embryo cells and requires high levels of expression to induce myogenesis (Boyer et al., 1993). Transgenic mice overexpressing Ski with a murine sarcoma virus (MSV) long terminal repeat (LTR) promoter have also indicated that Ski has a role in myogenesis (Sutrave et al., 1990b). This particular model system resulted in a selective hypertrophy of type II fast skeletal muscle fibers. The researchers also found an increase in muscle fiber diameter but not an increase in fiber number. This would indicate that Ski has a myogenic effect on the muscle rather than a mitogenic effect. The muscle fiber structure of transgenic mice overexpressing Ski was examined in detail and it was discovered that there was a reduction in the fraction of contractile material, poor intracellular alignment of Z-discs, and a concomitant reduction in contractile force per cross-sectional area (Bruusgaard et al., 2005). However, the promoter used to create the transgenic mice appears to have a large impact on the resultant phenotype (Sutrave et al., 2000). Transgenic mice with either a skeletal actin alpha (α_{sk} -actin) promoter or an albumin promoter controlling Ski failed to produce a muscular phenotype. A transgenic bovine was also generated using pronuclei

microinjection (Bowen et al., 1994). The single surviving transgenic calf was normal at birth but then developed muscular hypertrophy 8 weeks after birth and subsequently experienced muscle degeneration. At ten weeks of age the animal had periods of weakness to the point that it could not stand without support. At fifteen weeks the animal was euthanized for humane considerations. Histological examination of the animal revealed hypertrophy of most muscles. There were also a small number of degenerating fibers and mononuclear cells at the periphery of the fibers.

One of the mechanisms behind the myogenic activities of Ski and SnoN appears to be related to their functions as transcription factors. v-Ski was found to induce MyoD and myogenin expression and induce myotube formation (Colmenares et al., 1991). However, a mutated v-Ski that also induced MyoD and myogenin expression failed to induce myotube formation, indicating that another function is required for myotube formation. It has been found that Ski has the ability to upregulate myosin light chain 1/3 (MLC) and muscle creatine kinase (CK) gene transcription (Engert et al., 1995). Ski can also increase the gene transcription of the transcription factor myogenin (Ichikawa et al., 1997). The domain of Ski that is responsible for the regulation of myogenin was determined to be in exon 1 (Zheng et al., 1997b). This domain was found to form a globular structure and share a portion of its sequence with the domain that is responsible for transformation. Recent studies indicate that Ski's myogenic activities are independent of its interaction with the TGF β signaling pathway (Kobayashi et al., 2007). Proteins of the myoD family and the myocyte enhancer factor E (MEF2) family are also

believed to form a complex with Ski in order to repress myogenin mRNA transcription (Kobayashi et al., 2007). Ski's ability to suppress histone deacetylases (HDAC), specifically HDAC1, is also thought to be involved in the regulation of myogenin expression (Kobayashi et al., 2007). Another possible mechanism for the skeletal muscle hypertrophy is a low rate of proteolysis in the affected muscles. Ski overexpressing transgenic mice were found to have decreased expression and activity of proteolytic systems in fast twitch muscle compared to normal controls (Costelli et al., 2003). Protein synthesis does not appear to be affected in the transgenic mice.

Ski has also been implicated in skeletal muscle regeneration (Soeta et al., 2001). A mouse model of muscle injury was created by injecting mice with 500 μ L of hypertonic saline solution in the femoral muscle. mRNA levels of Ski increased three-fold, two days after injury and returned to normal by two weeks post-injury. This involvement may contribute to some of the symptoms that are seen in overexpressing Ski transgenic animals. As mentioned before, fiber degeneration occurs after the hypertrophic muscle phenotype presents. MyoD null mice have defective satellite cell differentiation so they are unable to produce new myoblasts in skeletal muscle (Megeny et al., 1996; Sabourin et al., 1999). When MyoD-null mice are used to generate Ski transgenics, the hypertrophic muscle phenotype is increased and there is also an increase in the degeneration of muscle fibers (Charge et al., 2002). This would indicate that satellite cells are able to repair the degeneration and that Ski may be involved in the process.

Less is known about SnoN and its involvement in myogenesis. SnoN is a much weaker myogenic factor than Ski and requires a much higher expression level to induce myogenesis (Boyer et al., 1993). Transgenic mice expressing SnoN under control of a simian virus 40 (SV40) enhancer/promoter displayed a variety of phenotypes (Kano et al., 1998). One of the mice had smaller soleus muscles and a decrease in type I skeletal muscle fiber compared to a normal mouse. This compares with the Ski transgenic mice that showed hypertrophy of the type II fibers. Another one of the mice had retarded growth. Recently it was discovered that SnoN is sumoylated at lysines 50 and 383 by Ubc9, PIAS1, and PIASx (Hsu et al., 2006; Wrighton et al., 2007). This post-translational modification has the greatest effect on SnoN's myogenic properties, specifically a decrease in myogenin transcription when sumoylation is blocked. There was little effect on the ability of SnoN to repress TGF β - induced signaling indicating that SnoN's myogenic properties, like Ski's, are likely separate from its ability to repress TGF β signaling.

Transcription levels of Ski and SnoN have also been shown to change during myogenesis. When C2C12 mouse myoblast cells are grown to confluence and serum deprived, mRNA levels of SnoN transiently increased 25 fold over basal levels as demonstrated by competitive RT-PCR (Mimura et al., 1996). The same increase in SnoN transcripts was seen in C3H10T 1/2 fibroblast cells, although they do not differentiate into myotubes. It is thought that the increase in SnoN may be necessary for entry into the G0 phase of the cell cycle.

1.3.4 Development

Not all of the functions of Ski and SnoN in development have been ascertained. Many knockout, knockdown, and overexpression studies have greatly increased our knowledge in this area. Much of this work has been performed in mice, although the different splice variants in mice compared to humans suggest disparate functions. Homozygous Ski knockout mice demonstrated an abnormal curvature of the spine, a 10% decrease in size, a flat, foreshortened snout, an abnormal jaw, and had prenatal lethality (Berk et al., 1997). 85% of the homozygous mouse pups showed exencephaly, abnormal posture, and an abnormal facial morphology. The other 15% also showed facial clefting defects that ranged from complete frontonasal clefting to mild clefting of the lip and nose. All of the homozygous knockouts died shortly after birth regardless of the severity of the defect. There was also a lower than expected number of homozygous pups, indicating an approximately 25% embryonic lethality from the knockout. A small percentage of the heterozygous knockouts had exencephaly or facial clefting. A decrease in skeletal muscle mass was seen in 50-60% of homozygous knockouts, while there was no loss of skeletal muscle mass in heterozygous knockouts. Histologically, the mice with reduced muscle mass had smaller diameter muscle fibers and more space between the fibers. Many of the fibers also appeared to be shorter and disorganized. Terminal differentiation did not appear to be affected, as mRNA expression of myogenic regulatory genes such as *myoD*, *myogenin*, *MRF-4*, and *p21^{Cip}* was not different in the homozygous knockouts compared to normal control littermates. The authors commented that the skeletal muscle phenotype is closest to a myogenin mutation

where secondary myogenesis is disrupted. However, they hypothesized that Ski may help to regulate the balance between proliferation, terminal differentiation, and survival of both skeletal muscle and neural lineages. The homozygous knockouts also had skeletal abnormalities including malformed and undersized or absent bones. The defects to the vertebrae represented a phenocopy of a Hox4 mutation. Another observation in this study was that there was increased apoptosis in the cranial mesenchyme and the cranial neural tube and that the timing of the apoptosis occurs at the same time as the neural tube should be closing in normal mice. Normal mice also showed high expression of Ski mRNA in these areas at this time in development.

A follow-up study examined the effect that the strain of mouse has on the phenotype of the Ski knockout (Colmenares et al., 2002). This study examined 129P2 and Swiss black backcrosses, and 129P2 and C57BL/6 backcrosses. Homozygous knockouts that were backcrossed on a 129 background were much more likely to have exencephaly than a facial cleft. Conversely, homozygous knockout mice backcrossed on a C57BL background were more likely to have a facial cleft than exencephaly. In both cases only a small percentage of the homozygous knockouts showed no craniofacial defects. The percentages of different phenotypes that were obtained from additional intercrosses indicate that a complex expression system consisting of several modifier genes was involved rather than a single modifier.

Another study looked at ocular abnormalities in a Ski knockout mouse (McGannon et al., 2006). They found that 100% of the homozygous negative mice had

persistent hyperplastic primary vitreous (PHPV). This is a defect in ocular development where fetal vitreous tissue and vasculature involute. The etiology of PGPV is not well defined, but there is some evidence that abnormal retinoic acid signaling may be involved. The mice also showed other eye abnormalities including anterior segment and lens dysgenesis, retinal folds, chorioretinal coloboma, and Peters anomaly. This phenotype resembles that of animals lacking retinoic acid receptor genes, or animals exposed to excess retinoic acid during gestation. The authors hypothesized that this phenotype may be due to Ski's interaction with retinoic acid receptors.

There is one Ski knockdown study where a novel vector was used to express a long double-stranded RNA from an RNA polymerase II promoter (Shinagawa and Ishii, 2003). This method generates transcripts that lack a 5'-cap structure and a 3'-poly(A) tail, facilitating transport to the cytoplasm and leading to a more robust siRNA response with decreased likelihood of an interferon response. Embryos from C57BL mice were used in this study and defects in neural tube closure and eye formation that are phenotypic of Ski homozygous knockouts were found in the mice.

A condition known as 1p36 monosomy is a rare disorder in humans where the end of one of chromosome 1 is missing. The missing region contains, among other genes, the *SKI* gene. This deletion syndrome is associated with cleft lip and/or cleft palate, large anterior fontanelle, hypotonia, moderate to severe mental retardation, seizures, growth delay, pointed chin, eye and vision problems, deep-set eyes, hearing deficits, low-set ears, clinodactyly, flat nasal bridge, abusive behavior,

and thickened ear helices. Specific eye and vision abnormalities found in 1p36 deletion syndrome are strabismus, sixth-nerve palsy, amblyopia, hyperopia, myopia, astigmatism, anomalous optic disks, and lacrimal defects. Unlike the mouse knockout studies, this condition is a heterozygous loss and is complicated by the loss of other genes. Nevertheless, some of the same symptoms that are present have been found in the mouse knockouts.

The developmental defects seen in the Ski knockout mice may be partially explained by Ski's interaction with the bone morphogenetic protein (BMP) pathway. BMP receptors are members of the TGF β family. They signal via Smad proteins and their signaling can be modified by Ski (Luo, 2003). Ski has been shown to modify the expression of two BMP responsive genes, *Vent1/2* is repressed and *NCAM* is induced by Ski overexpression (Wang et al., 2000). *Vent1/2* are important in ventral patterning in *Xenopus laevis* (Wang et al., 2000), while *NCAM* is a neural specific adhesion protein that is important in neurodevelopment (Paratcha and Ledda, 2008).

The expression pattern of Ski in mice shows correlations between the pattern and the proposed function of Ski (Lyons et al., 1994; Namciu et al., 1995). The first large increase in Ski expression occurs at the time of neural tube closure and migration of neural crest cells. The second large increase in Ski expression correlates with skeletal muscle development. Additional evidence of Ski's function in neural development was found when Ski mRNA was injected into endodermal cells in *Xenopus laevis* embryos, and an ectopic neural tube-like structure formed (Amaravadi et al., 1997). Cells derived

from the injected cells were found in the spinal cord. These data provide more supporting evidence for Ski's role in neural development.

SnoN also appears to be important in at least some developmental pathways. One line of SnoN homozygous knockout mice are embryonic lethal and die before implantation (Shinagawa et al., 2000). Analysis indicates that they fail to continue beyond the 16-cell stage and do not develop normal blastocysts. It is clear from this work and the work on Ski knockouts that Ski and SnoN have different functions in development. Heterozygous SnoN knockouts appeared healthy at birth, but displayed severe wasting after 4 months. These mice also developed spontaneous lymphomas and an increased frequency of tumors relative to normal mice when challenged with a carcinogen. There is a decreased sensitivity to apoptosis and cell cycle arrest in B cells, T cells, and fibroblasts of these mice as well. These data indicate that SnoN also acts as a tumor suppressor. Two other lines of SnoN homozygous knockout mice were found to be viable and only showed defects in T cell activation (Pearson-White and McDuffie, 2003). These defects could be overcome by incubation with either anti-TGF β antibodies or exogenous interleukin-2. It is not clear why there is a discrepancy between the two studies.

There is growing evidence that Ski also has a role in hematopoiesis (Beug et al., 1995; Dahl et al., 1998a; Larsen et al., 1993; Namciu et al., 1994; Pearson-White et al., 1995; Ueki et al., 2008). The first indication of this role was the ability of v-ski and either ts-v-sea or c-kit to transform myeloid cells that arise either from the monocyte and/or

granulocyte lineages or erythroid cells (Larsen et al., 1993). The cells did not display a transformed phenotype when v-ski was transfected into the cells without one of the other genes. This data led to studies examining the long-term effect of v-ski and c-kit expression in primary avian multipotent progenitors (Beug et al., 1995). The cells were immortalized and had a life span of over 100 generations. Further examination of the immortalized cells showed that Ski associates with the retinoic acid receptor (RAR) complex and can act as a repressor of a retinoic acid response element (Dahl et al., 1998a). Using high concentrations of RAR α -specific ligand they were able to abolish the v-ski mediated transformation of the multipotent progenitors. Ski mRNA expression was also found to be upregulated in myeloid cell lines that were exposed to phorbol 12-myristate 13-acetate (PMA) (Namciu et al., 1994). This study showed that Ski mRNA levels only increase in the cell lines that respond by differentiating along the megakaryocyte lineage and that the increase is proportional to the concentration of PMA used. An extensive study of both Ski and Sno mRNA expression was performed in hemolymphopoietic lineages (Pearson-White et al., 1995). The researchers found that the two proteins are differentially expressed with Sno being expressed more widely in myeloid progenitors and Ski being expressed in dual-lineage megakaryocyte/erythrocytes. Another protein that interacts with Ski is the transcription factor PU.1, which is a myeloid and B-cell specific E26 transformation specific (ETS) family member (Ueki et al., 2008). Ski recruits histone deacetylase 3 to PU.1-occupied promoters, blocking transcription activation and subsequent differentiation into macrophage colony-stimulating factor receptor (M-SCFR)-positive macrophages. It is

evident from these studies that the functions of Ski and SnoN in hematopoiesis are beginning to be elucidated. More work is still needed to clarify the distinct roles of each in this developmental process.

1.3.5 Cancer

Ski and SnoN were first posited to be involved in oncogenesis because of their transforming abilities in chicken embryo and quail embryo cells (Li et al., 1986). Originally, *SKI* was incorrectly mapped to the region of 1q22-q24 and implicated in several different cancers with chromosome abnormalities in that region. It has been reported that non-Hodgkin's lymphomas often have chromosomal translocations involving this region (Chaganti et al., 1986). *SKI* was also associated with Merkel cell carcinoma, in which 89% of the tumor samples examined showed chromosomal abnormalities (Koduru et al., 1989). Many of the samples had chromosomal rearrangements in the region of 1q22. Another chromosome 1 translocation that was found in a case of pre-B acute lymphoblastic leukemia (ALL) was thought to involve *SKI* because of the 1q23 mapping of the translocation (Kees et al., 1990). Finally, another study examined a ganglioneuroblastoma patient with a t(1;13)(q22;q12) translocation (Michalski et al., 1992). All of these studies only performed chromosome analysis and did not look at either gene expression or protein quantification. No mutations were found in the *SKI* gene in any of these studies.

