The Cyclin-Dependent Kinase Inhibitor and Tumor Suppressor Locus *p16/INK4A-p14ARF* and Regulation of the Transition Into and Out of the Cell Cycle

by

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Abstract

p16/INK4A/CDKN2A is an important tumor suppressor gene located in the *INK4A/ARF* locus, which encodes a 16 kDa protein known as p16, and a 14 kDa protein known as p14ARF in humans. p16 arrests cell cycle in early G1 phase thereby inhibiting the binding of cyclin dependent kinase 4/6 with cyclinD1. This leaves the retinoblastoma protein (pRb) tumor suppressor hypo-phosphorylated and S phase transcription factor E2F bound and inactive. p14ARF expression up-regulates cyclin dependent kinase inhibitor p21, which inhibits the G1/S phase transition by stabilizing p53 expression upon disassociation from mdm2. We hypothesized that p16 has a role in exit from the cell cycle, becomes defective in cancer cells and has binding partners other than CDK4/CDK6 in quiescent or differentiated cells when their canonical target proteins are thought to be nonfunctional. We have hypothesized that INK4A/ARF encoded proteins perform important regulatory roles that are defective in canine mammary cancer and may cause loss of differentiation potential. Well characterized p16-defective canine mammary cancer cell lines, normal canine fibroblasts, and CMT-derived p16-transfected CMT cell clones, are used to investigate expression of p16 after serum starvation into quiescence followed by re-feeding to induce cell cycle re-entry. We have successfully demonstrated cell cycle arrest and synchronous cell cycle re-entry in CMT28 and NCF cells as well as p16 transfected CMT27A, CMT27H, CMT28A, and CMT28F cells, which is confirmed by ^{3}H-thymidine incorporation and flow cytometric analysis of cell cycle phase distribution. NCF, CMT27A, and CMT28F cells expressed up-regulated levels of p27

mRNA coincidently with elevated expression of p16 mRNA, as cells exited cell cycle and entered quiescence. To find alternate binding partners of p16, co-immunoprecipitation was performed in quiescent CMT27A cells, which resulted in the unique co-immunoprecipitation of the p53-associated and putative tumor suppressor 14-3-3σ protein only in quiescent CMT27A cells in comparison to exponential cells. Levels of 14-3-3σ mRNA expression also rose along with p16 in quiescent NCF cells. We differentiated 3T3-L1 fibroblasts into adipocytes for investigating the *p16* and related gene expression profiles during differentiation. This study is the first to report the predicted mRNA and protein sequence of canine p14ARF and *p14ARF* mRNA expression in canine cells.

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Dedication

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List of Abbreviations

ADP	Adenosine Diphosphate
cAMP	Cyclic Adenosine Monophosphate
ARF	Alternate Reading Frame
BRCA	Breact Cancer Type Susceptibility Protein
CDC6	Cell Division Cycle 6
CDK	Cyclin Dependent Kinase
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
cDNA	Complementery DNA
CIP/KIP	CDK Interacting Protein/ Kinase Inhibitory Protein
CKI	Cyclin Dependent Kinase Inhibitor
CMT	Canine Mammary Tumor
CPD	Cumulative Population Doubling
ΔΔCt	ΔΔ Cycle Threshold
CtBP	C-Terminal Binding Protein
DMP1	Dentin matrix acidic phosphoprotein 1
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ER/PR	Estrogen Receptor/Progestrone Receptor

FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HaCaT	Human Keratinocyte Cell Line
HATs	Histone Acetyltransferases
HBS	HEPES Buffered Saline
HCl	Hydrochloric Acid
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HEK27	Human Embryonic Kidney Cells
HER-2	Human Epidermal Growth Receptor-2
HIF1-α	Hypoxia Inducible Factor-α
HMER1	Human Mammary Epithelial Receptor
IBMX	3-Isobutyl-1-Methylxanthine
Id	Inhibitor of DNA-Binding
INK4	Inhibitor of CDK4
ISOC2	Isochorismatase Domain Containing 2
KSHV	Kaposi's Sarcoma-Associated Herpesvirus
MAPK	Mitogen-Activated Protein Kinases
MCM	Minichromosome Maintenance
MITF	Micropthalmia-Associated Transcription Factor
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
MyC	Myelocytomatosis
NaCl	Sodium Chloride

NCF	Normal Canine Fibroblast
NPM	Nucleophosmine Gene
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral Blood Mononuclear cells
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PI3 kinases	Phosphatidylinositol 3-Kinases
PMSF	Phenylmethanesulfonylfluoride
PRC2	Polycomb Repressive Complex 2
PreRC	Pre Replication Complex
PVDF	Polyvinylidene Fluoride
q-PCR	Quantitaive Polmerase Chain Reaction
Rb	Retinoblastoma
pRb	Retinoblastoma Protein
RNA	Ribonucleic acid
RS	Replicative Senescence
rt-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SSC	Standard Saline Citrate
TBS	Tris-Buffered Saline
TBX2	T-Box Transcription Factor

TGF	Transforming Growth Factor
TERT	Telomerase Reverse Transcriptase

Chapter 1: Literature Review

Section-1 Cell Growth and Development

Normal growth and development requires the combined processes of cell reproduction followed by commitment and differentiation of cells. An initial phase of rapid proliferation is followed by declining rate of replication in the course of cellular development. A cell undergoes a finite number of cumulative population doublings (CPDs) in culture before it enters replicative senescence (RS), which varies between species and is proportional to species lifespan. According to Hayflick's replicative senescence model, cell division is governed by its own internal mechanism to check growth and proper development (Hayflick, 1965). Human cells undergo roughly 50 cell divisions before reaching RS. The Hayflick limit has been linked to the shortening of telomeres, a region of DNA at the end of chromosomes (Harley *et al.*, 1990), as absence of telomerase activity leads to telomere shortening, which induces replicative senescence (Bell and Sharpless, 2007).

Parent cells replicate by dividing into two daughter cells. Cell replication or growth is controlled by a complex network of signals that control the cell cycle, the orderly sequence of events that all cells pass through as they grow to approximately twice their size, copy their chromosomes, and divide into two new cells. The cell cycle consists of 4 phases; G1, S, G2, and M phase (Enoch and Nurse, 1991). DNA duplication takes place in S phase, and chromosome seperation and cytokinesis in M phase. G1 and G2 are

gap phases which provide the time for the cell to ensure suitability of the external and internal environment and to prepare for DNA duplication and division. Cell cycle progression from one phase to another is controlled principally by two major classes of cell cycle proteins: 1) cyclins and 2) cyclin dependent kinases (CDKs), a family of serine/threonine kinases (Evans *et al.*, 1983; Norbury and Nurse, 1991; Afshari and Barrett, 1993). Cyclins are the cell cycle proteins, which bind to CDKs and activate them to function and enhance cell cycle progression (Pines and Hunter, 1991). Cyclin/CDK complexes are specific for each phase transition. In complex eukaryotic cells there are twenty CDK related proteins. Complex combinations of the different CDKs and cyclins in different phases of the cell cycle provide tightly regulated control of cell cycle progression (Satyanarayana and Kaldis, 2009). The levels of CDKs in cells vary little throughout the cell cycle but cyclins, in contrast, are synthesized and destroyed in a timely periodic manner to better regulate CDK activity during each cell cycle phase (Malumbres and Barbacid, 2009).

Early G1 phase progression is facilitated by CDK4/6 binding with cyclinD family proteins. This complex phosphorylates members of the retinoblastoma protein (pRb) family (pRb, p130, and p107) (Sherr and Roberts, 1999). Phosphorylation of pRb results in release of E2F protein, which otherwise binds to pRb. E2F is a transcription factor that activates E2F responsive genes that are required for further cell-cycle progression (Weinberg, 1995). CyclinE/CDK2 complexes complete pRb phosphorylation and further progression of cell cycle through late G1 phase. These complexes further activate E2F mediated transcription and passage through the restriction point to complete the G1/S phase transition (Sherr and Roberts, 1999). At the onset of S phase, cyclinA is

synthesized. In S phase, it forms a complex with CDK2, which in turn phosphorylates proteins involved in DNA replication (Petersen *et al.*, 1999).

During replication of DNA in the S phase of the cell cycle, CDC6 and Cdt1 are recruited to origin recognition complex (a multisubunit DNA binding complex). Together these factors help in the recruitment of mini-chromosome maintenance (MCM) proteins to pre-replicative complexes (preRC) (Lei and Tye, 2001). In early S phase, preRC recruits the functional replication complex including DNA polymerase and associated processivity factors such as proliferating cell nuclear antigen (PCNA) to origins of replication (Celis *et al.*, 1987). Subsequent cell cycle transition takes place through the activity of the CDK1/cyclinA complex which initiates prophase of mitosis (Furuno *et al.*, 1999). Finally, CDK1/cyclinB complex activity completes entry into mitosis (Riabowol *et al.*, 1989).