Melanoma is the sixth most common cancer in the U.S. and has the worst prognosis of any of the skin cancers (Jemal et al., 2008). Ski was examined in six

melanoma cell lines and normal melanocytes. No mutations in the *SKI* gene were detected but Ski mRNA was expressed at a higher level in melanoma cell lines than in normal melanocytes (Fumagalli et al., 1993). Further inspection of the Ski protein in melanoma led to the finding that Ski is predominately nuclear in preinvasive melanomas, but present in both the nucleus and the cytoplasm of primary invasive and metastatic melanoma tumors (Reed et al., 2001). The importance of Ski in melanomas was then ascertained by using an antisense Ski vector to knock down the high endogenous Ski expression in melanoma cell lines, restoring TGF β mediated growth inhibition. Ski has been previously shown before to interact with activated Smad complexes in the nucleus and repress Smad mediated TGF β signaling (Luo et al., 1999). In melanoma, Ski interacts with Smad3 in the cytoplasm and prevents its nuclear transport. Blocking Smad nuclear transport effectively inhibits Smad-mediated TGF β signaling in the cytoplasm (Reed et al., 2001). These data suggest that the inhibition is mediated by the up-regulation of cyclin-dependent kinase inhibitor p21 (Waf-1) and inhibition of cyclin-dependent kinase 2 activity is due to high levels of Ski. It was then discovered that Ski activates the Wnt/beta catenin signaling pathway, presumably through its interaction with the transcription factor FHL2 (Chen et al., 2003). Gene targets of this interaction include microphthalmia-associated transcription factor and Nr-CAM. These proteins are associated with melanoma cell survival, growth, motility, and transformation.

SnoN has also been found to be highly expressed at both the mRNA and the protein levels in melanoma cell lines (Poser et al., 2005). However, only one of the cell lines examined was positive for Ski expression. A stable cell line was generated expressing an antisense SnoN vector in one of the Ski-negative cell lines. This cell line demonstrated reduced proliferation, indicating that high SnoN expression was making the cells unresponsive to TGF β mediated growth inhibition. Unlike Ski, data indicate that SnoN does not regulate p21 expression. However, a target gene of TGF β , the transcription factor Id1, is repressed by SnoN. These results indicate that, although both Ski and SnoN are involved in melanoma progression, they may exert this influence by different mechanisms.

There is growing evidence that Ski and SnoN may be involved in the progression of esophageal squamous cell carcinoma (ESCC). Ski protein levels in 80 surgical samples and 6 cell lines were examined by immunohistochemistry and Western blotting and compared to normal epithelium and an immortalized esophageal keratinocyte cell line (Fukuchi et al., 2004). Although SnoN expression was not significantly correlated with any of the clinicopathological characteristics of the patient samples, Ski showed significant correlation with depth of invasion, pathological stage, and TGF β expression. Five of the six cell lines had high Ski expression and concomitant low expression of p21, which was hypothesized to be caused by Ski's repression of TGF β signaling. Support for the involvement of SnoN in the progression of esophageal cancer was demonstrated in studies involving a growth inhibition-resistant esophageal cancer cell line. It was found

that SnoN forms complexes with Smad3 in the cell lines that lack TGF β signaling (Edmiston et al., 2005). This result can be explained by subsequent research that showed that the cell line that was used had lost the E3 ubiquitin ligase Arkadia (Levy et al., 2007). This protein marks SnoN for degradation only when Arkadia is in a complex with activated Smad2 or Smad3; thus, Arkadia downregulates SnoN upon Smad-mediated TGF β signaling. In the absence of Arkadia, SnoN represses c-myc transcription, preventing growth arrest and leading to oncogenesis.

A recent study examined SnoN protein levels by immunohistochemistry in 59 ESCC patient samples and found higher levels of SnoN in primary ESCC compared to corresponding normal epithelia (Akagi et al., 2008). The protein levels had significant correlation with depth of invasion and recurrence. In addition, 52.5% of the samples were positive for SnoN and the positive samples were correlated with more unfavorable outcomes. A common amplification in these samples at 3q26, the approximate chromosomal location of *SKIL*, is thought to be responsible for the high levels of SnoN in these cases.

Another recent study examined the protein levels of Ski and SnoN by immunohistochemistry in patients with Barrett's esophagus (BE) and found that, although neither protein was expressed in normal esophageal epithelium, both were strongly expressed in BE (Villanacci et al., 2008). The positive samples displayed cytoplasmic staining, a decrease of staining in dysplastic areas in patients with low-

grade dysplasia, and an absence of staining in patients with high-grade dysplasia or high-grade dysplasia/adenocarcinoma.

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by t(9;22), known as the Philadelphia Chromosome. This translocation produces the Bcr-Abl fusion protein and leads to excessive neutrophil production. In one study looking at Ski in CML, Ski was one of 158 genes that were significantly overexpressed in CML CD34(+) versus normal CD34(+) cells when examined using a cDNA array and verified with real-time RT-PCR (Kronenwett et al., 2005). The significance of this finding was discussed in the context of studies looking at Ski in hematopoiesis, where myeloid cells transformed with v-ski and c-kit were immortalized (Dahl et al., 1998a). The researchers propose that repression of the RAR signaling pathway by Ski may be responsible for the oncogenic progression and that Ski should be considered as a potential therapeutic target in CML.

In the United States, colorectal cancer is the third most common cancer among both men and women (SEER). Both incidence and mortality rates have been slowly decreasing over the last twenty years, with decreases of 2.6% and 4.4%, respectively. Ski was examined in the context of colorectal cancer and amplification of the SKI gene was found to be a negative prognostic marker in early colorectal cancer (Buess et al., 2004). However, deletion of neither of the *SKI* nor *SKIL* genes was correlated with cancer progression.

Rhabdomyosarcoma (RMS) is predominately a childhood cancer and the most common soft tissue cancer in children under 14, with an incidence rate of 4.6 cases per million (Loeb et al., 2008). RMS is classified into two major groups by histology, embryonal (eRMS) and alveolar (aRMS). eRMS histologically resembles immature skeletal muscle, while aRMS resembles normal lung parenchyma. The alveolar subtype has chromosome translocations in over 75% of cases, resulting in either t(2;13)(q35;q14) with the PAX 3/FKHR fusion protein or t(1;13)(p36;q14) with the PAX 7 /FKHR fusion protein (Loeb et al., 2008). The later translocation results in a break in the region of the *SKI* gene which is located at 1p36.3. Both varieties have been found to express muscle specific markers such as desmin and MyoD (Loeb et al., 2008). There is scant data concerning involvement of Ski and/or SnoN in RMS. An early study looking at Ski and SnoN expression in a variety of cell lines found that Ski mRNA is expressed in the eRMS cell line named RD (Colmenares and Stavnezer, 1990). Another paper reported the discovery of a novel SnoN splicing variant, SnoI, and the expression of its mRNA in RMS tissue (Pearson-White, 1993). The only other mention of Ski in RMS is from a study of *PATCHED1* mutant mice in which heterozygous knockouts develop medulloblastoma and RMS (Eichenmuller et al., 2007). These mice showed an increased level of transcription of a number of genes including Ski and TGF β . Although there has been little research into the involvement of Ski and SnoN in RMS, I believe that there may be a functional link. RMS is a cancer in which many of the cells express myogenic factors and structural skeletal muscle proteins. As both Ski and SnoN have been shown to have

involvement in not only a growing list of different cancers but also in myogenesis, it would seem likely that they would be involved in RMS.

The mechanism(s) of Ski's and SnoN's contribution to oncogenesis is not clear. As mentioned previously, some of the pathways involved are beginning to be deciphered. One of the interactions that may be important is the repression of the transcription factor c-Myb (Nomura et al., 2004). c-Myb has been associated with both myeloid leukemia and cisplatin resistance in colon cancer (Funato et al., 2001; Jahagirdar et al., 2001). Another protein-protein interaction that may be involved is the interaction of Ski with pRb. Overexpression of Ski has been shown to lead to hyperphosphorylation of pRb and cellular proliferation (Jacob et al., 2008). Ski and pRB are then colocalized in the cytoplasm. Overexpression of Ski and SnoN may also lead to low affinity interactions that would not otherwise occur and produce oncogenic effects. TGF β signaling suppresses the ability of Ski to inhibit tumor metastasis by inducing Ski's degradation through a complex formed with phospho-Smad2 or Smad3 and Arkadia (Le Scolan et al., 2008). Therefore, the loss of either TGF β signaling or Arkadia will inhibit Ski degradation and will lead to the deregulation of Ski. As mentioned in the context of melanoma, the interaction of Ski with the Wnt/ β -catenin signaling pathway may also prove to be important in tumor progression. Since the inactivation of this pathway is common in colorectal cancers, it was hypothesized that this may be a mechanism of Ski's function in colorectal cancer (Buess et al., 2004). Data also supports the function of Ski and SnoN as tumor suppressors. Both Ski and Sno heterozygous mouse knockouts have an increased

susceptibility to tumorigenesis (Shinagawa et al., 2000; Shinagawa et al., 2001). Based on the studies summarized above, it is apparent that Ski and SnoN have a role in oncogenesis; however, more data is needed to determine how they are involved and if they are a viable therapeutic target in specific cancers.

1.4 Hypothesis & Approaches

Ski and SnoN are important in several aspects of development including myogenesis. Knockouts of either gene led to developmental defects in heterozygous models and lethality in double knockouts. Animal models overexpressing Ski or SnoN demonstrate muscle hypertrophy and muscle weakness. Knockouts of these genes do not have myogenic defects, although it is possible that one can compensate for the loss of the other. It is also apparent that Ski and SnoN overexpression and/or mislocalization is found in many types of cancer. This information led me to investigate the mechanism of Ski localization and the possible involvement of Ski and SnoN in a cancer with skeletal muscle characteristics, RMS.

My first hypothesis is that Ski contains a functional nuclear localization signal (NLS) that is both necessary and sufficient for nuclear localization. Recent studies have shown that, in some cancers, Ski is located in the cytoplasm in addition to its normal nuclear location. Defining the NLS will aid in further research into this mislocalization. In order to obtain putative candidates for an NLS, we used computer algorithms to find likely sequences. I then used a combination of site-directed mutagenesis and restriction enzymes to create plasmids with green fluorescence protein (GFP) fused to various

mutants of Ski. These fusion proteins were expressed in a variety of cell lines and the subcellular localization of the fusion protein was monitored by fluorescence microscopy. I determined that when a putative NLS predicted by my computer analysis to be similar to the SV40 large T antigen was mutated by *in vitro* mutagenesis, Ski was located almost exclusively in the cytoplasm. I also discovered that when the non-mutated NLS is fused to GFP it is located mostly in the nucleus. However, the mutated NLS fused to GFP was found almost exclusively in the cytoplasm. These results show that this NLS is sufficient and necessary for Ski to localize to the nucleus.

My second hypothesis is that Ski and SnoN expression is prognostic in the pediatric cancer RMS. Because Ski and SnoN are involved in myogenesis, and RMS tumors exhibit skeletal muscle markers, I posit that they may be involved in the pathogenesis of RMS. We examined both gene expression by real-time RT-PCR and protein levels in cell lines derived from the RMS and in tumor tissue samples. I also used immunofluorescence microscopy to determine the quantity location of Ski and SnoN in the tumor samples. I found a large variation in the subcellular localization of Ski and SnoN in the cancer cell lines. I did not see any correlation between the localization of the proteins with the expression levels in the rhabdomyosarcoma cell lines. However, my data do show that Ski protein level in rhabdomyosarcoma tumor tissue is negatively correlated with tumor group.

My third hypothesis is that Ski phosphorylation is specific to its subcellular location. There are several reports of Ski being phosphorylated and it is believed that

this modification could be responsible for regulating subcellular localization. I used subcellular fractionation, immunoprecipitation, and Western blotting to determine the location of phosphorylated Ski. My data show that Ski is phosphorylated when it is localized to the cytoplasm, but Ski is unphosphorylated in the nucleus.

Chapter 2: Characterization of the Nuclear Localization Signal in Ski

2.1 Abstract

Ski is a transcription factor that acts as a co-repressor of the Smad-mediated transforming growth factor beta (TGF β) signaling pathway. Ski is usually found in the nucleus where it can recruit histone deacetylases (HDACs) and form complexes with activated Smad proteins on Smad binding elements within promoters on DNA. Data from Ski knockout and overexpression experiments indicate that Ski may have other functions as well. In metastatic melanoma, Ski is located in both the nucleus and the cytoplasm in contrast to its normal nuclear localization. Under the assumption that the mechanism of Ski subcellular localization is important in the progression of melanoma, we decided to look into how this is regulated. Our hypothesis is that the putative NLS is an SV-40 large T antigen-like sequence and that Ski localization is determined by phosphorylation state. We have found that Ski has a functional nuclear localization signal (NLS) that is sufficient and necessary for Ski to localize to the nucleus. This finding has been confirmed by another paper using a different mutagenesis strategy. We have also found that serine phosphorylated Ski is only found in the cytoplasm.

2.2 Background

Proteins are often localized to specific subcellular compartments to carry out their normal function. This localization often requires specific sequences that are recognized by transport proteins. An example is the nuclear localization of the simian

virus 40 (SV40) large T antigen via karyopherin proteins and the RAN GTPase system. The SV40 large T antigen contains a nuclear localization sequence or NLS (PKKKRKV) that has been shown to be necessary and sufficient for localization to the nucleus (Smith et al., 1985). This SV40 NLS or related sequences have been shown to interact with the karyopherin importin α (Yoneda et al., 1999). The SV40-bound importin α then binds to importin β , which facilitates translocation into the nucleus via interactions with nucleoporins within the nuclear pore complex. Another class of NLS contains a bipartite signal (Boulikas, 1993), which consists of two clusters of basic amino acids that are usually separated by a spacer of around 10 amino acids. Importin α also interacts with the bipartite NLS. We used an NLS prediction computer algorithm to search for a putative NLS in Ski. The algorithm identified both an SV40 large T antigen-like NLS and a bipartite signal. We investigated both of these and have determined that only the SV40-like NLS appears to be functional.

2.3 Methods

2.3.1 Antibodies

The antibody to SnoN was purchased from Cell Signaling Technology (cat. #4973, Danvers, MA) and used at dilution of 1:200. The phosphoserine antibody was purchased from Abcam (cat. #ab9332, Cambridge, MA) and used at a dilution of 1:500 for Western blots and 10 μ l per 80 μ l of cellular extract for IPs. The anti-Ski antibody is a rabbit polyclonal generated against the peptide CRVSSEPPASIRPKTDDTSS. Both the peptide and the antibody were purchased from New England Peptide (Gardner, MA). The

antibody was purified by affinity chromatography by the manufacturer. The antibody was used at a dilution of 1:250 for IF, 1:1000 for Western blots, and 10µl per 80 µl of cellular extract for IPs. Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA) and used at a dilution of 1:250 for IF and 1:5000 for Western blots.

2.3.2 Cell Culture and Transfection

HeLa or SJRH30 rhabdomyosarcoma cells were obtained from ATCC (Manassa, VA) and were cultured in DMEM (cat. #12430-104, Gibco, Carlsbad, CA) with 10% FBS (cat. #16140-071, Gibco, Carlsbad, CA) 100U/ml Penicillin and 100µg/ml Streptomycin (cat. #15140-122, Gibco, Carlsbad, CA). Low passage number cells were seeded on 22mm coverslips, allowed to attach and grow for 24hrs, and then transfected using Fugene6 (cat. #1814443, Roche, Basel, CN) according to the manufacturer's directions.

2.3.3 Mutagenesis and Plasmid Construction

SKI was cloned from HeLa cells into pGEM-T Easy (cat. #A1380, Promega, Madison, WI) using the following primers:

5'-AACTGGAGCATGGAGGCGGCGGCAGGCGGC-3'and

5'-AAGGATCCCGGCTCCAGCTCCGCAGCGCC-3'

The *SKI* gene was then cloned into the EGFP-N3 plasmid (cat. # 6080-1, Clontech, Palo Alto, CA) by digesting both plasmids with *Xho*I and *Bam*HI, gel purifying the fragments, and ligating the *Ski* fragment into the GFP plasmid.

The putative SV-40 large T antigen-like NLS of *SKI* was mutated by using PCR to mutate a lysine to an alanine in the Ski-GFP plasmid. The mutation was generated with the first round of PCR using the following primers pairs and Ski-GFP plasmid as the template:

5'-TGGCCGGCTCTTCCAATA-3'

5'-CTGGGTGCAAGTGGCGAGAGGCTCGGGGGCCCGGGAGAC-3'

and

5'-CTCGCCAATTGCACCCAGGTGGACACCCCAGGAGCCCCA-3'

5'-AGCTCCTCCACCACCTTCTC-3'

The two overlapping fragments were combined by using them as a template and amplifying with the flanking primers. The resulting full-size fragment was cloned into a Ski-GFP plasmid that had been digested with *EcoRI* enzyme to remove the corresponding unmutated sequence.

The Ski-GFP 1-490 was constructed by digesting the Ski-GFP plasmid with *XhoI* and *EcoRI* and gel purified the fragment. The EGFP-C2 (cat. # 6083-1, Clontech, Palo Alto, CA) plasmid was digested with *XhoI* and *EcoRI* and the Ski fragment (1-490) was ligated into the EGFP-C2 plasmid creating the Ski (1-490)-GFP plasmid.

To create the GFP-NLS-GFP and GFP-NLS-SDM-GFP constructs we digested EGFP-N3 with *NhoI* and *BamHI* removing the MSC from the plasmid. EGFP-C3 (cat. # 6082-1, Clontech, Palo Alto, CA) was digested with *XhoI* and *EcoRI* to prepare the plasmid for the NLS

sequence. Both fragments were gel purified. The following single stranded nucleotide sequences were designed for the NLS and NLS-SMD constructs:

5'-TCGAACCTCGGAAGCGGAAGCTGACTGTC-3'

5'-AATTGACAGTCAGCTTCCGCTTCCGACGT-3'

5'-TCGAACCTCGGAAGCGGGCCCTGACTGTC-3'

5'-AATTGACAGTCAGGGCCCGCTTCCGACGT-3'

These were used to construct GFP-NLS-GFP and GFP-NLS-SDM-GFP, respectively. Each pair was duplexed by combining them in equal molar amounts in STE buffer and heating them to 95°C for 10min. They were then allowed to cool to room temperature for 1 hr. A 5' phosphate was added to the duplex DNA by incubating the DNA in 2X rapid ligation buffer with T4PNK at 37°C for 1hr. The DNA was gel purified and ligated to EGFP-C3. The ligated plasmid was digested with *Nho*I and *Bam*HI and gel purified. This fragment was ligated into the digested EGFP-N3 plasmid which had been digested with the same restriction enzymes. The resultant plasmid was electroporated into JM109 cells and plasmid was purified using Qiagen Plasmid Maxi kit (cat. #12163, Qiagen, Valencia, CA).

All restriction enzymes were from New England Biolabs (Ipswich, MA) and all oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA).

2.3.4 Immunofluorescence

Cells were grown on 22mm coverslips until nearly confluent. Cells were then fixed in 4% formaldehyde in PBS for 20min and washed 3X in PBS. Cells were permeabilized in 0.4% Triton X-100 in PBS with 1% NGS at 4°C for 5min and washed 3X in PBS with 1% NGS.

The samples were then incubated for 1hr with primary antibody in PBS with 1% NGS and then washed 3X in PBS with 1% NGS. The coverslips were incubated in the appropriate fluorophore-conjugated secondary antibody for 1hr in PBS with 1% NGS, washed 4X in HEPES Buffered Saline, mounted with Vectashield containing DAPI (cat. #H-1200, Vector Laboratories, Burlingame, CA), and sealed with nail polish. Images were acquired with a CoolSNAP HQ camera (Photometrics, Tucson, AZ) on a Zeiss Axioplan2 (Thornwood, NY) using a Xenon light source and 63X (Plan-Apo, 1.4 NA) lens with Slidebook software Version 4.2 (Intelligent Imaging Innovations, Denver, CO).

2.3.5 Protein Extraction

Cells were grown to near confluence in 75cm² flasks and washed with PBS and detached using trypsin-EDTA. FBS was added to inactivate the trypsin and the cells were pelleted by centrifugation and washed with PBS. Cytoplasmic and nuclear proteins were obtained using the NE-PER (cat. #78833, Pierce, Rockford, IL) kit according to the manufacturer's instructions. HALT phosphatase inhibitor (cat. #78420, Pierce, Rockford, IL) and Complete Mini Protease Inhibitor Cocktail tablets (cat. #11836170001, Roche, Basel, CH) were used according to the manufacturer's directions.

2.3.6 Immunoprecipitation

40µl of EZview Red Protein A Affinity Gel (cat #P6486, Sigma, St. Louis, MO) was washed 3 times in the appropriate final nuclear or cytoplasmic buffer from the NE-PER kit. 80µl of protein extract (see previous section) was added to the washed beads to pre-clear the extracts of non-specific binding proteins, and incubated at 4°C for 1hr on a rocking

platform. The samples were centrifuged and the extract was transferred to a tube containing 10 μ l of either anti-Ski or anti-phosphoserine, and incubated at 4°C for 1hr on a rocking platform. Then 80 μ l of fresh washed EZview Gel was added to each sample and incubated at 4°C overnight on a rocking platform. The samples were centrifuged and the EZview Gel was washed 3X in the corresponding final buffer from the NE-PER kit. After the final wash, 100 μ l of Laemmli buffer (Bio-Rad, Hercules, CA) was added to the gel, the samples were vortexed, snap frozen in liquid nitrogen and stored at -80°C until needed.

2.3.7 Protein Electrophoresis and Western Blots

Protein samples from the IP were boiled for 10min in Laemmli buffer (Bio-Rad, Hercules, CA), 30 μ l was loaded per lane on a 7.5% Tris-HCl Criterion gel (cat. #345-0006, Bio-Rad, Hercules, CA) and then electrophoresed for 1 hour at 200V. The samples were transferred to a PVDF membrane (cat. #88518, Pierce, Rockford, IL) using a Criterion blot apparatus (Bio-Rad, Hercules, CA) with Towbin buffer containing 10% methanol at 100V for 1.25hrs using pre-chilled reagents and a gel ice block inside the blot apparatus. The blot was then blocked in TBS with 3% BSA (cat. #A2153, Sigma, St. Louis, MO) and 0.1% gelatin for 1hr. After washing the blot 3X in TBST (TBS with 0.1% Tween-20), it was incubated overnight in primary antibody in TBST with 0.5% BSA at 4°C. The blot was washed 3X in TBST and incubated 1hr in HRP-conjugated secondary antibody in TBST. The blot was washed 3X in TBST and Pierce ECL Western Blotting Substrate (cat. #32106, Pierce, Rockford, IL) applied according to the manufacturer's directions. The blots were

then exposed to BioMax MR film (Kodak, New Haven, CT) and developed in a film developer.

2.4 Results

2.4.1 GFP Studies

Various GFP-Ski fusion constructs were generated to examine the function of a putative NLS in Ski. For initial studies we created a Ski-GFP fusion construct using the Clontech Living Colors GFP plasmid, which is under control of the CMV promoter, and examined its location in different cell lines. The fusion protein was predominately nuclear in all of the cells, with none of the cells having the fusion protein excluded from the nucleus (see Figure 7, A & B). We next examined the construct that contained only the N-terminal 490 amino acids of Ski fused to GFP (Ski-GFP 1-490). This construct contains the putative SV40 NLS and was also predominately nuclear, although the punctate staining pattern in the nucleus was different than that of the full size Ski-GFP fusion protein (see Figure 7, C & D). The construct with the K454A mutation within the putative NLS (Ski-GFP K454A) demonstrated nuclear exclusion in most of the cells, indicating that the NLS was disrupted (see Figure 7, E & F). To confirm this result, we generated GFP fusion constructs using the NLS fused to 2 copies of GFP to ensure that the resulting fusion protein was large enough to prevent easy diffusion out of the nucleus. The distribution of the GFP-NLS-GFP fusion protein was similar to that of the Ski-GFP fusion protein, localizing predominately to the nucleus (see Figure 8, C & D).

However, an NLS that had the K454A mutation fused to two copies of GFP (GFP-NLS-SDM-GFP) localized predominately to the cytoplasm (see Figure 8, E & F).

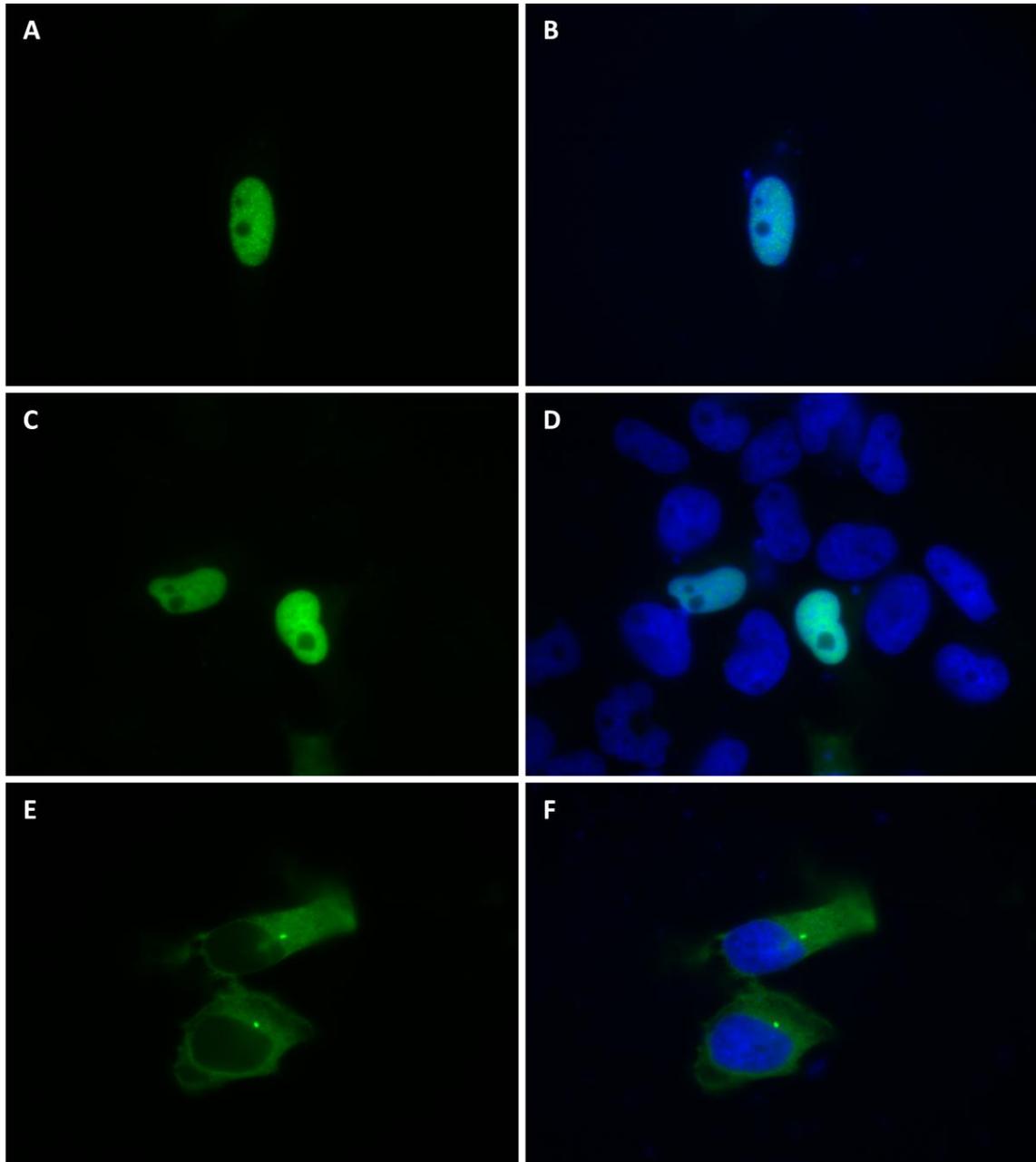


Figure 7: Representative images of the Ski-GFP fusion protein, alone on the left and with a DAPI overlay on the right. A & B are full-length Ski-GFP. C & D are Ski (1-490)-GFP containing the N-terminal 490 amino acids and the SV40-like NLS. E & F are Ski-SDM-GFP with the full-length Ski and a site-directed mutation in the SV40-like NLS. All images shown are SJRH30 cells.

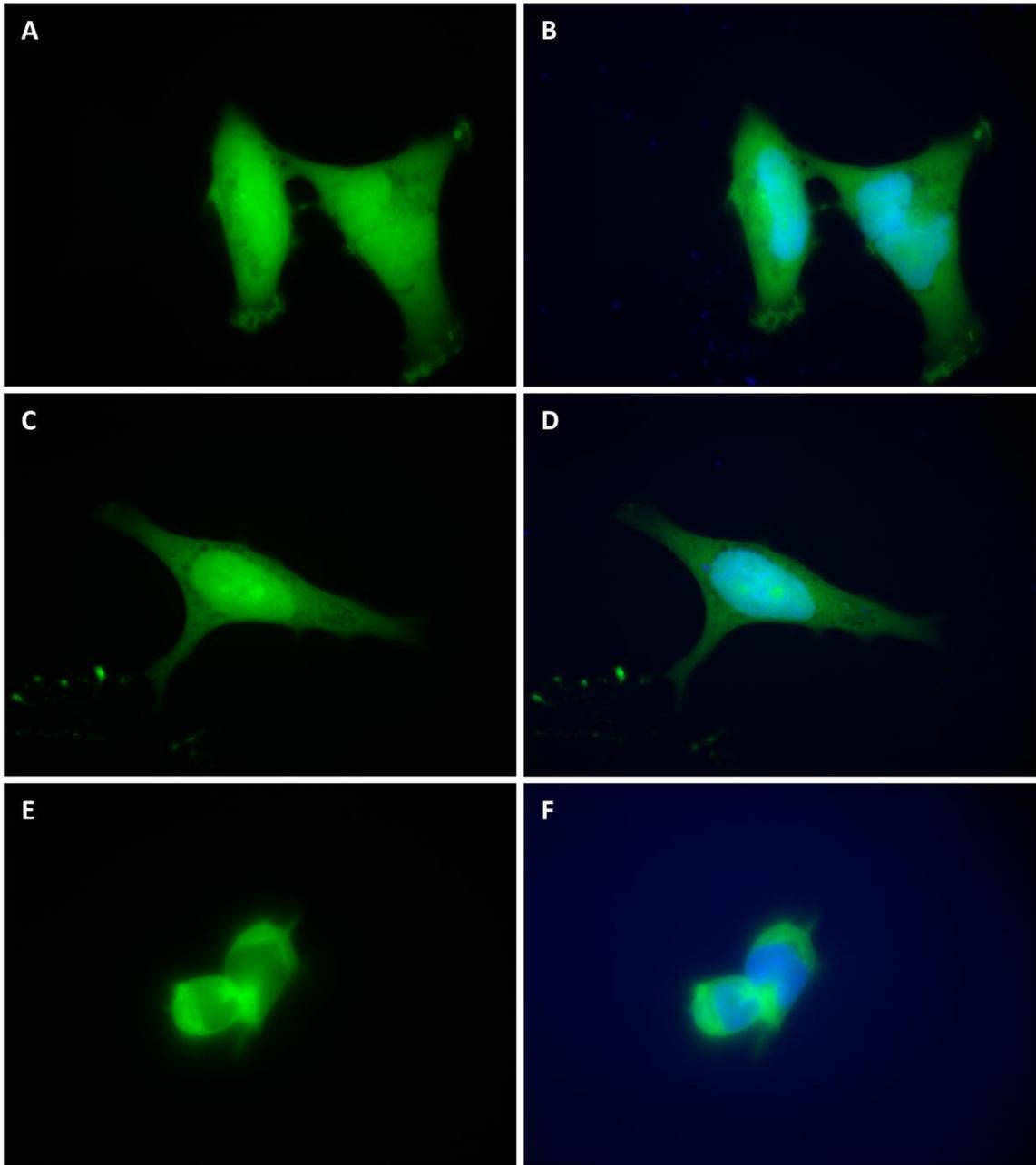


Figure 8: Representative images of the GFP fusion protein, alone on the left and with a DAPI overlay on the right. A & B are GFP-GFP. C & D are GFP-NLS-GFP containing the SV40-like NLS from Ski. E & F are GFP-NLS-SDM-GFP containing the SV40-like NLS from Ski with a site-directed mutation. All images shown are SJRH30 cells.