Along with the cyclins and CDKs, other proteins such as the tumor suppressor genes retinoblastoma protein (pRb), p53, and transcription factors such as the E2F proteins, play important roles in regulating cell cycle progression. The cell cycle has two important check points that occur at the G1/S and G2/M phase transitions (Hartwell and Weinert, 1989). These check points control cell cycle progression during normal proliferation and during stress, DNA damage, and other types of cellular dysfunction. At these cell cycle check points, cellular CDKs can be inhibited by cyclin-dependent kinase inhibitors (CKIs); thus, inhibiting and regulating cell cycle progression (Morgan, 1997).

pRb arrests cell cycle by inhibiting E2F from inducing DNA replication factors such as CDK2, which results in loss of PCNA activity (Angus *et al.*, 2004). pRb is

important and thought to be dispensable in self-renewal and differentiation such as occurs in hematopoietic stem cells (Walkley and Orkin, 2006). pRb is required for normal development as mutation of *Rb* leads to multiple defects in mouse embryo development (Lee *et al.*, 1992). Homozygous mutation of the *Rb* gene causes defects as distinct as neuronal cell death and defective erythropoiesis leading to embryo death between the 14^{th} and 15^{th} days of gestation that is approximately the time first differentiated cells appear (Jacks *et al.*, 1992). pRb is also required for appropriate exit of retinal progenitor cells from the cell cycle and for rod development in a cell-autonomous manner (Zhang *et al.*, 2004). Entry of human fibroblasts into senescence is dependent on inactivation of p110Rb (unphosphorylated form of pRb) as well (Stein *et al.*, 1990). Inactivation of pRb-dependent tumor suppressor pathways is very common in human cancers. It is not surprising, that pRb pathway defects are among the most common defects found in breast cancer.

Two CKI families which play important roles in regulating cell division (Vidal and Koff, 2000) are the INK4 family and the KIP/CIP family. INK4 family inhibitors inhibit CDK4 and CDK6, while KIPs inhibit CDK1, CDK2 and CDK4. The INK4 family consists of p16 (INK4A), p15 (INK4B), p18 (INK4C), and p19 (INK4D). The KIP family consists of p21 (CIP1), p27 (KIP1), and p57 (KIP2). All of these CKI members are known or suspected tumor suppressor genes (Vidal and Koff, 2000; Mainprize *et al.*, 2001).

Section-2 Tumor Suppressor Genes and Cancer

Tumor suppressor genes and oncogenes are important factors for regulating transitions in and out of the cell cycle and have a role in regulating the gateway to terminal differentiation (Tripathy and Benz, 1992). Defects in tumor suppressor genes and proto-oncogenes result in uncontrolled cell division, which leads to cancer (Tripathy and Benz, 1992). Proto-oncogenes have a role in malignancy of tumors and most frequently regulate cell cycle re-entry. Gain-of-function (to gain a new and abnormal function) mutations result in transformation of proto-oncogenes into dominant oncogenes. In contrast tumor suppressor genes encode proteins that suppress cell growth and most frequently result in exit from the cell cycle. Loss-of-function mutations in tumor suppressor genes result in tumor malignancy and can account in some cases for hereditary cancers or in some individuals, a higher predisposition for cancer. Every gene has two alleles present in the genome (with a few exceptions on the hemizygous regions of the sex chromosomes). In individuals heterozygous for tumor suppressor genes, for suppression to be inactivated, deletion of one allele or somatic mutation of the other allele is required, resulting in a loss of heterozygosity (Swellam *et al.*, 2004), or somatic deletion of both of the alleles is required resulting in a complete loss of homozygosity (Quelle *et al.*, 1997). Tumor suppressor genes can also be inactivated by hypermethylation so that genes can not be transcribed (Herman *et al.*, 1997).

Cancer is caused by the progressive accumulation of multiple gene mutations (Bartek and Lukas, 2001), epigenetic dysregulation of gene mechanisms and protein pathways (Neumeister *et al.*, 2002), and abnormal function and regulation of cell cycle proteins (Nigg, 1995). According to the American Cancer Society (2011), one of the most

prevalent cancers in women is breast cancer. Currently, approximately one out of every three cancers diagnosed in women in the US will be breast cancer. There were estimated to be 230,480 new invasive cases in 2011 and an estimated 39,520 deaths occur due to breast cancer, in the US alone, every year.

Breast cancer is the heterogeneous form of cancer derived from mammary epithelial cells. Mammary glands include two different types of epithelial cells; basal-like and luminal epithelial cells each of which have distinct cytokeratin expression patterns. Most breast cancers originate from luminal epithelium while only 3%-15% of breast cancers originate from basal-like epithelium (Schneider *et al.*, 2008). There are five distinct subtypes of breast cancer that have been characterized in humans; normal-like tumors that resemble normal breast tissue, HER-2-like tumors that overexpress HER-2 receptors, luminal A and B tumors that are estrogen receptor positive, and basal-like tumors that are triple negative (estrogen-receptor negative, progesterone-receptor negative, and HER-2 receptor negative; i.e. all three important breast cancer causing receptors are absent) (Foulkes *et al.*, 2003). Distinct tumor subtypes are associated with different risk factors which manifest different biological behavior and progression.

Hormonal and reproductive factors such as pregnancy history as well as ages of menarche and menopause are major contributors to breast tumor risk (Colditz *et al.*, 2006), Genetic components also have a major role in the onset of breast cancer. Research is ongoing to find the molecular mechanisms involved in breast cancer progression and to discover genetic alterations informative in the early detection, diagnosis, and treatment of breast cancer. It has also been found that 5-10% of breast cancer patients have inherited mutations in breast cancer susceptibility genes such as BRCA1 and BRCA2 (Easton *et*

al., 1995). Immediate relatives of breast cancer patients also have increased risk of developing breast tumors due to the inherited mutation of these breast cancer susceptibility genes (O'Brien, 2000). Individuals carrying single *BRCA1* or *2* mutations have a 40-80% chance of developing breast cancer, which makes them major predictors of hereditary predisposition to breast tumor (Easton *et al.*, 1995). BRCAs proteins are associated with and function within the DNA repair mechanism.

Absence of BRCA proteins results in non-conservative (non-homologous end joining and single strand annealing) and error-prone DNA repair, and increased frequency of non-homologous DNA end joining, which leads to genomic instability (Turner *et al.*, 2005). BRCA1 tumors are usually high grade (rapidly growing and spreading cancer cells) and triple negative (estrogen, progesterone, and human epidermal growth receptor-2 negative) (Turner *et al.*, 2005). BRCA1/2 deficient cells are sensitive to interstrand DNA crosslinking agents such as cisplatin, carboplatin, and poly (ADP-ribose) polymerase inhibitors but, due to secondary *BRCA1/2* mutations, cancer cells become resistant to these drugs (Dhillon *et al.*, 2011). Secondary mutations in BRCA1/2 deficient cancer cells are an example of genetic reversion promoting cell survival. *Smad3* gene encodes a regulatory protein in the transforming growth factor-beta signaling pathway, contributes to increased risk of breast cancer in *BRCA2* mutation carriers (Walker *et al.*, 2010). Allelic imbalance of BRCA1/2 expression contributes to familial ovarian cancer (Shen *et al.*, 2011). Mutation of *BRCA2* results in inhibition of reloading of RAD51 recombinase onto telomeres, eventually resulting in telomere dysfunction and genetic instability in BRCA2 deficient tumor cells (Badie *et al.*, 2010).

Primary tumors are initially benign, but can become malignant and then metastatic where tumors migrate to secondary locations. Metastasis in breast cancer often results in death if left untreated. About 7% of women have metastatic tumors when they are first diagnosed with breast cancer. Breast carcinoma is also the most common spontaneous malignancy in unspayed female dogs, which comprise approximately 52% of all neoplasms in female dogs (MacEwen and Kurzman, 1996). Breast cancer in both women and female dogs has the same etiology (origin of disease). In both species a majority of tumor cases are estrogen receptor or progesterone receptor (ER/PR) dependent. Dogs have no known retroviruses and there is no viral etiology in canine mammary tumors (Bird *et al.*, 2008). Ovariohysterectomy prior to the fourth estrus cycle in dogs reduces risk to 0.05% from 25% (MacEwen, 1990) and canine breast cancers involve the same tissue types as human breast cancer. Both canine and human patients have poor clinical outcomes particularly when metastases have developed. This makes development of novel strategies to better treat and manage mammary cancer a high priority.

Section-3 p16/INK4A/CDKN2A

All of the CKIs are proven tumor suppressor genes or are suspected of having this potential. One of the most important CKIs is p16 a 16 kDa protein. *p16* is a tumor suppressor gene and is encoded on the 9p21 region of the human genome (Serrano *et al.*, 1993; Kamb *et al.*, 1994) at the *INK4A/ARF/INK4B* gene locus. This locus is a 35kb multigene region which encodes three distinct tumor suppressor proteins p15 (15 kDa), p14ARF (14 kDa), and p16 (16 kDa) proteins (Sherr and Weber, 2000). *p15* has its own open reading frame and is physically distinct, but *p14ARF* and *p16* share a common

second and third exon although each has a different and unique first exon (Kim and Sharpless, 2006). p16 is encoded by exon1α of that gene locus, while p14ARF is encoded by exon1β of the same gene locus. p16 is a 16kDa and p14ARF is a 14kDa protein. The proteins are encoded by alternative reading frames; therefore, the proteins are different; they have no amino acid homology, despite sharing substantial nucleic acid sequence. INK4A/ARF locus remains the only such example in eukaryotes outside of their viruses.