2.4.2 Phosphorylation Studies

We next examined the phosphorylation status of nuclear and cytoplasmic Ski. We obtained nuclear and cytoplasmic fractions of the SJRH30 rhabdomyosarcoma cell line, immunoprecipitated with an anti-Ski antibody, and electrophoresed the resultant protein fractions. After transfer to a PVDF membrane, the membrane was probed with an antibody against phosphorylated serine. This produced a band in the cytoplasmic fraction with an approximate molecular weight of 98kDa (Figure 9). The blot was stripped and reprobed with an anti-Ski antibody and the same cytoplasmic band was detected. To confirm this result we repeated the experiment using the antibody to phosphorylated serine for the IP and the antibody to Ski to probe the blot. We found the same molecular weight band in the cytoplasmic fraction. A control experiment for assessing non-specific interactions was carried out using rabbit Ig G in the IP step and anti-Ski antibody for the Western blot. No bands were detected in either the nuclear or the cytoplasmic IP fractions (Figure 10).

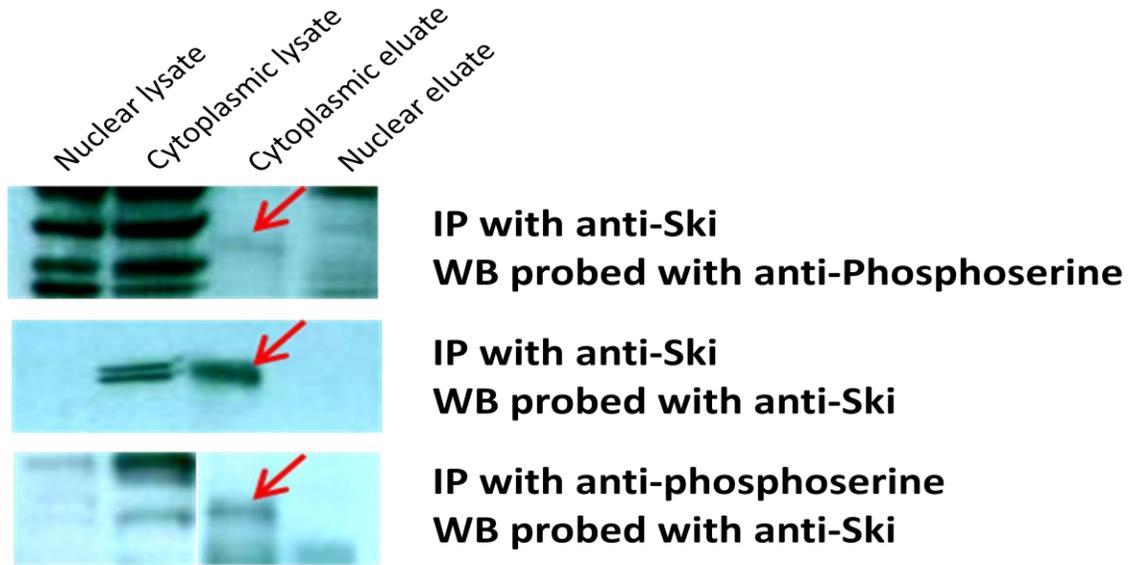


Figure 9: Western blots of lysates and IP eluates. The red arrows indicate the phospho-Ski band. The data indicate that Ski is phosphorelated in the cytoplasm but not in the nucleus.

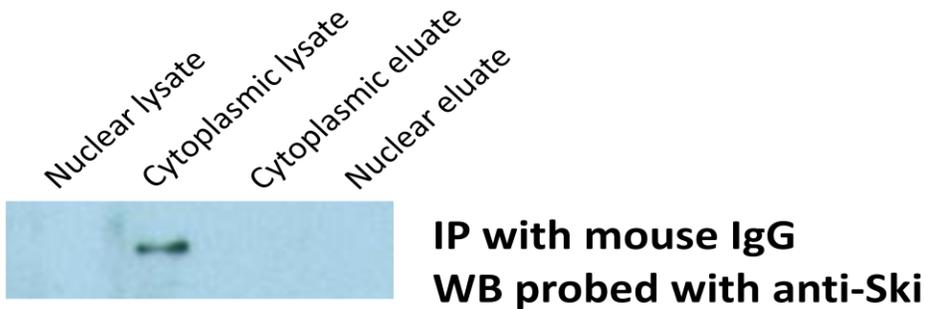


Figure 10: Control Western Blot with Mouse IgG.

<i>Homo sapiens</i>	APPAQQKVVSSPPCAA AVSRAPEPLATCTQPRKRKLTVDTPGAPETLAPVAA
<i>Equus caballus</i>	APPAQQKVVSSPPCATVVSRAPEPLATCVQPRKRKLAVDTPGAPETPVPVAA
<i>Mus musculus</i>	APPTQQKVVNSPPCTTVVSRAPEPLTTCIQPRKRKLTLDTAGAPDMLTPVAA
<i>Rattus norvegicus</i>	APPTQQKVVNSPPCTTVVSRAPEPLTTCIQPRKRKLTMDAPGAPDMLTPVAA
<i>Canis lupis familiaris</i>	APPAQQKVVSSPPCATVVSRAPEPLATCLQPRKRKLTVDTPGAPETPAPAAA
<i>Macaca mulatta</i>	APPAQQKVVSSPPCTAAVSRAPPEPLATCTQPRKRKLTVDTPGAPETLAPVAA
<i>Bos Taurus</i>	APPAQHKAVSSPPCATVVSRAPEPLPACIQPRKRKLPA DTPGAPETPAPGPA
<i>Gallus gallus</i>	APPAQQKVVSNPPCATVVSRSSEPPSSAAQPRKRKHAAETPAVPEPVATVTA
<i>Monodelphis domestica</i>	APPPQ-KIVSSPPCTTMVARAPEPLPTSIQPRKRKLPMETLGPVETLVPAAA
<i>Xenopus laevis</i>	TPPVQQKVITSPPCV PALPRSTQSSGSPPOSKRRRPTAELPIVPEAPAPPAP

Figure 11: Protein alignment of Ski in various species from Kalign at EMBL-EBI (<http://www.ebi.ac.uk/Tools/kalign/index.html>). The NLS is colored red and the mutated arginine is colored green.

2.5 Discussion

Our data support the hypothesis that the SV40 large T antigen-like Ski NLS is necessary and sufficient for nuclear localization of Ski. This sequence is highly conserved through evolution as can be seen in Figure 11. Insects have only one ortholog to Ski and SnoN and this insect ortholog has less homology to either the mammalian Ski or SnoN proteins than they have to each other (da Graca et al., 2004). The *Drosophila melanogaster* SnoN protein does not contain the SV40-like NLS that we found in the human Ski. This NLS is also not found in the human SnoN protein. In fact, an NLS was recently discovered in the N-terminus of SnoN that is not conserved in the Ski protein (Krakowski et al., 2005).

The data from the phosphorylation experiments suggest that phosphorylation may also be important in Ski subcellular localization. Previous research suggest that Ski is phosphorylated at certain times during the cell cycle, although the specific phosphorylation status appeared to vary in different cell lines (Marcelain and Hayman, 2005). This research also indicates that cdc2 (Cdk1) can phosphorylate Ski *in vitro*. In the rhabdomyosarcoma cell line that we used, Ski was localized to both the nucleus and the cytoplasm. This localization pattern is also found in other types of cancer such as metastatic melanoma and esophageal squamous cell carcinoma (Fukuchi et al., 2004; Reed et al., 2001). Cyclin-dependent kinases (Cdks) have been found to be frequently mutated in cancers (Malumbres et al., 2003). It would be plausible that the cytoplasmic localization of Ski is the result of a Cdk mutation or deregulation leading to

phosphorylation of Ski and its subsequent cytoplasmic localization. Future studies will be directed at determining the phosphorylation sites on Ski and their functions.

Chapter 3: Protein Expression and Subcellular Localization of the Oncoprotein Ski in Rhabdomyosarcoma

3.1 Abstract

Ski is a transcription factor that acts as a co-repressor of the Smad-mediated TGF β signaling pathway through its interaction with Smad complexes and histone deacetylases. It has been reported that this normally nuclear protein is localized to both the nucleus and the cytoplasm in metastatic melanoma and in esophageal squamous cell carcinomas. In metastatic melanoma cell lines, Ski has been found to sequester activated Smads while in the cytoplasm, preventing them from translocating to the nucleus. Ski has been implicated in myogenesis but has not been studied in rhabdomyosarcoma (RMS), which is a cancer with characteristics of skeletal muscle. We examined the location of Ski in several different cancer cell lines, in RMS tissue samples, and in differentiating myoblasts. The RMS tumor group was found to be inversely correlated with Ski protein intensity in the tissue samples. We also found a slight shift towards Ski cytoplasmic localization in the differentiated myotubes, with discrete areas of intense Ski staining. Fluorescence resonance energy transfer (FRET) was performed to examine the co-localization and intracellular interaction of Ski with the Ski family member SnoN. We detected FRET between Ski and SnoN in both the nucleus and the cytoplasm.

3.2 Introduction

The chicken homolog of Ski was the focus of early studies since the group at Sloan Kettering Institute that discovered Ski was working with avian viruses and chicken embryos (Barkas et al., 1986; Sutrave and Hughes, 1989). These studies found that when the embryos were transfected with retroviruses expressing chicken Ski, the protein was located in a speckled pattern in the nucleus while being excluded from the nucleolus (Sutrave and Hughes, 1989). Ski was also found to associate with condensed chromatin in mitotic cells. The idea of Ski being a nuclear protein was further reinforced when Ski was found to act as a co-repressor of Smad-mediated TGF β signaling by forming complexes on DNA (Akiyoshi et al., 1999). Ski's association with melanoma was uncovered when Ski was found to be predominately nuclear in preinvasive melanomas, but in both the nucleus and the cytoplasm of primary invasive and metastatic melanoma (Reed et al., 2001). In addition, Ski has been found to be cytoplasmic in esophageal squamous cell carcinoma (Fukuchi et al., 2004). Cytoplasmic Ski has subsequently been found to be functional, sequestering activated Smad complexes in the cytoplasm, which inhibits Smad-mediated TGF β signaling (Reed et al., 2001). There is evidence that Ski interacts with hyperphosphorylated pRb in the cytoplasm of Schwann cells and influences cell proliferation (Jacob et al., 2008). RMS is a pediatric cancer with characteristics of skeletal muscle. Early research showed that *SKI* and *SKIL* are expressed in RMS tissue but their proteins have not been studied in this context. Ski and SnoN increase the transcription of myogenin promoting skeletal muscle myogenesis so we

thought that this would be an interesting cancer to examine (Deheuninck and Luo, 2009).

The Ski and SnoN proteins interact forming heterodimers and trimers that are thought to be important for transcription regulation. This interaction was determined when researchers immunoprecipitated them together from reticulocyte lysates in which they had been overexpressed together (Nagase et al., 1993). These experiments were done before Ski was found to be mislocalized in some cancers and so the subcellular location of this interaction was not examined. We hypothesized that Ski and SnoN interact in the cytoplasm when they are both in that subcellular location as seen in some cancers. We used fluorescence resonance energy transfer (FRET) microscopy to determine if Ski and SnoN interact in the nucleus or the cytoplasm.

FRET is a very sensitive tool that is useful in measuring protein-protein interactions when the interacting proteins are within 10nm of each other. The more important criteria are that the fluorophores involved should be within 10nm and in the correct orientation to allow dipole-dipole coupling (Kenworthy, 2001). One method to measure FRET is by directly exciting the donor fluorophore and measuring the fluorescence emitted from the acceptor fluorophore. This method requires specific FRET filter sets for the fluorescence microscope as well as software for acquisition and mathematical FRET determination. We chose to use the acceptor photobleaching method where images of the donor and acceptor are acquired before and after photobleaching the acceptor (Kenworthy, 2001). The FRET is determined by measuring

the increase in fluorescence of the donor. This occurs because energy transfer from the donor to the acceptor that occurs in FRET results in quenching of the donor fluorescence. When the acceptor is photobleached, the quenching is halted and the energy from the donor that had been exciting the acceptor is now visible fluorescence. The general equation to determine FRET efficiency is:

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

where E is the efficiency, r is the distance between the donor and acceptor, and R₀ is the distance between the donor and acceptor where FRET efficiency is 50% (Kenworthy, 2001). The equation used to determine the ratio of FRET in acceptor photobleaching is:

$$\%FRET = \left(\frac{I_{Donor\ PostBleaching} - I_{Donor\ PreBleaching}}{I_{Donor\ PostBleaching}} \right) \times 100\%$$

The variable “I” represents the individual pixel intensity (Stepensky, 2007). Although most FRET experiments are performed by expressing fluorescently tagged proteins such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) as the donor and acceptor fluorophores, there have been reports of FRET experiments using endogenous proteins labeled with Cy3 and Cy5-conjugated antibodies (Kenworthy, 2001). Our preliminary studies expressing Ski-GFP proteins in cell lines indicated that the fusion protein did not match the endogenous localization, with the fusion protein being mostly nuclear in cell lines that had cytoplasmic expression of endogenous Ski. In order to obtain what we believe to be the most accurate *in vivo* result we decided to use

antibodies to visualize interactions between the endogenous proteins instead of GFP fusion proteins.

Ski and SnoN were close enough to produce FRET in both compartments although we detected a higher prevalence of FRET in the cytoplasm than in the nucleus. This would mean that they are within 10nm of each other and in an orientation that would allow for efficient FRET. We also looked at Ski protein level and localization in cancer cell lines, rhabdomyosarcoma (RMS) tumor tissue, and differentiating myoblasts to determine the localization of Ski in these samples and found that the localization varied among the cell lines and increased slightly during myogenesis. The data with the RMS tumor tissue indicate that Ski protein level has a significant negative correlation with RMS tumor group.

3.3 Methods

3.3.1 Antibodies

The antibody to SnoN (#4973) was purchased from Cell Signaling Technology (Beverly, MA) and used at a dilution of 1:100 for paraffin sections and 1:200 for cell lines. The antibody to Ski is a rabbit polyclonal generated against the peptide CRVSSEPPASIRPKTDDTSS. Both the peptide and the antibody were custom made by New England Peptide (Gardner, MA). The antibody was used at a dilution of 1:250 in cell lines. For FRET studies the Ski antibody was covalently linked to Cy5 using the Amersham (Chalfont St. Giles, UK) Cy5 antibody labeling kit (PA3500). In paraffin sections the antibody was used at a dilution of 1:100 and in cell lines it was used at a

dilution of 1:150. Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA) and used at a dilution of 1:250.

3.3.2 Cell lines and Cell Culture

HeLa, SJRH30, RD, HS729t, A204, and A673 cell lines were purchased from ATCC (Manassas, VA). RMZ-RC2 cells were kindly provided by Drs. Lorena Landuzzi and Pier-Luigi Lollini at the Laboratory of Immunology and Biology of Metastasis, Cancer Research Section, Department of Experimental Pathology in Bologna Italy. MCF-7 and MDA-MB-231 cells were kindly provided by Dr. Rebecca Hartley at the UNM HSC. LNCaP cells were kindly provided by Dr. Marco Bisoffi at the UNM HSC. HSMM cells were purchased from Cambrex (Walkersville, MD).

HeLa, SJRH30, RMZ-RC2, RD, HS729t, A204, A673 LNCaP, and MCF-7 cells were cultured in DMEM (cat. #12430-104, Gibco, Carlsbad, CA) with 10% FBS (cat. #16140-071, Gibco) and 100U/ml Penicillin and 100µg/ml Streptomycin (cat. #15140-122, Gibco). Clonetics HSMM cells were grown in Sk-GM-2 media (cat. #CC-3245, Cambrex, Walkersville, MD). HSMM cells were differentiated in DMEM with 2% horse serum (cat. #26050-070, Gibco) and 100U/ml Penicillin and 100µg/ml Streptomycin. MDA-MB-231 cells were cultured in DMEM/F12 (cat. #11320-082, Gibco) with 10% FBS and 100U/ml Penicillin and 100µg/ml Streptomycin. Low passage number cells of each cell line were seeded on #1.5 22mm coverslips, allowed to attach and grown until nearly confluent.

3.3.3 Tumor Tissue

The RMS tumor samples were acquired from the Cooperative Human Tissue Network (CHTN) at the National Cancer Institute by Dr. Stuart Winter. Coding information was provided by Dr. James Anderson. Samples were obtained from 18 children and young adults who were diagnosed with rhabdomyosarcoma and consented to have their tumor samples stored in the Children's Oncology Group (COG) Cell Bank (Columbus, OH). The patients then received treatment on COG studies D9502 (n = 1), D9602 (n = 7), D9802 (n = 1), or D9803 (n = 9), depending on the treatment era at the time of diagnosis. Molecular and protein findings were compared with the original histological diagnoses, annotated patient demographic features, staging, grouping and event free survival associated with the treatment studies.

3.3.4 Immunofluorescence

Cells were grown on #1.5 22mm coverslips until nearly confluent. Cells were then fixed in 4% formaldehyde in PBS for 20min and washed 3X in PBS. Cells were permeabilized in 0.4% Triton X-100 in PBS with 1% NGS at 4°C for 5min and then washed 3X in PBS with 1% NGS. Cells were incubated for 1hr with primary antibody in PBS with 1% NGS and then washed 3X in PBS with 1% NGS. Cells were incubated in the appropriate fluorophore-conjugated secondary antibody for 1hr in PBS with 1% NGS. Cells were washed 4X in HEPES buffered saline and then mounted with Vectashield containing DAPI (cat. # H-1200, Vector Laboratories, Burlingame, CA) and sealed with nail polish. Cell

images were acquired with a CoolSNAP HQ (Photometrics, Tuscon, AZ) on a Zeiss Axioplan2 (Thornwood, NY) using a Xenon light source and 63X (Plan-Apo, 1.4NA) lens with Slidebook software Version 4.2 (Intelligent Imaging Innovations, Denver, CO).

Paraffin-embedded rhabdomyosarcoma tissue samples were obtained and mounted on slides. The samples were deparaffinized and rehydrated by taking the slides through two changes of each of the following baths: Citrisolve, 100% ethanol, 95% ethanol, 80% ethanol, 70% ethanol, dH₂O, and PBS. Antigen retrieval was performed by placing the slides into pre-warmed 10mM sodium citrate buffer and keeping them at 97°C for 10min. The slides were allowed to cool at room temperature for 30min and then were washed 3X for 5min each in dH₂O and 3X for 5min each in PBS. Samples were then blocked in PBS with 0.3% Triton X-100 and 5% NGS for 1hr. Blocking buffer was replaced with primary antibody diluted in PBS with 0.3% Triton X-100. The samples were incubated in primary antibody overnight at 4°C. Samples were then washed 3X in PBS with 0.1% Tween-20 and incubated in secondary antibody diluted in PBS with 0.3% Triton X-100. Samples were washed 3X in PBS with 0.1% Tween-20 and counterstained with DAPI for 5min. The slides were washed 4X in HEPES buffered saline, mounted with Mowiol, and sealed with nail polish.

3.3.5 Fluorescence Resonance Energy Transfer

Cells were grown on #1.5 22mm coverslips until nearly confluent. Cells were then fixed in 4% formaldehyde in PBS for 20min and then washed 3X in PBS. Cells were permeabilized in 0.4% Triton X-100 in PBS with 1% NGS at 4°C for 5min and then washed

3X in PBS with 1% NGS. Cells were incubated for 1hr in anti-SnoN antibody in PBS with 1% NGS and then washed 3X in PBS with 1% NGS. Cells were incubated in the Cy3-conjugated secondary antibody for 1hr in PBS with 1% NGS. Cells were washed 3X in PBS with 1% NGS and then incubated in Cy5 conjugated anti-Ski antibody for 1hr. Cells were washed 3X in PBS and counter-stained with DAPI for 5min. Cells were washed 4X in HEPES buffered saline and then mounted with Mowiol and sealed with nailpolish. Cell images were acquired with a CoolSNAP HQ camera (Photometrics, Tuscon, AZ) on a Zeiss Axioplan2 (Thornwood, NY) with a Xenon light source and 63X (Plan-Apo, 1.4NA) lens with Slidebook software Version 4.2 (Intelligent Imaging Innovations, Denver, CO). Images of the donor (Cy3) and the acceptor (Cy5) were obtained and then the acceptor was photobleached for 2min. Post-photobleaching images were then obtained of each channel. FRET calculations were performed using the Image J FRETcalc plugin using the acceptor photobleaching method (Stepensky, 2007).

3.3.6 Statistics

Student's t test and Spearman's coefficient were performed in Microsoft Excel 2007 (Redmond, WA) using a spreadsheet from the *Handbook of Biological Statistics* with assistance from Dr. Betty Skipper at the University of New Mexico (McDonald, 2007).

3.4 Results:

I performed immunofluorescence microscopy studies to determine the subcellular distribution of Ski in different cell lines. Representative images can be seen in Figures 12-14. We found that Ski was located primarily in the nucleus in A204

(undifferentiated sarcoma) and LNCaP (prostate cancer) cell lines. In propagating HSMM (human myoblast), HeLa (cervical cancer), RMZ-RC2 (alveolar RMS), Hs729T (RMS), and RD (embryonal RMS) cells there was more Ski signal in the nucleus than in the cytoplasm, but there was still considerable staining in the cytoplasm. SJRH30 (alveolar RMS) and A673 (Ewing's sarcoma) cells had as much Ski labeling in the cytoplasm as in the nucleus and it was no longer easy to define the nucleus based solely on the Ski staining. We looked at the staining intensity of Ski at several time points during myogenic differentiation of the HSMM cell line. We allowed the cells to grow to confluence and then changed them to a growth media consisting of 2% horse serum in DMEM. We detected a slight gradual increase in cytoplasmic Ski staining as the differentiation progressed. Cells at the 20 day time point had several areas of intense cytoplasmic staining. However, in these cells it is still possible to delineate the nucleus as it had more staining than the surrounding cytoplasm. There were also differences in protein levels and localization between the RMS tumor samples. The images were graded from 0 to 6 based on level of staining intensity ranging from no staining to very high intensity, respectively. A Spearman's rank correlation was performed as we were dealing with non-parametric categories. We found that there is a statistically significant negative correlation ($p=0.0138$) between Ski protein staining intensity and RMS tumor group, while SnoN protein staining intensity had a non-significant positive correlation ($p=0.0926$) with RMS tumor group (see Figures 15 and 16).

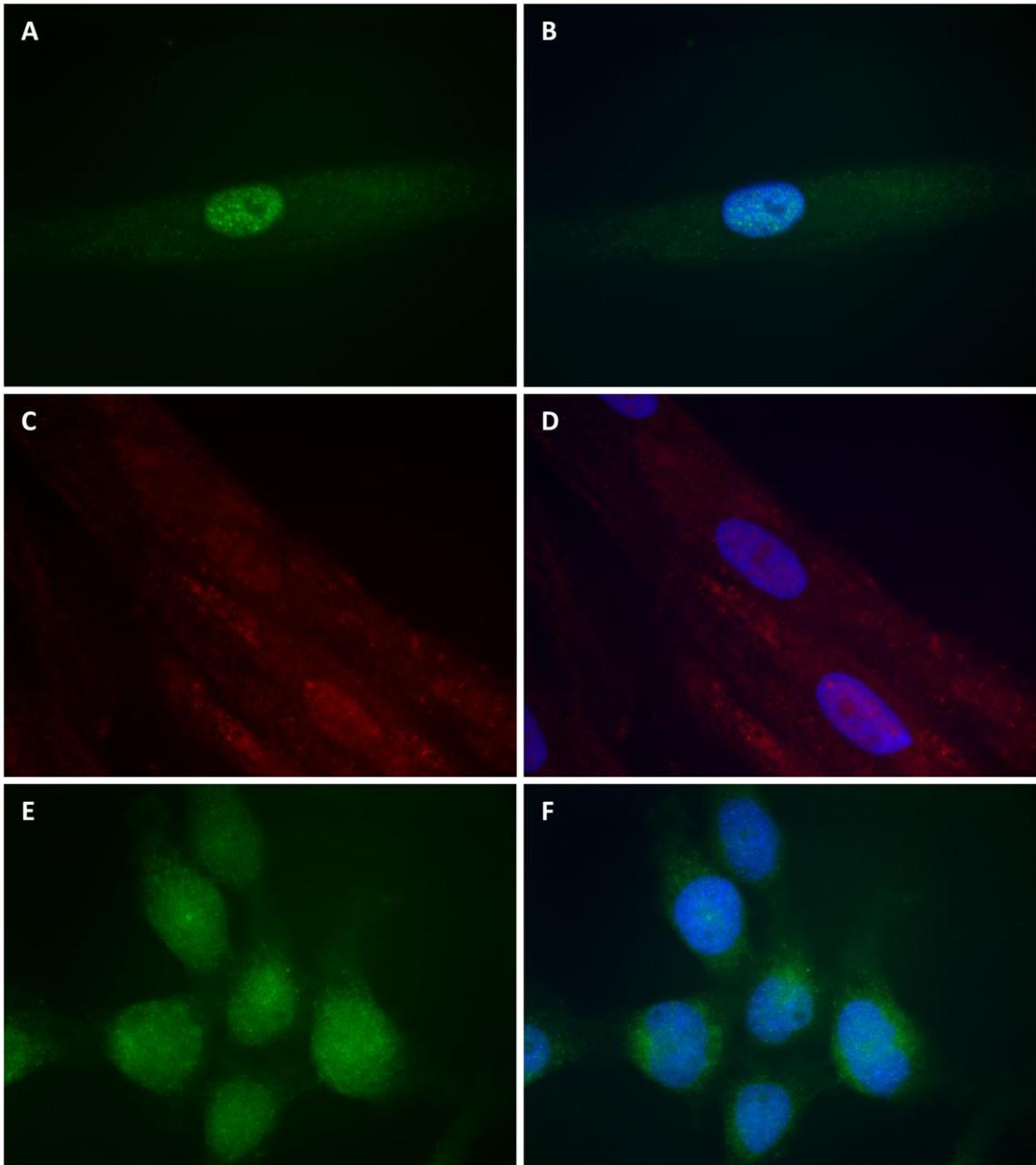


Figure 12: Indirect immunofluorescence images of Ski labeled with either FITC or Cy3 secondary antibodies are on the left and corresponding images with DAPI overlay are on the right. A & B are propagating HSMM primary human skeletal muscle cells. C & D are HSMM cells after 20 days of differentiation. E & F are SJRH30 RMS cells.

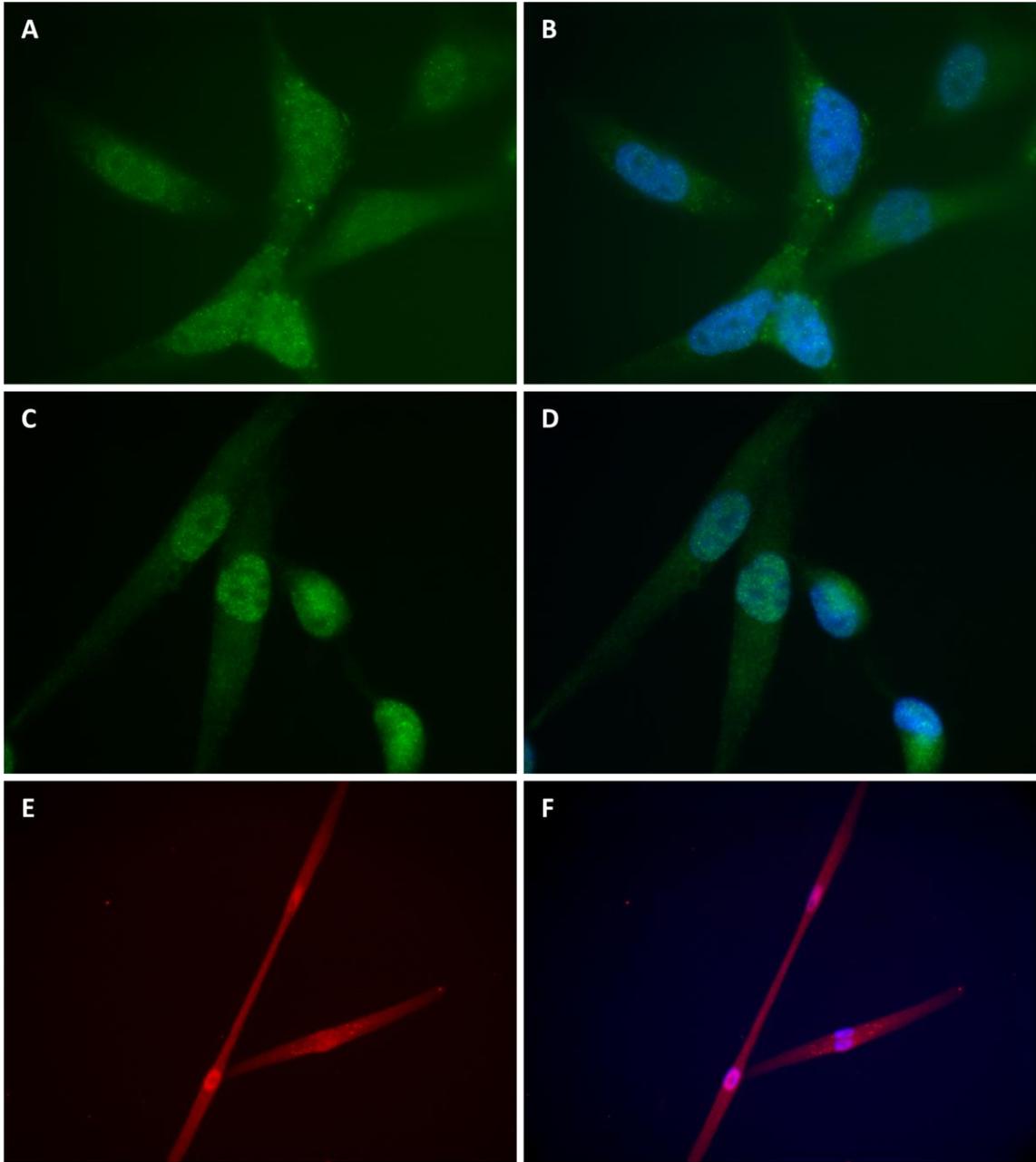


Figure 13: Indirect immunofluorescence images of Ski labeled with either FITC or Cy3 secondary antibodies are on the left and corresponding images with DAPI overlay are on the right. A & B are RD RMS cells. C & D are HS729t RMS cells. E & F are RMZ-RC2 RMS cells.