p16/INK4A/CDKN2A checks the cell cycle in early G1 phase and inhibits further transition of the cell cycle from G1 to S phase as a component of a multi-protein regulatory complex. During G1 phase, CDK4 and CDK6 form complexes with cyclinD1 which in turn phosphorylate the Rb protein family. This results in additional phosphorylation by cyclinE/CDK complexes and release of the E2F transcription factor from pRb/E2F complexes. pRb otherwise inhibits transcription factor E2F arresting the cell cycle progression (Weinberg, 1995). E2F is a transcription factor that initiates transcription of genes required for S phase such as DNA polymerase, thymidine kinase, dihydrofolate reductase, replication origin binding protein HsOrc1 and MCM (Lukas *et al.*, 1996). The action of p16 inhibits binding of CDK4/6 with cyclinD1, which leaves pRb and pRb related proteins such as p107 and p103 un-phosphorylated, and E2F bound and inactive (Serrano *et al.*, 1993; Walkley and Orkin, 2006). p16 binds opposite to the cyclin binding site, next to the ATP binding site of the catalytic cleft of CDK, which results in a structural change in the cyclin binding-site (Russo *et al.*, 1998). p16/INK4A targets CDK4 and CDK6, rather than the cyclin subunit and actually competes with cyclinD1 for CDK binding. Binding of p16 results in changes in conformation of CDK proteins so that they can no longer bind cyclinD1 (Russo *et al.*, 1998). It does so by

distorting the kinase catalytic cleft, thereby interfering with ATP binding. In this manner it may also deactivate pre-assembled CDK4/6-cyclinD1 complexes, blocking their function (Russo *et al.*, 1998). Binding sites for p16 and cyclinD1 on CDK4 are overlapping in some cases and are present near the amino terminus where a majority of the mutations in CDK4 are found. Mutations in the p16 binding site result in diminished capability of p16 binding to CDK4, and compromise the binding of cyclinD1 to CDK4, which can lead to melanoma (Coleman *et al.*, 1997; Tsao *et al.*, 1998).

p16 encodes four or five ankyrin repeats (Russo *et al.*, 1998). Ankyrin repeats are 30 amino acid structural motifs that resemble the letter 'L' with a stem made of a pair of anti-parallel helices with a beta-hairpin region forming the base (Russo *et al.*, 1998). p16 interacts with the N and C terminal lobes of CDK6 and binds to one side of the catalytic cleft opposite to the cyclin binding site. CDK6 bound to p16 is inactive, because it cannot bind to cyclin and is not phosphorylated; thus, proliferation is suppressed (Russo *et al.*, 1998). p16/INK4A also exerts transcriptional control over cyclinD1. Inactivation of cyclinD1 by p16 is independent of its CDK4 inactivating properties and requires a cAMP-response element/activating transcription factor-2-binding site (D'Amico *et al.*, 2004).

p14ARF inhibits MDM2, which results in stabilization of the important tumor suppressor p53. p53 is a transcription factor, which activates expression of proteins required for cell-cycle inhibition and apoptosis and is itself a tumor suppressor gene (Boehme and Blattner, 2009). One of the downstream regulatory protein activations mediated by p53 is p21 up-regulation. p21 upregualation stops the cell cycle late in G1/S phase transition. p53 also acts as a transcription repressor of other genes (Gomez-Lazaro

et al., 2004). p53 is more stable in human mammary epithelial cells than in human fibroblasts, which underscores the importance of p53 in mammary epithelial cell growth (Delmolino *et al.*, 1993). Under normal conditions, p53 is rapidly degraded to keep its protein level low, mediated through the E3 ubiquitin ligase MDM2. Under conditions of cellular stress, or any cellular dysfunction, p14ARF binds to MDM2; thus, releasing and stabilizing p53 by blocking MDM2. Wild type p53-induced phosphatase 1 (Wip1/Ppm1d) stabilizes MDM2 and downregulates p53 expression (Lin *et al.*, 2007). Disruption of Wip1 activates p53, p16, and p14ARF pathways through p38MAPK signaling, and suppresses mouse embryo fibroblast transformation by oncogenes *in-vivo* (Bulavin *et al.*, 2004).

p14ARF expression is not directly involved in the response to DNA damage although p53 negatively regulates p14ARF expression and both of them have an inverse correlation with each other with respect to activity (Stott *et al.*, 1998). The function of p14ARF is not limited to p53 because p14ARF also has other independent roles such as vascular regression of the developing eye (McKeller *et al.*, 2002) and arrest of cell cycle in embryo fibroblasts in the absence of p53 (Weber *et al.*, 2000). Other than MDM2, p14ARF also binds to E2F-1, MDMX, HIF1-α, topoisomerase I, myc, and nucleophosmine (NPM) (Boehme and Blattner, 2009). p19ARF (the mouse ortholog of human p14ARF) is able to induce cell cycle arrest in mammalian fibroblasts analogous to p16 (Quelle *et al.*, 1995). E2F induces cell proliferation by activating S phase regulatory proteins but, according to one report, may have other activities. For instance, E2F induces senescence in human diploid fibroblasts by inducing p14ARF expression, which is required for p53 stabilization (Dimri *et al.*, 2000). *Id1* (inhibitor of DNA-binding)

encodes a helix-loop-helix transcription factor that is overexpressed in high grade breast tumors and estrogen receptor-negative diseases (Gupta *et al.*, 2007). Overexpression of Id1 or inactivation of the p14ARF-p53-p21 pathway can also reverse senescence induced by ras signaling in mouse mammary carcinoma (Swarbrick *et al.*, 2008).

Other than p16 and p14ARF transcription from the INK4A locus, there is one more alternative transcript that is derived from this gene in human lymphoblastic leukemia which is named *p16 gamma* (p16γ) (Lin *et al.*, 2007). p16γ has been demonstrated to be expressed at both transcriptional and translational levels confirming its functional potential (Lin *et al.*, 2007). *p16γ* shares the same exon 1α, exon 2, and exon 3 as p16 but with a 197 bp insertion between exon 2 and 3, due to an alternative splicing event that included sequence from the intron. p16γ is also an ankyrin-repeat protein and interacts with CDK4. p16γ suppresses E2F activity and induces cell cycle arrest like p16/INK4A. It is still not known what functional characteristics distinguishes these 2 transcripts.

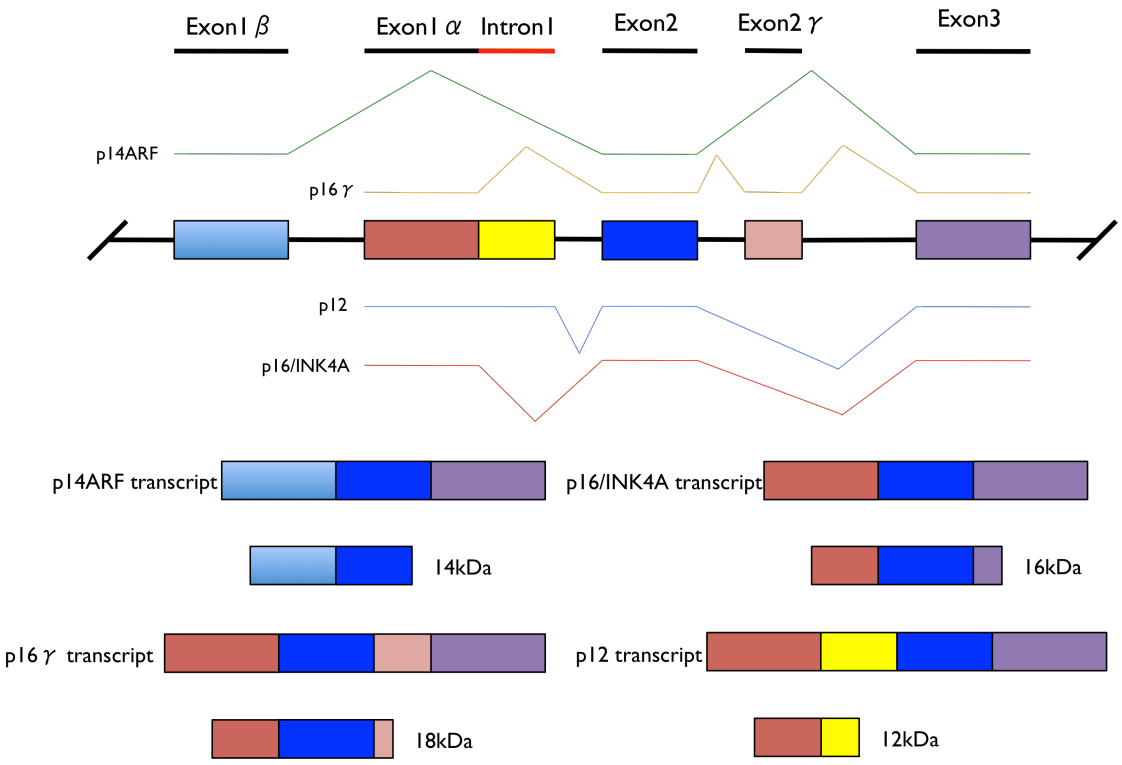

Figure 1: Alternative transcription from the *INK4A/ARF* gene locus.

Schematic presentation of all the four different transcripts, transcribed from the common *INK4A/ARF* gene locus. *p14ARF* and *p16/INK4A* share exon 2 and exon 3, but differ in their first exons. *p14ARF* includes exon 1β while p16 includes exon 1α. The stop codon for p14ARF reading frame is located in exon2. The stop codon for the p16/INK4A is located in exon 3. Another transcript transcribed from this locus is *p16γ*, which has an extra exon (exon2γ) along with all three exons of the *p16/INK4A* transcript. Exon2γ (197bp) is located in intron 2 between exon 2 and exon 3. *p16γ* encodes a 18 kDa protein. The smallest 12 kDa protein encoded from INK4A locus is p12. *p12* shares the first exon, exon1α with *p16*, but first exon of *p12* transcribes little longer in intron 1 to give an additional 274bp sequence. The stop codon for p12 is located in the additional intron 1α sequence. It introduces an earlier stop codon and encoding a 12 kDa protein inspite of having a longer transcript than p16/INK4A.