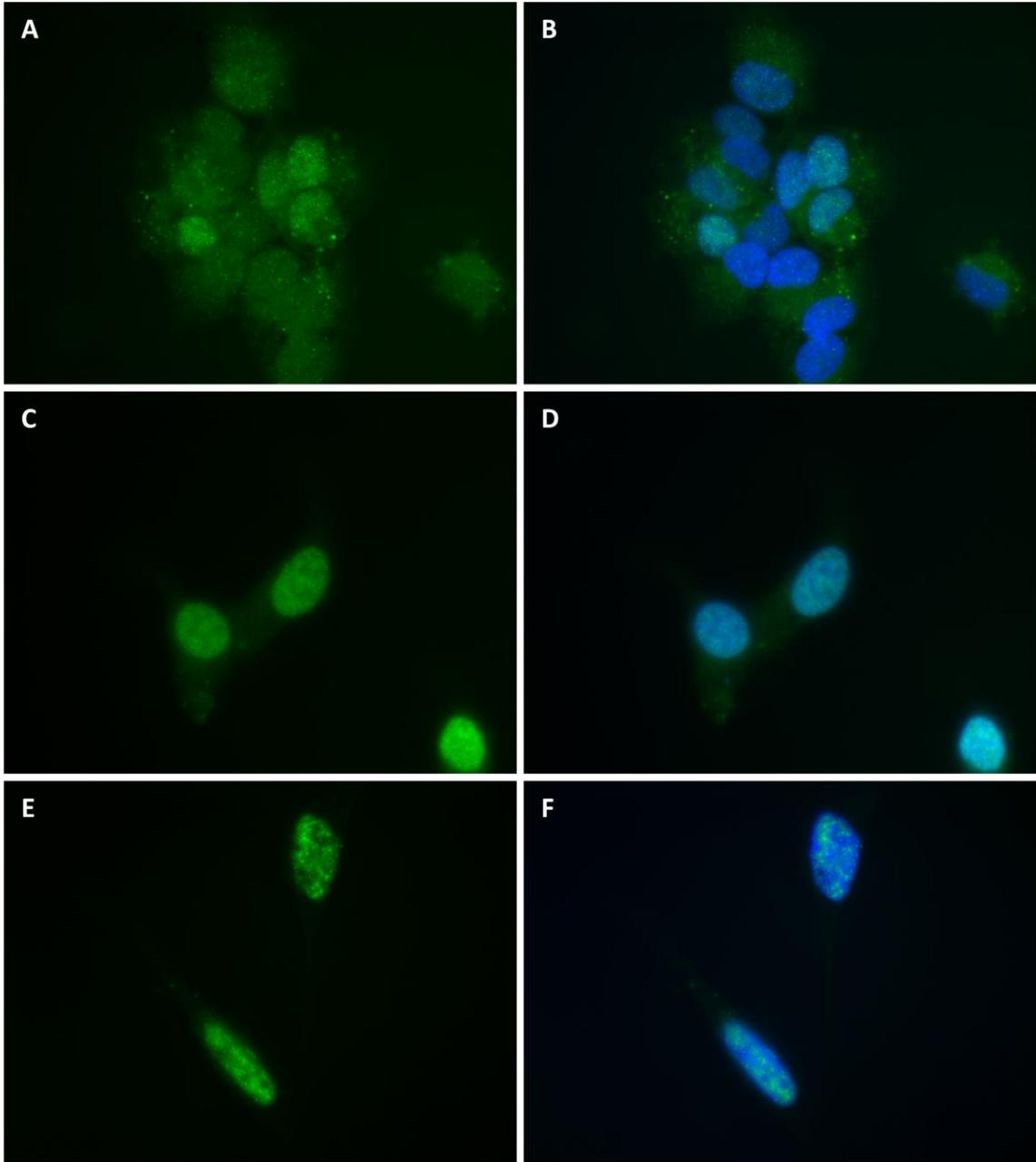


Figure 14: Indirect immunofluorescence images of Ski labeled with FITC secondary antibodies are on the left and corresponding images with DAPI overlay are on the right. A & B are A673 Ewing's sarcoma cells. C & D are LNCaP prostate cancer cells. E & F are A204 sarcoma cells.

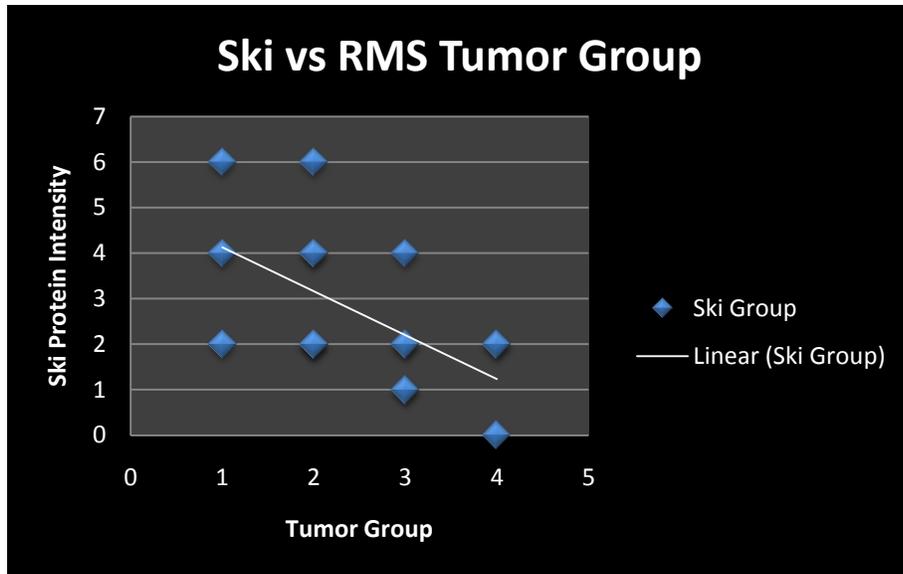


Figure 15: Graphical representation of Ski protein intensity vs RMS tumor group illustrating a statistically significant negative correlation ($p=0.0138$).

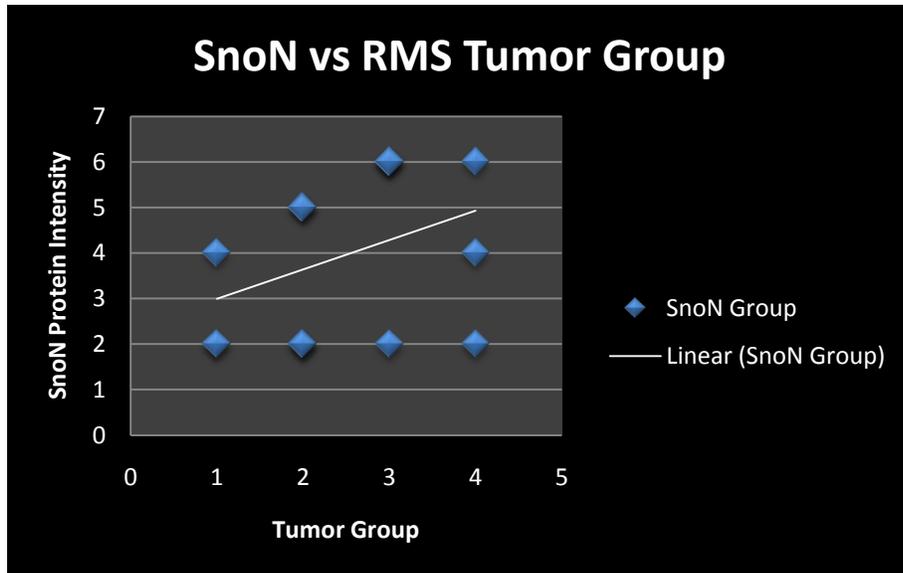


Figure 16: Graphical representation of SnoN protein intensity vs tumor group illustrating a positive, but not statistically significant, correlation ($p=0.0926$).

Next we performed fluorescence resonance energy transfer (FRET) to determine if Ski and SnoN were in close proximity to one another. We found differences between cell lines as well as differences between the nucleus and the cytoplasm. In SJRH30 cells we found that there was an average FRET value of 21 in the cytoplasm and 11 in the nucleus. A Student's T-test was performed to determine if the difference was significant and we obtained a p-value of 0.003 (see Figure 17). However we found that in HeLa cells the FRET value was less, although there was still a significant difference between cytoplasmic and nuclear FRET with a p-value of 9.23×10^{-6} (see Figure 17). These results indicate that there is more Ski/SnoN colocalization in the cytoplasm than in the nucleus.

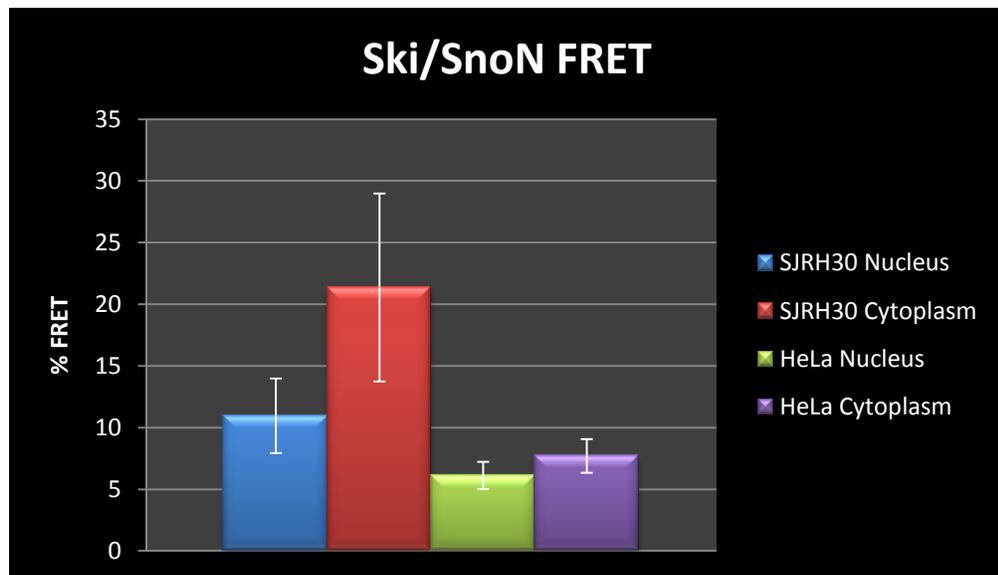


Figure 17: FRET percentage in the nucleus and cytoplasm in SJRH30 RMS cells and HeLa cervical cancer cells illustrating an increased level of FRET in the cytoplasm of both cell lines.

3.5 Discussion

We found that the subcellular localization of Ski varied in the different cell lines that we examined. Ski localization in the RMS cell lines was in all cases at least partially

cytoplasmic. The SJRH30 cell line in particular had strong cytoplasmic staining. This is an alveolar RMS cell that has the t(2;13) translocation and an amplicon that includes CDK4 and GLI with an overexpression of CDK4 mRNA and protein (Khatib et al., 1993). Alveolar RMS tumors as a group have a worse prognosis than other types of RMS tumors (Loeb et al., 2008). The other three RMS cell lines that we examined also have Ski staining in the cytoplasm but there was still noticeably more Ski in the nucleus. Of these cell lines, one was an embryonal (RD), one was not classified (Hs729t), and the other cell line was alveolar (RMZ-RC2) although it contains the t(1;13) translocation instead of the t(2;13) translocation (Nanni et al., 1986). The A673 Ewing's sarcoma cell line is the other cell line that we found to have intense cytoplasmic Ski staining. Interestingly, this cell line was established from a patient with a primary RMS although cytogenetics has shown that it has the EWS/FLI1 fusion gene and therefore is a Ewing's tumor cell line (Martinez-Ramirez et al., 2003).

The intensity of Ski staining in the RMS tumor tissue has a statistically significant negative correlation with tumor group. The intensity of SnoN staining in these samples trended to a positive correlation with tumor group but failed to be statistically significant. Tumor group refers to the postsurgical status of the cancer. Group I is localized tumor tissue that is removed with clean margins. Group II is localized tumor tissue that has microscopic amounts of tumor left, or cancer that has spread to local lymph nodes and may or may not have microscopic cancer remaining after surgery. Group III has macroscopic tumor tissue remaining after surgery for local cancer. Group

IV has metastatic tumor tissue at the time of diagnosis. This result suggests that lower quantities of the Ski protein are found in higher tumor group RMS cancers, which are more likely to have metastasized. And conversely, high Ski protein levels are correlated with tumors that are less likely to act in an aggressive manner. This would imply that either Ski is involved in the early stages of tumorigenesis or that the loss of Ski expression is a precursor to tumorigenesis.

The HSMM myoblast cell line will differentiate into myotubes if allowed to grow to confluence and placed in a low serum media (Linardic et al., 2005). This cell line has also been converted to embryonal RMS-like cells when infected with retroviruses encoding *T/t-Ags*, *hTERT*, and *H-Ras*^{V12G} (Linardic et al., 2005). We found mostly nuclear Ski staining with a small amount in the cytoplasm while propagating. After 20 days of low serum differentiation, Ski is found in both the nucleus and the cytoplasm, although the overall intensity of staining appears to be less than in the propagating myoblasts. Ski has been shown to be involved in regulating the transcription of muscle specific transcription factors, regulating myogenesis (Colmenares et al., 1991; Ichikawa et al., 1997). Ski is expressed in myoblasts and regulates terminal differentiation by activating the transcription of *MyoG* through interactions with Six1 and Eya3 (Zhang and Stavnezer, 2008). With the shift of Ski protein to the cytoplasm in late myogenesis it is possible that Ski has a function during myogenesis other than acting as a transcription factor.

My FRET results indicate that Ski and SnoN are often within 10nm of each other in both the nucleus and the cytoplasm. This result corroborates earlier studies that found that when overexpressed, Ski and SnoN can be cross-linked and pulled down together (Heyman and Stavnezer, 1994; Nagase et al., 1993; Zheng et al., 1997a). However, my results show that in cells with only endogenous proteins, Ski and SnoN are in close enough proximity to have functional interactions. The results also surprisingly indicate that this interaction is occurring not only in the nucleus, but also in the cytoplasm. This suggests that the cytoplasmic interaction of Ski and SnoN may have important functional consequences. Ski and SnoN have each been shown to separately interact with Smad proteins and pRb in the cytoplasm, but this is the first evidence of an interaction between Ski and SnoN in the cytoplasm (Jacob et al., 2008; Krakowski et al., 2005; Reed et al., 2001).