There is an alternative splice variant of p16 present in human pancreas as well, known as p12 (Robertson and Jones, 1999). p12 is a 12 kDa size protein, which is encoded from the same *INK4/ARF* locus. The p12 gene shares the p16 promoter, 5'UTR, ATG-start codon and exon 1α, and uses the alternative splice donor site to splice to exon 2. The extra sequence encodes a premature stop codon that results in a smaller protein. p12 shares the first ankyrin repeat with p16 but is not predicted to bind to CDK4 or CDK6 based on crystal structure studies. p12 is reported to suppress cell growth but in a pRb-independent mechanism (Sharpless, 2005). When the effect of ectopic expression of all the three transcripts, p16, p14ARF, and p12 was compared, p16 had the most inhibitory effects on cell growth of the human lung cancer cell line A549 (Zhang *et al.*, 2010).

Less is known regarding the functions of other INK4 family members. Expression of other members of the *INK4* gene family, such as *p18* and *p19*, have been shown to predominate during early to mid-gestation in mouse development (Zindy *et al.*, 1997) while expression of p15 has been found in later stages of gestation (Zindy *et al.*, 1997). Circumstantially, it appears that different INK4 proteins are not functionally redundant as they appear to be expressed during different periods of development and may also be expressed in distinct tissue-specific profiles. Expression of p15 is down-regulated during human lymhocyte mitogenesis with a marked increase in retinablastoma kinase activity providing a potential role for p15 in cell cycle arrest. p15 and p27 levels were decreased during lymphocyte activation and appear important in maintaining cell quiescence (Lois *et al.*, 1995). Although p15 acts as a tumor suppressor, the frequency of mutations and defects in p15 in tumor cells appears lower than p16 (Stone *et al.*, 1995). Overexpression

of p15 can induce cell cycle arrest in cancer cells (Thullberg *et al.*, 2000), TGF-β-mediated cell cycle arrest in human keratenocytes (HaCaT) (Hannon and Beach, 1994), and cell cycle arrest by the pyrido-pyrimidine derivative JTP-70902 in the human colon cancer cell line HT-29 (Yamaguchi *et al.*, 2007). p18 inhibits the CDK-cyclin binding site by distorting the ATP binding site and by misaligning catalytic residues. p18 can also distort the cyclin-binding site of CDKs by reducing the size of the interface of bound cyclin (Jeffrey *et al.*, 2000).

Section-4 Cell Cycle fate

Although expression appears temporarily distinct during development, the INK4 and CIP cyclin dependent kinase inhibitor families appears to have overlapping roles of cell cycle arrest in mouse embryonic fibroblasts. Loss of both INK4 (p15, p16, and p18) and CIP (p21) promotes pRB inactivation, cell immortalization, and H-rasV12/c-myc-induced loss of contact inhibition. However, loss of both families of CKIs is still only weakly able to cause cell immortalization, largely due to an accompanying induction of active apoptosis (Carbone *et al.*, 2007).

It is hypothesized that cells have an internal clock mechanism, which directs the cell toward four possible fates: senescence, differentiation, quiescence, or the death pathway (apoptosis). Differentiation is the developmental process through which cells gradually restrict their fate to a single terminal post-mitotic cell type. Cells keep on dividing until they reach one of the above listed non-proliferative states. Oncogenes normally promote cell cycle re-entry and progression and their inhibition is required to

exit cell cycle. Cyclin-dependent kinase inhibitors (CKIs) and other tumor suppressor genes promote cell cycle exit and thus oppose the activities of many oncogenes.

A majority of the cells in the adult organisms's body remain in the quiescent or differentiated cell stage such as post-mitotic neuronal cells or skeletal muscle cells. This shows the importance of cell cycle exit in multi-cellular organisms and cell differentiation. Cell fate begins to be decided, and is progressively narrowed during the early embryonic stages of the developmental pathway as cell fate is closely linked to developmental stage and position. Although much remains to discovered, as proliferating cells decide to progress from G1 to S phase, the decision to proceed through another cell cycle to complete mitosis or to exit the cell cycle must be made.

Determining the distinct roles of individual genes in cell fate during the course of differentiation has great significance in understanding normal cell growth and development, as well as the development of cancer. In adults, cell damage and death are compensated as cell populations are maintained at constant levels through division of adult tissue somatic stem cells. Somatic stem cells are undifferentiated cells found among differentiated cells in tissues. Regeneration of cells in tissues and organs from somatic stem cells occurs in two ways: 1) monophasic or 2) biphasic regenerative cell proliferation and differentiation. Stem cells of skin and intestine undergo monophasic regenerative cycles in which the rate of cell proliferation is approximately equal to the rate of cell death and shedding (Barker *et al.*, 2008). This results in almost no variation in the size of tissues and organs throughout the life span of the organisms once adulthood is reached. On the other hand throughout female reproductive life, the lactogenic portion of the mammary gland undergoes biphasic regenerative cycles of cell proliferation and

differentiation which results in significant expansion and contraction of the breast epithelia (Smalley and Ashworth, 2003; Harmes and DiRenzo, 2009).

During regeneration from adult tissue stem cells, one stem cell divides into two progeny cells, each of which have distinct cell fates due to asymmetric cell mitosis (Lin, 2008). One of the daughter cells undergoes proliferation and differentiation to fulfill the need for tissue cell replacement and loses self-renewing capacity. The other daughter cell preserves its replicative capacity, but becomes quiescent and resists differentiation. Thus, it restores the stem cell pool of the tissue. Quiescence of stem cells, coupled with simultaneous evasion of differentiation signals, suggests a close and perhaps reciprocal link between cell quiescence and differentiation (Harmes and DiRenzo, 2009). Stem cells in monophasic regenerative cell cycles proliferate continuously, expressing cell proliferation markers Ki67 and telomerase reverse transcriptase (TERT) (Davenport *et al.*, 2003; Flores *et al.*, 2005). On the other hand, in mammary tissues, regeneration occurs in a narrow window of cell expansion followed by retirement to cell quiescence to insure future regenerative cycles. Mammary stem cells in the resting stage have low or undetectable Ki-67 and hTERT expression, characteristic of a nonproliferative cell compartment (Kolquist *et al.*, 1998). Mammary stem cells utilize quiescence for preservation of self-renewal. The micro-environment surrounding the stem cells in mammary tissues plays an important role in establishment of cell quiescence and physical dislocation of cells from this niche, which results in cell proliferation and differentiation. The point at which cell fate determination takes place between quiescence and differentiation and the mechanisms that regulate it are still unknown. Quiescence in breast tissue resists terminal differentiation, confering a longer-term resting, self-

renewing capacity. The preserved replicative capacity of breast tissue and its resistance to differentiation over an extended period, may promote accumulation of mutations as well as making breast tissue stem cells vulnerable to breast tumor initiation (Pardal *et al.*, 2003).

Cell Quiescence/Cell Cycle Exit

The quiescent stage of any cells is characterized by an absence of DNA synthesis, lower metabolism, and smaller cell size (Yusuf and Fruman, 2003). Cell quiescence can be induced experimentally by serum-starvation, contact inhibition, and loss of adhesion (Coller *et al.*, 2006). During the G1 to S phase transition, a cell must pass through the restriction point (R) before it can retire to the G0 phase. Once cells have crossed the R point, they are destined to reach S phase even in the absence of mitogens (Zetterberg *et al.*, 1995; Boonstra, 2003). The most likely purpose of the R point mechanism is to regulate the threshold of hyper-phosphorylated pRb. pRb is non-phosphorylated in G0 phase, hypo-phosphorylated (~2 mol of PO_4 to 1 mol of pRb) during G1 progression, and hyper-phosphorylated (~10 mol of PO_4 to 1 mol of pRb) in late G1 phase, when it becomes inactive (Ezhevsky *et al.*, 2001).

Another hallmark of cell quiescence is a decrease in cyclinD1 concentration (Boonstra, 2003; Pajalunga *et al.*, 2007) which contributes to the unphosphorylated state of pRb in the absence of cyclinD1/CDK complexes. CyclinD1 expression is generally required for S-phase re-entry (as for example in neonatal vestibular hair cells in mice) and thus cyclinD1 suppression is critical for promoting cell quiescence (Laine *et al.*, 2010). pRb family members also have a significant role in cell quiescence. This is

particularly the pRb family member p130. p130 is expressed at high levels in quiescent cells as is accumulation of unphosphorylated pRb which also occurs in the quiescent phase (Smith *et al.*, 1996; Mayol and Grana, 1998; Stevaux and Dyson, 2002). Like pRb, p130 also binds E2F4 and other E2F family members which has a repressor effect on genes required for S phase entry. As *Rb* knockout mice showed a loss of function in promoting quiescence of cells, both pRb and p130 appear necessary to maintain quiescence (Sage *et al.*, 2000).