Ski and SnoN are unique proto-oncoproteins in that they can induce both oncogenic transformation and terminal muscle differentiation when expressed at high levels (Colmenares and Stavnezer, 1990; Fumagalli et al., 1993; Mimura et al., 1996). The function of Ski in myogenesis is regulating the transcription of skeletal muscle transcription factors (Zhang and Stavnezer, 2008). Ski and SnoN are also co-repressors of TGF β signaling and aberrant signaling within this pathway has been linked to tumorigenesis in a number of solid tumors (Buijs et al., 2007). I originally hypothesize that since RMS cells have characteristics of skeletal muscle, Ski may also be involved in RMS tumorigenesis and/or progression (Loeb et al., 2008). Both of these cell types

express Ski and the protein is localized to both the nucleus and the cytoplasm. There did not appear to be a correlation between Ski localization and RMS subtype although this may be because the genotype of tumors in each subtype is not identical. The difference in Ski localization in myoblasts and RMS cells may indicate a different function for Ski in each cell type. The co-localization of Ski and SnoN in the cytoplasm of the SJRH30 cells may be necessary for this function. Determining the functional consequences of this interaction will allow us to better understand the molecular basis of RMS and possibly define new therapeutic targets. Additional studies will be required to determine if the high amount of FRET in the SJRH30 cell line is RMS-specific or if it is specific to this particular cell line. I have performed preliminary experiments with the MDA-MB-231 breast cancer cell line and the results are similar to those from the HeLa cell line. Performing FRET analysis on differentiating myoblasts may help to determine if Ski and SnoN may have a functional interaction during myogenesis.

Chapter 4: Gene Expression

4.1 Abstract

Ski and SnoN are transcription factors, encoded by the *SKI* and *SKIL* genes, respectively, that act as co-repressors of the Smad-mediated TGF β signaling pathway. The literature reports that Ski and SnoN are involved in several aspects of development including neural tube formation, hematopoiesis, and myogenesis. Recent studies have indicated that *SKI* is overexpressed in some cancers. We have examined the expression of *SKI* and *SKIL* in several cancer cell lines, human myoblasts during differentiation, and in rhabdomyosarcoma (RMS) tumor tissue. We confirm earlier findings that *SKIL* is expressed at a higher level in ER-positive breast cell lines than in ER-negative cell lines. We also found that there is a difference between the expression profiles in differentiating primary human myoblasts and differentiating mouse myoblasts of the C2C12 cell line. The gene expression profiles of the RMS tumor tissue showed trends that may be significant with a larger sample size.

4.2 Introduction

Ski and SnoN are essential transcription factors that are involved in development of muscle and neuronal cell lineages, double knockouts of either one have been found to be lethal (Amaravadi et al., 1997; Berk et al., 1997; Kaufman et al., 2000; Shinagawa et al., 2000). They are unique proto-oncoproteins in that they can induce both oncogenic transformation and terminal muscle differentiation when expressed at high levels (Ambrose et al., 1995; Barkas et al., 1986). Both proteins are prognostic in

melanoma (Reed et al., 2001), esophageal squamous cell carcinoma (Akagi et al., 2008), colorectal cancer (Buess et al., 2004) and breast cancer (Zhang et al., 2003). Several potential mechanisms for Ski's and SnoN's involvement in cancer have been identified. They are both co-repressors of Smad-mediated TGF β signaling (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999; Vignais, 2000) in which Smad proteins are activated by TGF β receptors that have been bound by the appropriate ligand (Miyazono, 2000). The Smads then shuttle to the nucleus where they form complexes on DNA, either activating or repressing transcription, depending on what other proteins are within the complex. Ski and SnoN have been shown to interact with histone deacetylases, promoting changes in chromatin structure and subsequently inhibiting transcription (Nomura et al., 1999). Both Ski and SnoN have been shown to sequester Smad proteins in the cytoplasm (Kokura et al., 2003; Krakowski et al., 2005). This inhibits Smad-mediated TGF β signaling before the Smads can shuttle to the nucleus. Ski has also been shown to activate the Wnt/ β -catenin signaling pathway in melanoma (Chen et al., 2003). Although Ski and SnoN have been found to be either overexpressed and/or located in the cytoplasm in many types of cancer, little attention has been given to rhabdomyosarcoma (RMS). This is predominately a cancer of childhood and the most common soft-tissue sarcoma in children (Loeb et al., 2008). RMS has characteristics of skeletal muscle, displaying muscle specific markers. As Ski and SnoN are involved in myogenesis, we hypothesized that they may also be involved in RMS. To investigate this issue, we examined the expression of *SKI* and *SKIL* mRNAs by performing quantitative reverse transcriptase PCR (qRT-PCR) on several cancer cell lines and on RMS tumor

tissue samples. We found that both genes are expressed at varying levels in both RMS cell lines and tumor tissue. We also examined SKI and SKIL gene expression in differentiating myoblasts to see if the gene expression was similar to that seen in the RMS tumors.

4.3 Methods

4.3.1 Cell Lines and Cell Culture

HeLa, SJRH30, RD, HS729t, A204, and A673 cell lines were purchased from ATCC (Manassas, VA). RMZ-RC2 cells were kindly provided by Drs. Lorena Landuzzi and Pier-Luigi Lollini at the Laboratory of Immunology and Biology of Metastasis, Cancer Research Section, Department of Experimental Pathology in Bologna, Italy. MCF-7 and MDA-MB-231 cells were kindly provided by Dr. Rebecca Hartley at the UNM HSC. LNCaP cells were kindly provided by Dr. Marco Bisoffi at the UNM HSC. HSMM cells were purchased from Cambrex (Walkersville, MD).

Hela, SJRH30, RMZ-RC2, RD, HS729t, A204, A673 LNCaP, and MCF-7 cells were cultured in DMEM (cat. #12430-104, Gibco, Carlsbad, CA) with 10% FBS (cat. #16140-071, Gibco) 100U/ml Penicillin and 100µg/ml Streptomycin (cat. #15140-122, Gibco). Clonetics HSMM cells were grown in Sk-GM-2 media (cat. #CC-3245, Cambrex, Walkersville, MD). HSMM cells were differentiated in DMEM with 2% horse serum (cat. #26050-070, Gibco) and 100U/ml Penicillin and 100µg/ml Streptomycin. MDA-MB-231 cells were cultured in DMEM/F12 (cat. #11320-082, Gibco) with 10% FBS and 100U/ml Penicillin and 100µg/ml

Streptomycin. Low passage number cells of each cell line were seeded on #1.5 22mm coverslips, allowed to attach and grow until nearly confluent.

4.3.2 Tumor Tissue

The RMS tumor samples were acquired from the Cooperative Human Tissue Network (CHTN) at the National Cancer Institute by Dr. Stuart Winter. Coding information was provided by Dr. James Anderson. Samples were obtained from 18 children and young adults who were diagnosed with rhabdomyosarcoma and consented to have their tumor samples stored in the Children's Oncology Group (COG) Cell Bank (Columbus, OH). The patients then received treatment on COG studies D9502 (n = 1), D9602 (n = 7), D9802 (n = 1), or D9803 (n = 9), depending on the treatment regiment at the time of diagnosis. Molecular and protein findings were compared with the original histological diagnoses, annotated patient demographic features, staging, grouping and event free survival associated with the treatment studies.

4.3.3 RNA Extraction

Cells were grown to near confluence in T-75cm² flasks and then rinsed with PBS. RNA was then collected using Trizol (cat. #15596-026, Invitrogen, Carlsbad, CA) according to the manufacturer's directions. RNA was snap frozen in liquid nitrogen and stored at -80°C until needed. Tumor tissue was handled at -80°C until placed in Trizol. Pieces of ~200mg tissue were cut off with a razor blade and placed in a prechilled microfuge tube. The piece was immediately processed with a Tissue Terror until all visible pieces were

gone (~1min). The samples were then processed according to the Trizol directions, snap frozen, and stored at -80°C.

4.3.4 RT-PCR

RNA concentration was calculated using a spectrophotometer and 10µg was used in a 60µl reaction with the Turbo DNA-free kit (cat. #AM1907, Ambion, Austin, TX) to normalize RNA levels. 4µl of that reaction was used in an RT-PCR reaction using the AccuScript kit (cat. #6000184, Stratagene, La Jolla, CA) with the following modifications: The reaction contained 1M Betaine (cat. #B0300, Sigma, St. Louis, MO) and 5% DMSO (cat. #D9170, Sigma, St. Louis, MO) and the PCR program was 25°C for 5min, 40°C for 60min, 50°C for 10min, and then 95°C for 5min. Random primers supplied in the kit were used for transcription.

4.3.5 Quantitative Reverse Transcriptase PCR

Quantitative reverse transcriptase PCR (qRT-PCR) was performed using TaqMan primers and the TaqMan Gene Expression Master Mix. All reagents, biologicals, and disposables were purchased from Applied Biosystems (Foster City, CA). The primers used were SKI (Hs00161707_m1), SKI (Mm00448744_m1), SKIL (Hs00180524_m1), SKIL (Mm00456917_m1), and Eukaryotic 18S rRNA endogenous control (4333760F). All reactions were done in triplicate and run in 96-well fast optical 0.1ml plates using the AB 7500 Fast System with the normal program.

4.3.6 Statistics

Statistical analyses were performed using two-tailed Student's t test and Spearman's coefficient for non-parametric testing using Microsoft Excel 2007 (Redmond, WA).

4.4 Results

We first examined the mRNA expression levels of *SKI* and *SKIL* in several different human cancer cell lines using TaqMan primers for qRT-PCR and 18S rRNA as an endogenous control (see Figure 18). All of the data in the gene expression studies were normalized relative to HSMM day 0 expression levels. We found the highest expression of *SKI* in the Ewing's sarcoma cell line A673. The highest expression of *SKIL* was found in the RMS cell line HS729t. The RMZ-RC2 alveolar RMS cell line had high *SKIL* expression, although the *SKI* expression was roughly half of the other RMS cell lines. Our data from the breast cancer cell lines showed that *SKIL* expression in the ER/PR positive cell lines MCF-7 and T47D is higher than in the ER/PR negative cell lines MDA-MB-231 and non-tumorigenic MCF-10A. Generally speaking, we found that most of the cancer cell lines have higher *SKIL* expression than *SKI* expression.

Next, we examined mRNA expression during myogenic differentiation in both a mouse myoblast cell line (C2C12) and a human skeletal muscle myoblast cell line (HSMM). In the C2C12 cell line we saw a gradual increase in both *SKI* and *SKIL* mRNA expression relative to time 0 that was sustained throughout the 15 days of differentiation (see Figure 19). In contrast, the HSMM cell line demonstrated a decrease

in *SKI* and *SKIL* expression (see Figure 20). *SKIL* expression levels returned to baseline by day three while *SKI* remained lower than baseline throughout differentiation.

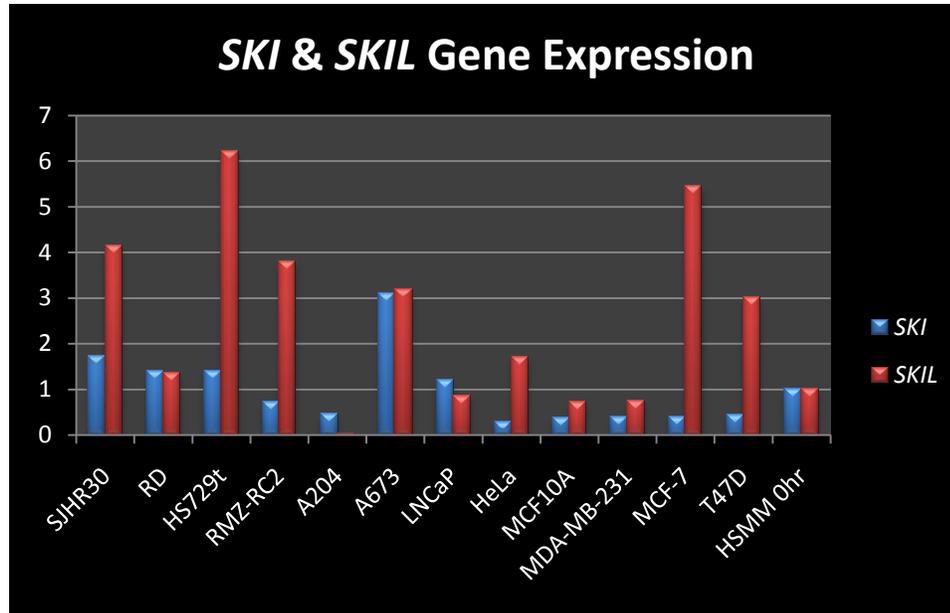


Figure 18: Real-Time PCR data using TaqMan primers and 18S rRNA endogenous control. Levels were normalized to HSMM Ohr samples. Most of the cancer cell lines tended to have higher relative *SKIL* expression than *SKI* expression.

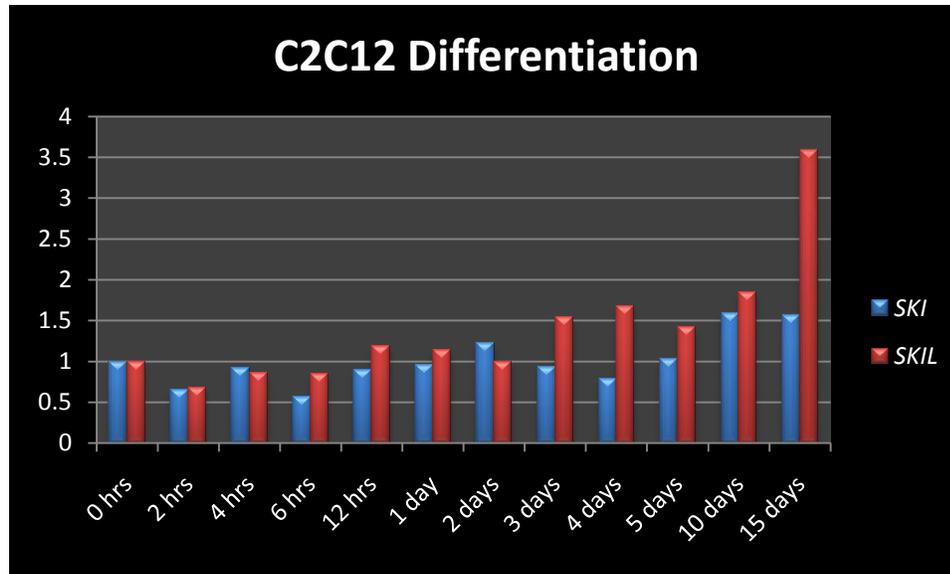


Figure 19: Real-Time PCR data using TaqMan primers and 18S rRNA endogenous control. Levels were normalized to HSMM 0hr samples. The expression of both *SKI* and *SKIL* are increasing throughout the differentiation process.

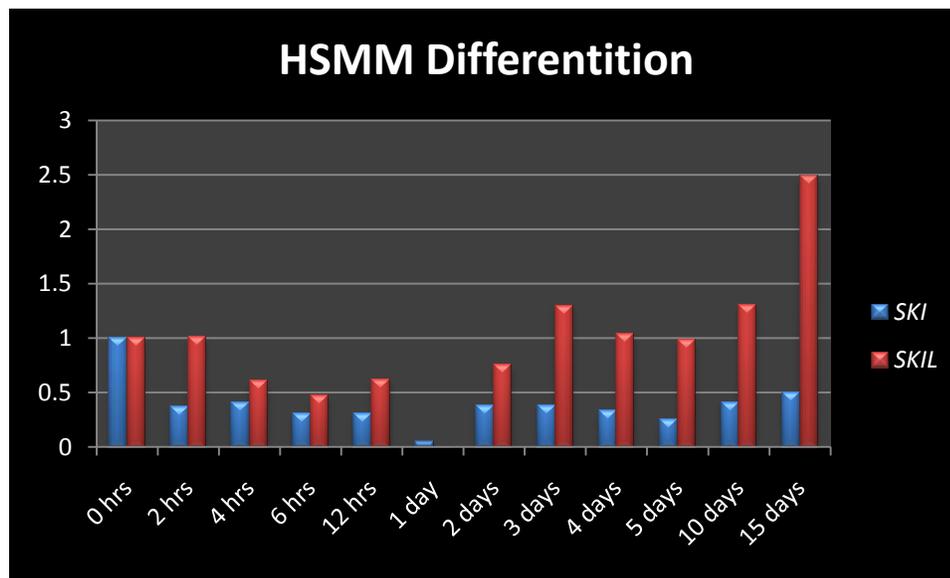


Figure 20: Real-Time PCR data using TaqMan primers and 18S rRNA endogenous control. Levels were normalized to HSMM 0hr samples. The expression of *Ski* drops after the initial time point and then stays level. The expression of *SKIL* is similar to the pattern seen in C2C12 cells.