Cyclin dependent kinase inhibitors (CKI) are important in maintaining cell quiescence, as they function as upstream regulators of the CDKs and cyclins. p27 functions by sequestering the CDK/cyclinD1 complex, which leaves pRb unphosphorylated resulting in cell quiescence. In quiescence, the level of p27 increases and then gradually decreases as cells re-enter the cell cycle and G1 progresses. Such modulations of the levels of p27 have been reported in a variety of cell types including peripheral blood T cells (Sherr and Roberts, 1995) and increased p27 has also been induced in serum-starved murine fibroblasts (Coats *et al.*, 1996; Rivard *et al.*, 1996). Reduction of p27 levels by antisense p27 treatment promotes S-phase re-entry in NIH 3T3 fibroblasts (Ladha *et al.*, 1998). Fibroblasts initiated proliferation when treated with antisense oligonucleotides targeting p27 mRNA suggesting it is required for cells to pass the R point. Up-regulated p27 maintains the quiescent stage of newly differentiated beta-cells generated during embryogenesis in mice (Georgia and Bhushan, 2006) and can induce quiescence in hydroxytamoxifen-treated breast cancer cells (Carroll *et al.*, 2003). Tamoxifen is a selective estrogen-receptor modulator which is effective in treatment and prevention of estrogen-receptor-positive breast cancer. Withdrawal of granulosa cells of

the ovaries from the cell cycle is possible only in the presence of CKI p27 and p21 and their expression is also required for luteal differentiation and possibly also for culture-induced senescence (Jirawatnotai *et al.*, 2003).

In T-cell mitogenesis, repression of p27 is required to activate the cyclin/CDK complex necessary for cell cycle progression (Nourse *et al.*, 1994). p27 downregulation is required for S phase re-entry from G0 phase. This downregulation can be achieved by sequestration of p27 by cyclinD2/CDK4 complex following increased expression of cyclinD1. CyclinD1 can be up-regulated by the action of a mitogen such as c-myc (Bouchard *et al.*, 1999). But cyclinD1 alone can not induce downregulation of p27, suggesting a role in regulation for cyclinD1 although other factors appear required (Ladha *et al.*, 1998). Downregulation of p27 is mediated by the PI3-kinase pathway through transcriptional inhibition and post-translational modification (Takuwa and Takuwa, 1997; Lea *et al.*, 2003). Id2 is a member of the helix-loop-helix transcription regulators which cause cell proliferation, induce tumorigenesis and suppress p21 and p27 expression. Re-expression of p27 can reverse both of these hyper-proliferative and tumorigenic phenotypes (Trabosh *et al.*, 2009).

CKI p21 is also important in cell quiescence and is an integral part of quiescence regulation in a variety of cell types including mouse hematopoietic stem cells (Cheng *et al.*, 2000). Depletion of p21 influences quiescence in human foreskin fibroblasts (Pajalunga *et al.*, 2007) while antisense p21 leads to cell proliferation in human fibroblasts (Nakanishi *et al.*, 1995). p27 and p21 are frequently functionally homologous and appear at least partially redundant as they can compensate for each other (Blomen and Boonstra, 2007). For example, p21 induces quiescence in p27 deficient mouse

hepatocytes (Kwon *et al.*, 2002) and p21 can also induce telomere shortening. Therefore, deletion of p21 can prolong life-span in telomerase deficient mice with dysfunctional telomeres (Choudhury *et al.*, 2007).

Cell Senescence

As cells approach their replicative limit they become senescent (Hayflick, 1965; Smith and Pereira-Smith, 1996; Beausejour *et al.*, 2003). Senescent cells cannot re-enter cell division under any physiological conditions. Unlike quiescent cells, senescent cells have a large flattened phenotype with high metabolism levels (Cristofalo *et al.*, 2004). Senescent cells have low cyclinD1 levels but have up-regulated lysozomal enzyme β-galactosidase (Stein *et al.*, 1999; Serrano and Blasco, 2001) which is therefore commonly recognized as a marker of senescence. Senescence is induced by DNA damage or telomerase depletion typical of aging or oncogenic stress (post-traumatic stress) (Serrano and Blasco, 2001). Senescence perevents cell proliferation and therefore is antagonistic to neoplastic growth. Senescent cells cease proliferation but do not die like apoptotic cells; rather they remain metabolically active. Senescent cells have low cyclinE and CDK2 kinase activities as well rendering them incapable of phophorylating pRb to block expression of late G1 phase genes such as cyclinA which are required for entry into S phase (Dulic *et al.*, 1993).

Two CKIs that also have an important role in senescence are p21 and p16. p21 acts as the mediator of cell cycle arrest prior to senescence, while p16 induces and maintains the senescent phenotype (Stein *et al.*, 1999). p53 also has a role in cell senescence in response to telomere depletion and hyper-mitogenic arrest because p53

upregulates p21 (Campisi, 2005). Cell quiescence can result from a lack of mitogen (a chemical substance that triggers mitosis) induction while senescence on the other hand is a result of over-stimulation of mitogens (Blagosklonny, 2006). Over-stimulation of mitogens activates MAPK pathways, which in turn induces cyclinD levels along with increased levels of CKIs. Increased levels of CKIs result in hyper-mitogenic cell cycle arrest which eventually leads to cell senescence; therefore, senescent cells are physiologically active, functionally metabolic, and larger in size than proliferating cells but merely irreversibly arrested with respect to cell cycle with the characteristics of late G1 cells (Blagosklonny, 2006).

Cell Differentiation

The third possible fate of cells in G1 phase is cell differentiation. Cell differentiation is also an irreversible non-proliferative state similar to senescence, but differentiated cells are distinct from senescent cells in that they are phenotypically specialized. Differentiated cells are no longer functionally comparable with their precursor cells after they acquire specialized functions. Like senescence and quiescence, differentiation is also induced by the activity of CKIs; mainly p27 and p21. Cells in the organ of Corti in the auditory neuroepithelium of the inner ear in mammals, require p27 expression for withdrawal from the cell cycle at just the right moment (between embryonic day 13 and E14) in differentiation (Chen and Segil, 1999). Similarly, in pituitary progenitor cells, p27 plays a significant role in cell differentiation (Bilodeau *et al.*, 2009). In *Xenopus* retinogenesis, p27XIc1 (the Xenopus counterpart of p27) functions in cell fate determination during gliogenesis and neurogenesis (Ohnuma *et al.*, 1999). p27 is also involved in differentiation of erythroid precursors (Denicourt and Dowdy, 2004).

Section-5 Role of p16 as Tumor Suppressor Gene

As noted earlier, CKI *p16* is an important tumor suppressor gene, defects of which are associated with cancer. *p16* defects are second in frequency only to those in *p53* for human malignancies (Baylin *et al.*, 1998). *p16* gene deletions are associated with, and appear permissive for, late-stage, high-grade cancers (Gruis *et al.*, 1995; Swellam *et al.*, 2004). Deletion of the 9p21 region, encoding p16 in humans, results in tumor formation in a wide range of cell types (Kamb *et al.*, 1994). Loss of heterozygosity (Swellam *et al.*, 2004), loss of homozygosity (Ranade *et al.*, 1995; Quelle *et al.*, 1997), and hypermethylation of the promoter (Herman *et al.*, 1997) in the 9p21 region, are all important mechanisms which result in loss of p16 expression and p16-associated neoplasms. Frequency of loss of p16 is high in pre-malignant lesions, suggesting the importance of loss of p16 activity as an early event in cancer progression (Liggett and Sidransky, 1998). The evaluation of p16 expression could have value as an early prognostic indicator for predicting cancer recurrence (Bartoletti *et al.*, 2007). Hypermethylation of CpG islands in the *p16* promoter can result in enhanced cell proliferation in human colorectal cancer and can activate DNA demethylation in the invasive region suppressing proliferation, but enhancing tumor invasion (Jie *et al.*, 2007). Additionally, *INK4A/ARF* region hypermethylation occurs frequently in mammary epithelial cells in high risk women with sporadic (occurring ocassionaly) breast cancer (Bean *et al.*, 2007; Jing *et al.*, 2007; Sharma *et al.*, 2007).

K-cyclin (ORF72) is a human homolog of cyclinD1 in Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8), which is oncogenic in immune-suppressed individuals. p16 inhibits the unphosphorylated CDK6-K-cyclin complex. The functional

availability of K-cyclin to induce tumorogenesis is largely dependent upon the balance between expression of p16 and CDK6 (Yoshioka *et al.*, 2010). This complex is normally resistant to p21 and p27 and can phosphorylate both of them explaining the important role of *p16* as a tumor suppressor gene in regulating malignancies induced by KSHV.

Mutations in the *p16* encoding gene have also been reported in other cancer types such as glioblastomas, melanoma-prone pedigrees, and pancreatic adenocarcinomas. Allelic variants of p16 in melanoma-prone pedigrees have also been found, which have lower affinity and thus lower activity in interactions with CDK4 and CDK6. These p16 allelic variants with decreased CDK interaction predispose individuals carrying these defective alleles to increased risk of cancer, reinforcing the important role of *p16* as a tumor suppressor gene (Reymond and Brent, 1995). Mutations in CDK4 prevent p16 binding to CDK4 and have been identified at several noncontiguous amino acid sequences. Mutated CDK4 has oncogenic potential and occurs spontaneously (naturally occurring over the lifespan of an organism) in melanomas and other neoplasms (Ceha *et al.*, 1998).