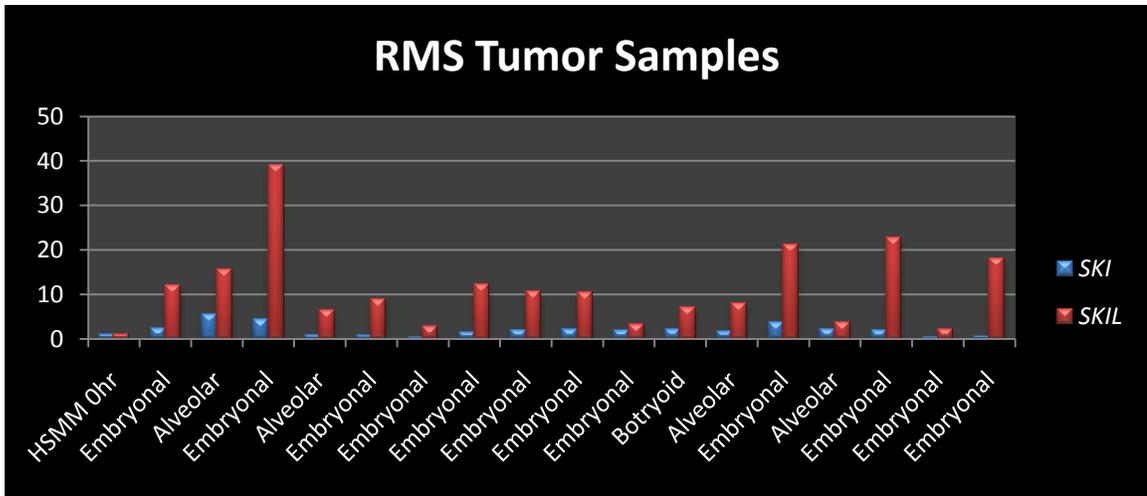


Figure 21: Real-Time PCR data using TaqMan primers and 18S rRNA endogenous control. Levels were normalized to HSM 0hr samples. There was no correlation between RMS subtype and the expression of *SKI* or *SKIL*.

Finally, we examined *SKI* and *SKIL* mRNA expression levels in RMS tumor samples to see if they correlated with tumor histology, site or extent of primary disease, and size of presenting tumor in a cohort of 18 patients with alveolar (n = 6), embryonal (n = 11) or botryoidal rhabdomyosarcomas (n = 1). The mRNA expression of the samples did not seem to correlate significantly with tumor subtype, age of the patient, or prognosis. There was a considerable variation of expression levels across the samples, with a 16-fold difference in *SKI* expression and an 18-fold difference in *SKIL* expression (see Figure 20). Again, we see that *SKIL* expression is higher than the expression of *SKI* in all of the samples. Next we investigated whether Ski and SnoN ratios were linked to the tumor histology. All Ski/SnoN ratios were less than 1.0. Using Student's T-test, we found no correlation between Ski and SnoN ratios with histologic features and, in addition, we found no significant correlation with the other annotated patient demographic data.

4.5 Discussion

Ski and SnoN are co-repressors of TGF β signaling and aberrant signaling within this pathway has been linked to tumorigenesis in a number of solid tumors (Buijs et al., 2007). Researchers have examined the gene expression of *SKI* and *SKIL* during myogenesis in mouse cells and in several varieties of tumor cells (Colmenares and Stavnezer, 1990; Fumagalli et al., 1993; Mimura et al., 1996). The only report of *SKI* or *SKIL* gene expression in RMS is in a description on a novel splicing variant from the *SKIL* gene that codes for the Sno1 protein (Pearson-White, 1993). The expression of *SKI* and

SKIL gene transcripts in several tissues including RMS tumor tissue, skeletal muscle, myofiber, and myoblasts was examined. They found *SKI* and *SKIL* gene expression in all of these samples, with the novel splicing variant in all but the skeletal muscle sample, but at a lower expression level than the other transcripts. Since RMS is a cancer with characteristics of skeletal muscle, and *Ski* and *SnoN* have been shown to be involved in myogenesis, we examined several RMS cell lines and found that they expressed *SKI* and *SKIL* transcripts. In order to further examine *SKI* and *SKIL* expression in RMS tissue, we obtained tumor samples from the CHTN. We did not find that mRNA levels of *Ski* or *SnoN* were associated with tumor histology, disease progression or site of location at the time of diagnosis, leading us to conclude that *Ski* and *SnoN* mRNA levels may not be clearly linked to tumorigenesis in rhabdomyosarcoma. However, because the TGF β pathway is vitally involved in muscle differentiation, and *Ski* and *SnoN* are commonly shared in rhabdomyosarcoma, these oncogenes remain to be considered for targeted therapy in this disease subtype. As observed, the *Ski* to *SnoN* ratio was always below 1.0. This may be skewed by our choice of normalizing cell line. It is also possible that the HSMM cell line has a considerably higher *SKI*, or lower *SKIL*, gene expression than other cells and tumor tissue. The Ct values from the Real-Time PCR data for *SKI* and *SKIL* are within three cycles of each other in the HSMM 0hr time point, with *SKI* having a lower Ct than *SKIL*. This particular cell line was chosen as the normalizer because we thought that it would be more representative of a normal cell than cancer cell lines.

The data from the cell lines illustrate that there is a wide range of *SKI* and *SKIL* gene expression among different varieties of cancer. Previous research has looked at *SKIL* expression in several breast cancer cell lines (Zhang et al., 2003). Our results confirmed that *SKIL* expression is higher in ER/PR positive cell lines than in ER/PR negative cell lines. The *SKI* expression was similar in all of the breast cancer cell lines. The A673 Ewing's sarcoma cell line is unique in that it has high expression of both *SKI* and *SKIL*. There are no reports in the literature of Ski or SnoN being associated with Ewing's sarcoma although there is a potential explanation. Ewing's sarcoma (including the A673 cell line) has a characteristic t(11;22) translocation that produces a fusion protein with a member of the ETS family (Hahm et al., 1999). This protein has been shown to inhibit the TGF β 2 receptor making the cells unresponsive to TGF β signaling (Hahm et al., 1999). It is possible that the disruption of this pathway has an effect on the expression of Ski and SnoN. The other interesting result in the cancer cell lines involves the RMZ-RC2 alveolar RMS cell line. This cell line contains the t(1;13) translocation that moves the *SKI* gene from chromosome 1 to chromosome 13 (Nanni et al., 1986). Since the data shows that *SKI* expression in this cell line is roughly half that of the other RMS cell lines, we speculate that the translocated gene may be silenced during tumorigenesis.

SKI and *SKIL* expression has been examined before in the C2C12 mouse myoblast cell line (Mimura et al., 1996). This is a cell line that will differentiate into myotubes when grown to confluence and switched to a low serum media. The researchers used

qRT-PCR to quantify the mRNA levels and found that *SKIL* expression showed a transient increase at 12 hours while *SKI* expression stayed relatively even. They hypothesized that the increase occurred at the entrance to G0 phase. We are not sure why we failed to replicate this result as the reported increase was rather large (25-fold). The most reasonable explanation is that different methods of quantification were employed. We used a very accurate reverse transcriptase enzyme (AccuScript) and added betaine and DMSO to the reaction as there are some GC-rich regions in the transcript that are difficult to transcribe. A stepped-temperature program was used to transcribe the cDNA (see methods). We then used TaqMan gene specific probes to amplify the cDNA in a qRT-PCR machine using 18S rRNA as the endogenous control. The other group used standard protocol and MMLV enzyme, followed by competitive PC and GAPDH as the endogenous control. In our initial experiments we examined 18S rRNA, GAPDH and several other controls. We found that the 18S rRNA has less variation in its levels in different cell lines relative to total RNA. Since the endogenous quantities of this RNA are much higher than the other controls, small variations in quantities between samples have less of an effect on the results. Therefore, we believe that our result is correct. As more is learned about the role of Ski and SnoN in myogenesis, the function of these proteins in RMS will become clearer. Our gene expression data show that they are expressed at high levels in some RMS tumors and it seems unlikely that this is merely coincidental.

Chapter 5: Conclusions

5.1 Potential Impact

Rhabdomyosarcoma is the most common pediatric soft tissue sarcoma, with 350 cases diagnosed each year (Loeb et al., 2008). The two main variants of RMS are alveolar and embryonal, with the latter accounting for about 60% of cases. The embryonal variant resembles immature skeletal muscle, while alveolar RMS resembles lung parenchyma. Both variants express muscle-specific antigens such as desmin and MyoD. Five-year survival rates have been slowly increasing but are still in the mid-70s%. Chromosome translocations are found in 77% of the alveolar variant cases and are of either the t(2;13) or the t(1;13) type. The t(1;13) translocation is of interest with regard to *Ski* because this translocation involves the *PAX7* gene located on chromosome 1 from p36.12-p36.2, just centromeric to the *SKI* gene at 1p36.3. In this situation the translocated *SKI* gene is moved to chromosome 13. While we do not expect that the gene would be damaged by translocation, it is possible that to be placed under the influence of a different promoter. We examined the RMZ-RC2 RMS cell line that reportedly contains this translocation (Nanni et al., 1986). We saw by qRT-PCR that the *SKI* gene expression in this cell line is about half the level of the other RMS cell lines. Based on these data, we believe that the translocated *SKI* gene is being at least partially silenced in the new location. The *Ski* protein staining via immunofluorescence microscopy was average in this cell line and there was moderate cytoplasmic localization. Further examination into the correlation between mRNA and protein levels

in this and the other RMS cell lines may provide us with more information into the regulation of Ski.

A significant finding in our research is that Ski protein levels in RMS tumor tissue are negatively correlated with tumor group. This suggests that higher Ski protein levels are found in tumors that tend to be less aggressive. There is non-significant trend with SnoN as a positive correlation. It is interesting that, in all of the RMS tumor samples and most of the cancer cell lines that we examined, SnoN mRNA expression was higher than Ski expression. However, when we examined the Ski to SnoN ratio in regard to the tumor histology, we did not find any statistical trends. It is also interesting that Ski and SnoN protein levels did not correspond well to gene expression. This would suggest that post-transcriptional and post-translational regulation are more important than the regulation of transcription.

Ski and SnoN are unusual in that they can both act as either oncoproteins or tumor suppressors (Liu et al., 2001; Shinagawa et al., 2001). Part of the explanation for this is that they have been found to interact with almost twenty different proteins (Medrano, 2007); see Figure 1 in Chapter 1. The high number of interacting proteins also means that the subcellular localization of Ski and SnoN needs to be carefully regulated to prevent unwanted interaction. We have localized the NLS in Ski via site-directed mutagenesis and the creation of Ski-GFP fusion constructs. The SV40 Large T Antigen-like NLS is necessary and sufficient for nuclear localization of Ski. This result agrees with

work that was concurrently performed in another lab using a different mutation strategy (Nagata et al., 2006).

The NLS results merge well with our data showing that Ski is phosphorylated when in the cytoplasm. Our immunoprecipitation data show that Ski is phosphorylated when located in the cytoplasm but not in the nucleus. In light of previous research indicating that Ski is phosphorylated in a cell cycle dependant manner, we cannot ignore that the cytoplasmic cell fractions also include whole cell fractions of cells undergoing mitosis after the nuclear membrane has broken down (Marcelain and Hayman, 2005). Because we are working with rapidly dividing cancer cells, it is possible that some of the phosphorylated Ski is derived from the nuclei of cells in the process of cell division. It may be interesting to synchronize the cells and see if Ski phosphorylation and localization correlate or change during the cell cycle.

We believe that this research contributes interesting data to the existing knowledge of Ski and SnoN. Completion of the studies on the mechanism of localization will be a key step in moving Ski and SnoN research forward. The potential involvement in of these proteins in RMS is intriguing and requires further attention. There is clearly much more work to be done.

5.2 Future Studies

The next step in studying Ski localization is to determine if phosphorylation regulates its localization. First, the residue(s) that are being phosphorylated need to be determined. A quick search with an on-line search tool such as NetPhos

(<http://www.cbs.dtu.dk/services/NetPhos/>), will identify likely residues that can then be mutated using techniques that we used to construct the GFP vectors. Finding the kinase(s) involved is a difficult process as kinases generally phosphorylate many proteins and determining which one is responsible is very complex and out of the scope of this lab.

In regard to the RMS tumor tissue work, future studies should be focused on increasing the sample size. We originally obtained twenty samples, although coding data for only 18 of these was available. With the samples we have we can perform protein extractions and Western blots to verify the immunofluorescence data. We attempted this once but did not obtain good quality protein samples from the extracts. A different protein extraction protocol may yield better results. Another area of interest would be to examine the DNA sequence of *SKI* and *SKIL* from the tumor samples. Although no mutations in *SKI* or *SKIL* have been reported, it would be interesting to sequence the samples to be certain. And finally, fluorescence *in situ* hybridization (FISH) would allow us to examine the gene copy number to see if there are any amplifications or translocations.

5.3 Critique

There is a potential issue with our NLS experiments. The endogenous Ski in SJRH30 cells is found in both the nucleus and the cytoplasm. However, the Ski-GFP fusion is almost exclusively localized to the nucleus when expressed in these same cells. It is possible that the *SKI* gene is mutated in this cell line, although this cell line has been

extensively used and there are no reports of any *SKI* gene mutations. Another possibility is that the GFP fused to Ski is interfering with the normal function of Ski and its ability to interact with the proteins necessary for nucleocytoplasmic transport.

While performing the initial immunofluorescence studies on the cancer cell lines, we also did some immuno-electron microscopy (EM) studies to determine Ski and SnoN subcellular localization. Our goal was to obtain high resolution images of Ski and SnoN localization using these techniques. The problem that we encountered was that the localization of Ski was much different using immuno-EM than when using immunofluorescence microscopy (IFM). Since our data with the IFM matched the data reported in the literature, we decided to abandon the EM work. The EM fixation method and the commercial antibody that we were using may have been the issue. We did not get a chance to repeat these experiments with the new polyclonal antibody to Ski that we had made.

FRET microscopy has become a common method to determine protein co-localization. Recent literature report that it is possible to use either a CCD camera obtaining images from a fluorescence microscope or confocal laser scanning microscope (CLSM) to perform these experiments (Kenworthy, 2001). We performed some trials with a Zeiss 510 META CLSM to determine if obtaining confocal slices would have any effect on the results and we did not find any difference in the quality of data that we obtained. However, obtaining FRET data on more cell lines may provide us with important information regarding the Ski/SnoN interaction and its role in cancer. We

attempted to use FRET with the CHTN samples, but the background fluorescence prohibited us from obtaining useful data. More controls for the FRET studies should also be performed including performing the technique with proteins that we know interact and with proteins that we know do not interact.

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