Matrix metalloproteinases (MMPs) are the zinc-dependent endopeptidases which are capable of degrading components of the extracellular matrix (Ketelhuth and Back, 2011). The MMP family is composed of at least 20 enzymes. One of the MMP family enzymes is MMP-2, which has been reported to be strongly linked with various types of human cancers, such as glioma (Uhm *et al.*, 1996) and astrogliomas (Qin *et al.*, 1998). p16 represses expression of MMP-2 and invasiveness of gliomas (Chintala *et al.*, 1997) by blocking the transcription factor Sp1 to inhibit gene transcription of MMP-2 (Wang *et al.*, 2006). Thus, along with inhibiting cyclin-CDK complexes to suppress the cell cycle,

p16 can also suppress tumor invasion through other independent cell regulatory functions.

Rab proteins are members of the Ras-related small GTPase family. Rab27A is also linked with human genetic diseases (Seabra *et al.*, 2002). Rab27A is associated with invasive and metastatic breast cancer which is facilitated by down-regulation of p16 and up-regulation of cyclinD1 (Wang *et al.*, 2008). Surprisingly, concomitant overexpression of p16 and p73 has oncogenic potential with poor tumor characteristics affecting the development and growth of breast carcinomas (Garcia *et al.*, 2004).

Alteration of p15 and p16 expression and overexpression of TGF-α have all been frequently found in schistosoma bladder cancers and squamous cell carcinomas (Swellam *et al.*, 2004). Additionally loss of p16 expression has prognostic value in predicting recurrence-free probability in patients affected by low-grade urothelial bladder cancer (Bartoletti *et al.*, 2007). p16 has been reported to be inactivated in human colorectal cancer, but p16 expression is elevated by the demethylation of the p16 promoter in invasive cancer cells, making these cells cease proliferation at the invasive front and thus characterizing p16 as a cell growth suppressor (Jie *et al.*, 2007).

β-Catenin is the key downstream effector of Wnt signaling pathway involved in embryogenesic and cancer. β-Catenin is a potent oncogene. β-Catenin can also inhibit cell proliferation by activating the p14ARF-p53-p21 pathway during squamous cell differentiation of endometrial carcinoma cells (Em Ca) (Saegusa *et al.*, 2005). p16 is also induced along with loss of pRb expression in trans-differentiation of endometrial carcinoma cells, mediated by β-catenin and p21 (Saegusa *et al.*, 2006). Deletion of p16

has been reported in high-grade B-cell non-Hodgkin's lymphoma (Fosmire *et al.*, 2007) along with increases in pRb phosphorylation at the CDK4 sites but is less prevalent in low-grade tumors (Modiano *et al.*, 2007). p16 inactivation is more frequent in blastoid mantle cell lymphoma.

Section-6 Role of p16 in Cell Senescence

p16 expression has been shown to promote premature cell senescence (Zindy *et al.*, 1997). The level of p16 expression increases as mouse embryonic fibroblasts reach replicative senescence in culture (Zindy *et al.*, 1997). Immortalized fibroblast (NIH3T3) and tumor cell lines frequently lack *p16* gene expression suggesting removal of p16 as a potential way to bypass senescence and also pointing toward the importance of p16 as a tumor suppressor gene (Kamb *et al.*, 1994; Nobori *et al.*, 1994; Zindy *et al.*, 1997). T box proteins (Tbx2) and polycomb proteins (BMI1, Cbx7, Mel18) have been reported to be repressors of all three genes of the *INK4* locus (*p16*, *p14ARF*, and *p15*) (Jacobs *et al.*, 1999; Angus *et al.*, 2004). *Bmi-1* encodes the polycomb protein, which represses *p16* and *p14ARF* and is linked with regulation of the replicative life span of human fibroblasts (Itahana *et al.*, 2003). BMI-1 represses *p16* by binding directly to the BMI-1 responding element (BRE), within the *p16* promoter (Meng *et al.*, 2010), and is dependent on the continued presence of EZH2-containing Polycomb-Repressive Complex 2 (PRC2) complex (Bracken *et al.*, 2007).

Under the stimulus of stress and senescence, downregulated EZH2 levels coincide with upregulated p16. PRC2 and PRC1 complexes, localized at the regulatory domain of the *p16* gene, are lost when cells enter senescence, which in turn results in decreased

levels of histone and increased expression of histone H3K27 trimethylation (H3K27me3) and histone demethylase Jmjd3 with the recruitment of the MLL1 protein (Agherbi *et al.*, 2009). Polycomb proteins are recruited to the *INK4/ARF* locus through CDC6 and, upon senescence with an increase in Jmjd3 levels, MLL1 protein is recruited to the locus provoking dissociation of polycomb protein from the INK4/ARF locus. This leads to transcription and replication of the *INK4/ARF* locus in early S phase (Agherbi *et al.*, 2009). CDC6 is an essential DNA replication regulator. CDC6 overexpression induces increased *INK4/ARF* tumor suppressor gene expression through epigenetic modification of chromatin at the *INK4/ARF* locus (Borlado and Mendez, 2008). COOH–terminal-binding protein (CtBP), a physiologically regulated co-repressor, has also been reported to have a significant role in *p16* repression. Several of the pathways noted above repress p16 via CtBP-mediated repression as CtBP forms bridges between proteins having PxDLS amino acid motifs, including several transcription factors and other proteins involved in transcription (Mroz *et al.*, 2008).

The levels of p27 and p16 proteins are significantly increased in contact-inhibited human fibroblasts although p16 levels were low in serum-deprived human fibroblasts. In both cases, even though the mechanisms of growth arrest are different, they both affect the same pathway involving CDK4, cyclinD1, and pRb (Dietrich *et al.*, 1997). Maintenance of p16 and p27 concentration contributes to the low levels of proliferation in normal blood vessels (Izzard *et al.*, 2002) and p16 mRNA and protein accumulate in human fibroblasts as they become senescent (Hara *et al.*, 1996). As noted earlier cell senescence is a permanent resting phase of cell and is related to cell aging (Smith and Pereira-Smith, 1996). Accumulation of p16 is also associated with replicative senescence.

Increased p16 expression has been found in lymphocytes only a few cell doublings before replicative senescence (Chebel *et al.*, 2007). Senescence can also be induced by DNA replication stress or by oncogene expression. Oncogene induced senescence is linked with elevated p16 and p14ARF expression (Serrano, 1997).

Section-7 Role of p16 in Cell Quiescence

p16 checks the cell cycle at the G1/S phase transition and thus has an important role in cell cycle exit and quiescence. Ectopic p16 expression prevents re-entry into the cell cycle (Lea *et al.*, 2003) and p16 expression can induce a G0-like state in hematopoietic cells (Furukawa *et al.*, 2000). p16 expression is up-regulated by oxidative stress, aging, UV exposure, ionizing radiation, chemotherapeutic agents, telomere dysfunction, and wound healing (Kim and Sharpless, 2006; Natarajan *et al.*, 2006). p16 is induced by MAPK activation in response to stimulation of the ERK/MAPK pathway through RAS/RAF signaling. RAS activation induces p16 expression through ERK mediated activation of Ets1/2 (Ohtani *et al.*, 2001) and induces p14ARF expression through Jun-mediated activation of DMP1 (Sreeramaneni *et al.*, 2005). Histone acetyltransferases (HATs), such as p300/CBP, are important transcriptional up-regulators. The GC-rich region in the p16 promoter is the putative binding site for transcription factor Sp1 (Gizard *et al.*, 2005). p300, in cooperation with Sp1, transcriptionally upregulates p16 expression and induces cell cycle arrest in HeLa cells (Kivinen *et al.*, 1999; Wang *et al.*, 2008). Smooth muscle cells in mature arteries in rat have low rates of proliferation. Suppression of proliferation is dependent on the up-regulated levels of p16 and p27 which makes these cells unable to activate cyclinD1 and cyclinE and their associated kinase activties (Izzard *et al.*, 2002).

p16 has also been associated with a variety of additional cell proliferation control proteins. SEI-1/p34/TRIP-Br1 protein induces CDK4-mediated pRb phosphorylation through physical binding, independent of p16 (Li *et al.*, 2005). SEI-1 facilitates CDK4 function, making it resistant to p16 inhibition. ISOC2 protein binds and co-localizes with p16 inhibiting the function of p16 (Huang *et al.*, 2007). Other than ISOC2, p16 protein has also been found to bind to proliferating cell nuclear antigen (PCNA) and minichromosome maintenance protein 6 (MCM6) (Souza-Rodrigues *et al.*, 2007). p16 interacts with DNA polymerase δ accessory protein PCNA, and thus inhibits the function of DNA polymerase.

p16 is normally localized in the nucleus where it functions as an inhibitor of CDK/cyclin complexes but it has also been reported that p16 is localized in the cytoplasm (Nilsson and Landberg, 2006) . Both cytoplasmic and nuclear p16 bind CDK6 and have a role in cell cycle arrest. Human melanocytes initiate differentiation by activation of the cytoplasmic cAMP synthesis pathway. Activation of the cAMP pathway results in increased association of p16 and p27 with CDK4 and CDK2, respectively, pRb phosphorylation failure and decreased expression of E2F proteins with decreased DNA-binding activity (Haddad *et al.*, 1999). Senescence induced by the cAMP pathway in these cells can be attributed to the complex formation of CKI/CDK complexes causing cell cycle exit.

It is known that p16 induces cell cycle arrest via pRb, but there is another way by which p16 can arrest cell cycle independent of pRb. IκBα is a specific inhibitor of NFκB, which competes with p16 for binding to CDK4 and inhibits its activity (Li, 2003). This may suggest that IκBα could substitute for p16 in CDK4 inhibition in malignant cells.

Other than that, in G1 phase, activity of CDKs is required for proper recruitment of mini-chromosome maintenance (MCMs) protein to the origin of replication recognition complex. p16 influences CDKs and thus influences prereplicative complexes (preRC), at the MCM level, resulting in arrest of cell cycle (Braden *et al.*, 2006).

c-Myc is a transcription factor that plays an important role in cell proliferation. c-Myc can induce cell cycle progression from G1 phase to S phase in quiescent cells (Eilers *et al.*, 1991). Oncogenic activity of CDK4 is frequently due to its inability to bind p16 thus failing to inhibit its enzymatic activity. The oncogenic activity of c-Myc and the CDK4/cyclinD1 complex are both required to effectively transform cells. CDK4 requires Myc protein for proper function and similarly, Myc requires the CDK4/cyclinD complex kinase activity to effect tumor cell transformation. p16 inhibits the transcription regulatory activity of c-Myc by blocking cyclinD1/CDK4 complex formation (Haas *et al.*, 1997).

Section-8 Role of p16 in Cell Differentiation

Other than senescence and quiescence, like other CKIs, p16 also has a role in cell differentiation. Expression of p16 increases by several fold in terminally differentiated human adult brain tissue and p16 is thought to play role in human brain development (Lois *et al.*, 1995). During differentiation of human embryonic teratocarcinoma cells (NT2) into post mitotic neurons, expression of p16 and p15 protein levels become elevated (Lois *et al.*, 1995). The role of p16 in melanocyte differentiation has also been investigated. Microphthalmia transcription factor (MITF) is able to induce cell cycle arrest prior to cell differentiation by activation of p16 protein (Loercher *et al.*, 2005).

A-type lamins are intermediate filaments which affect gene expression during differentiation and are thought to function through an pRb-dependent mechanism. pRb associates with a number of tissue-specific transcription factors in an E2F-independent manner and induces differentiation in those tissues. pRb associates with MyoD and Mef2 in skeletal muscle cells (Sellers *et al.*, 1998; Novitch *et al.*, 1999), CBFA1 and Runx2 in osteocytes (Thomas *et al.*, 2001; Thomas *et al.*, 2004), and C/EBP in adipocytes and during macrophage differentiation (Chen *et al.*, 1996). pRb is essential for muscle and fat cell differentiation (Korenjak and Brehm, 2005) and cellular senescence (Ohtani *et al.*, 2001). Cells lacking A-type lamin do not arrest in the presence of p16 in the absence of pRb activity (Nitta *et al.*, 2006). This report suggests a dependence of p16-induced cell cycle arrest on pRb and posits a role for A-type lamins in pRb-dependent cell cycle arrest. CyclinD1, the principle cofactor of p16, targets CDK4/6 and may participate in myoblast differentiation (Rao and Kohtz, 1995). Thus, p16 appears to play a key regulatory role in cell differentiation and senescence through management of cell cycle exit. CDK4/6 also regulates cell division at different stages of erythroid maturation (Malumbres *et al.*, 2004). *CDK4* knock-out mice lack post-natal homeostasis of pituitary somato/lactotrophs and pancreatic B-cells (Jirawatnotai *et al.*, 2004). *CDK6* knock-out mice also have mild defects in hematopoietic cell differentiation. Double deficiency of CDK4/6 in embryos appears to have no effect on organogenesis and associated cell proliferation although they are lethal due to defects in the erythroid lineage (Malumbres *et al.*, 2004). Thus, CDK4/6 are required for specific tissue differentiation along with cell cycle progression. It has also been reported that CDK4 activity is required for pancreatic

β-cell proliferation. Increased expression of p16, limits the regenerative capacity of β-cells with aging (Krishnamurthy *et al.*, 2006).

Cell proliferation inhibits cell differentiation while conversely, factors inducing cell cycle exit lead to differentiation. Cell cycle regulatory proteins are multifunctional and can also affect cell differentiation independent of their role in cell cycle. p21 deficient cells are defective in differentiation and differentiation can resume by transducing cells with p16 to compliment the mutation (Gius *et al.*, 1999). Cyclin-CDK complexes can inhibit differentiation in a kinase-dependent manner. The cyclinD1-CDK4 complex can phosphorylate and inhibit DMP1 or Mef2c transcription factors that are essential for differentiation of skeletal muscle and pre-hypertrophic chondrocytes (Hirai and Sherr, 1996; Lazaro *et al.*, 2002; Arnold *et al.*, 2007). CDK2 and CDK4 can inhibit TGF-β induced growth arrest by phosphorylating Smad3 (Matsuura *et al.*, 2004). p16, as an inhibitor of cyclinD1 and CDK4 complexes may thus have an important role in cell differentiation. Interaction of p16 with CDK4, in various p16 mutant cells, does not correlate with cell cycle regulatory functions suggesting that there must be cellular targets of p16 other than CDK4 (Becker *et al.*, 2001). In absence of cyclin/CDK complexes following cell cycle exit and the persistence of p16 expression strongly suggest the existence of alternative p16 partners following cell cycle exit.

Therefore, it is verly likely that p16, along with its role in cell senescence, may also have a role in cell quiescence and differentiation that depends on alternative substrate specificity from the canonical CDK4/6 complexes once cells have exited the cell cycle.

Chapter 2: Analysis of the Role of p16 in Cell Quiescence

Section-1 Introduction

Cell cycle progression is controlled principally by cyclins and cyclin-dependent kinases (CDKs) and their cofactors (Afshari and Barrett, 1993). Cyclins bind CDKs and activate them to promote cell cycle progression (Pines and Hunter, 1991). Along with the cyclins and CDKs other associated proteins, such as tumor suppressors pRb and p53, and transcription factors such as the E2F proteins, play important roles in regulating cell cycle progression.

Cyclin/CDK regulation is controlled by the activities of two important classes of genes, which play critical roles in regulating transitions into and out of the cell cycle and function as a gateway to terminal differentiation. These are the tumor suppressor genes and oncogenes (Hartwell and Weinert, 1989) and they are instrumental in regulating important cell cycle check points occurring at the G1/S and G2/M phase transitions Nurse (Nurse, 1990; Serrano *et al.*, 1993). At cell cycle checkpoints CDKs can be inhibited by cyclin-dependent kinase inhibitors (CKIs), inhibiting cell division (Kamb *et al.*, 1994). There are two important CKI families (Swellam *et al.*, 2004); the INK4 family and KIP/CIP family. The INK4 family inhibitors inhibit CDK4 and CDK6 in association with cyclinD, while KIPs inhibit CDK2 and CDK4 in association with cyclinD and cyclinE. The INK4 family consists of p16/INK4A, p15/INK4B, p18/INK4C, and p19/INK4D, while the KIP family consists of p21/CIP1, p27/KIP1, and p57/KIP2. All the

CKIs are proven tumor suppressor genes or suspected of having this potential. All of the INK4 family members are tumor suppressor genes, which are structurally similar, equally potent, and have cell lineage-specific or tissue-specific functions (Canepa *et al.*, 2007). Expression of some members of the INK4 gene family, such as p18 and p19, predominate during early-mid gestation in mouse development (Herman *et al.*, 1997) while expression of p15 has been found in later stages of gestation (Herman *et al.*, 1997). Circumstantially, it appears that different INK4 proteins may not be redundant as they appear to be differentially expressed during development.

p16/INK4A/CDKN2A checks the cell cycle in early G1 phase and stops further transition of the cell cycle from G1 to S phase as a component of a multi-protein regulatory complex. During G1 phase, CDK4 and CDK6 form complexes with cyclinD1 which in turn phosphorylate pRb and result in additional pRb phosporylation by cyclinE/CDK complexes until there is release of the E2F family transcription factors from pRb/E2F complexes. pRb otherwise inhibits transcription factor E2F inhibiting further cell cycle progression as E2F initiates transcription of genes required for S phase (Bean *et al.*, 2007; Sun *et al.*, 2007). The action of p16 inhibits binding of CDK4/6 to cyclinD1 which leaves pRb unphosphorylated and E2F bound and inactive (Vidal and Koff, 2000). p16/INK4A protein causes inhibitory structural changes to the CDK4 and CDK6 targets rather than the cyclin subunit and actually competes with cyclinD1 for CDK binding CDKs by blocking activating structural changes to bound CDKs. Binding of p16 results in changes in conformation of CDK proteins so that they can not bind cyclinD1 (Sharma *et al.*, 2007). p16 may also bind to pre-assembled CDK4/6-cyclinD1 complexes, blocking their function.

p16 expression changes are highly correlated with changes affecting cell proliferation. In a coordinated way, maintenance of p16 and p27 CKI levels contribute to low levels of proliferation in normal blood vessels (Izzard *et al.*, 2002) and p16 mRNA and proteins accumulate in human fibroblasts as they become senescent (Lukas *et al.*, 1996). Deletion of the human 9p21 region, encoding p16, also results in tumor formation in a wide range of tissues (Kamb *et al.*, 1994). Loss of heterozygosity (Swellam *et al.*, 2004), loss of homozygosity (Vidal and Koff, 2000; Izzard *et al.*, 2002), and hypermethylation of the promoter (Tripathy and Benz, 1992) in the 9p21 region are all important mechanisms which have been shown to result in loss of p16 expression. Hypermethylation of the *p16* promoter can promote cell proliferation in human colorectal cancer and can subsequently activate DNA demethylation in the tumor invasive region suppressing proliferation but enhancing tumor invasion (Hara *et al.*, 1996). Evaluation of p16 expression and function has been proposed to have value as a prognostic indicator in predicting recurrence of breast cancer (Wolfe *et al.*, 1986). *p16* gene defects are second in frequency only to those in *p53* for human malignancies (Baylin *et al.*, 1998). *p16* gene deletions are significantly associated with late-stage, high-grade cancers (Morgan, 1997).

Cells are directed toward four possible fates following cell cycle exit: senescence, quiescence, differentiation, or death. Quiescence is the temporary non-proliferating stage characterized by an absence of DNA synthesis, lower metabolism, and smaller cell size (Yusuf and Fruman, 2003). Differentiation is the developmental process through which cells gradually restrict their fate to a single terminal post-mitotic cell type. Senescence is the permament exit of cells from the cell cycle. Senescent cells cannot revert back to normal cell division under any physiological conditions. Unlike quiescent cells, senescent

cells have a large flattened phenotype with higher levels of metabolism and characterstic expression of β-galactosidase (Cristofalo *et al.*, 2004). CKIs and other tumor suppressor genes promote cell cycle exit. p16 appears to play a key regulatory role in cell quiescence, differentiation and senescence through management of cell cycle exit. p16 induces and maintaines the senescent phenotype (Stein *et al.*, 1999) and has been reported capable of promoting premature cell senescence (Zindy *et al.*, 1997). In contrast, little is known about the role of p16 in cell quiescence and differentiation. Overexpression of pRb, p27, and underexpression of cyclinD1 are important factors in cell quiescence (Sherr and Roberts, 1995; Stevaux and Dyson, 2002; Laine *et al.*, 2010). Accumulation of p16 in senescent cells and its inhibitory role in regulating CDK4/CDK6/cyclinD1 complex formation suggests a role p16 could play leading to arrest of cell cycle at G1 phase. It might be suggested that p16, in addition to its role in cell cycle senescence, may also play a similar role in quiescence and differentiation. We hypothesize that p16 plays a critical regulatory role that is required for cells to exit from the cell proliferation cycle and for maintenance of these non-proliferative and post-proliferative states.

Section-2 Materials and Methods

Cell culture

Canine mammary tumor cell lines (CMT12, 27, and 28) were obtained from Dr. L. Wolfe (Wolfe *et al.*, 1986). Single cell-derived clonal canine mammary tumor cell lines stably transfected with human p16 (CMT27A, CMT27H, CMT28A, and CMT28F) and NCF (normal canine fibroblast) cells were also used (DeInnocentes *et al.*, 2009). CMT12, 27, and 28, and NCF were cultured in L-15 medium (Gibco) with antibiotics (ampicillin) (Sigma), and 10% FBS (Hyclone) in tissue culture flasks (Corning) at 37^{o}C (air, 95%; CO_2, 5%) (DeInnocentes *et al.*, 2006). CMT cell clones (CMT27A, CMT27H, CMT28A, and CMT28F) were cultured in L-15 medium (Gibco) as described above but with the addition of geniticin (100 µg/ml, Invitrogen) (DeInnocentes *et al.*, 2009). Cells were serum-starved by changing FBS concentration from 10% to 0.5% in L-15 medium.

Preparation of RNA, primer design and semi-quantitative and quantitative rt-PCR

Cell cultures were grown to 75-80% confluence and total cellular RNA was isolated using RNA Stat 60 (Tel-Test, Inc.). Concentration of RNA was determined by absorbance at 260 nm (You and Bird, 1995). *p16*, *CDK4*, *CDK6*, *L37*, *cyclinD1*, and *p27* cDNA synthesis and amplification was done by reverse transcriptase semi-quantitative and quantitative PCR using specific primers (Table 1) (DeInnocentes *et al.*, 2009). Quantitative PCR was performed using a Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System and assays were performed using an ABsoluteTM QPCR SYBR® Green Fluorescein Mix (Thermo Scientific). Amplicons were gel purified, cloned into vector pCR2.1 (Invitrogen) and sequenced (Auburn University Genomics and

Sequencing Laboratory). The process of RNA extraction, semi-quantative analysis by rt-PCR, and sequencing was performed as previously described (DeInnocentes *et al.*, 2006).

Table 1: List of primers sequence used for reverse-transcriptase PCR for cell quiescence model.

Gene	Primer (5'-3')	Genbank Accession*	Amplicon Size (bp)
L37 sense	5'-AAGGGGACGTCATCGTTCGG-3'	XM_844999	194
L37 anti-sense	5'-AGGTGCCTCATTCGACCGGT-3'		
p16 sense	5'-AGCTGCTGCTGCTCCACGG -3'	FJ542309	103
p16 anti-sense	5'-ACCAGCGTGTCCAGGAAGCC-3'		
CyclinD1 sense	5'-AGGAGCAGAAGTGCGAGGAG-3'	AY620434	374
CyclinD1 anti-sense	5'-CACATCTGTGGCACAGAGCG-3'		
CDK4 sense	5'-AAGCCTCTCTTCTGTGGAA ACTCT-3'	XM_538252	223
CDK4 anti-sense	5'-AGATTCGCTTGTGTGGGTTA AA-3'		
CDK6 sense	5'-AGGGCATGCCGCTCTCCAC CATCC-3'	XM_847267	353
CDK6 anti-sense	5'-GATGCGGGCAAGGCCGAAG TCAGC-3'		
p27 sense	5'-CTGGAGCGGATGGACGCCA-3'	AY004255	280
p27 anti-sense	5'-TCTCCTGCGCCGGCACCT-3'		

* Primers were designed based upon the canine cDNA sequence present in the Genbank database.

Western blot

Cells were lysed with extraction buffer (1X PBS pH 7, 0.1% Tween-20, 0.1% NP-40, and 100 mM PMSF). Three freeze/thaw cycles were performed to release proteins (8 X 10^6). Cell lysates were cleared by centrifuged at 14,000xg for 10 min at 4^{o}C and supernatants stored at -80^{o}C (Bird and Deinnocentes, 2004). Supernatants were assayed for protein concentration using a BCA200 protein assay kit (Pierce). Protein extracted from each cell line was boiled in 5X Laemmli buffer (50% glycerol, 10% SDS, 2.56% β-mercaptoethanol, 2.13% 0.5M Tris-HCl-SDS pH 6.8, trace of bromophenol blue) for 10 min before loading onto a polyacrylamide electrophoresis gel (4-20% preciseTM protein gels, Pierce) along with Kaleidoscope markers (range 10-250 kD, BioRad). Electrophoresis was run in BupHTM Tris-HEPES-SDS running buffer (Pierce) for one hour (100V) and proteins were transferred to PVDF membranes (Immobilone®-P Transfer Membrane, Millipore) using a BupHTM Tris-Glycine Buffer Pack (Pierce) as transfer buffer for 2 hr at 4^{o}C at 300 mA. The membrane was washed in washing buffer (TBS-T Buffer, 20 mM Tris-HCl, 136 mM NaCl, 0.1 % v/v Tween 20, pH 7.6). The membrane was blocked with blocking buffer (Pierce) for 2 hr at room temperature. The membrane was incubated with multi-species, polyclonal anti-human rabbit p16 primary antibody (1:1000; Santa Cruz Biotechnology, Inc.) overnight at 4^{o}C. Membranes were washed with washing buffer 4 times for 10 min each. The membrane was incubated with Anti-rabbit IgG-HRP secondary antibody (1:5000; Santa Cruz Biotechnology, Inc.) for 1 hour at room temperature. The membrane was washed again 4 times for 10 min each. Western blot analysis was performed using the ECLTM western blotting analysis system (Amersham Biosciences) according to the manufacturer's instructions. For GAPDH

analysis, membrane was stripped by incubating with stripping buffer (Pierce) at room temperature. The membrane was then incubated with mouse GAPDH primary antibody (1:5000; AbD Serotec) for 4 hrs, and washed with wash buffer overnight. The membrane was incubated with anti-mouse IgG-HRP secondary antibody (1:10000; Jackson Immunoresearch) for 1 hr at room temperature. Western blot analysis was performed as described above.

^{3}H-thymidine incorporation assay

CMT cells, their p16 transfected clones, and NCF cells were grown as described above in 24 well cell culture plates (Corning) until 75% confluent. Medium was changed to 0.5% FBS serum starvation medium and 10 μCi/ml tritium-labeled thymidine (PerkinElmer) was added to individual wells at 24 hr intervals after starvation. Cells were incubated for 4 hr and washed with 1X HBS (HEPES buffered saline; 10 mM HEPES pH 7.2, 0.9% NaCl) buffer twice. Cells were lysed with TES (Tris-EDTA-SDS; 10 mM Tris-HCl pH 8, 2 mM EDTA, 1% SDS). Well contents were transferred to Whatman 540 paper discs and fixed (Bird *et al.*, 1988). Discs were washed in bulk in 300 ml for 20 min each in 20% (w/v) tricholoroacetic acid then in 10% (w/v) trichloroacetic acid, absolute ethanol, chloroform, and then absolute ethanol at room temperature. Discs were dried and immersed in scintillation liquid (ScintiSafe^{TM} Econo 1, Fisher) and acid precipitable radioactivity determined in a liquid scintillation counter (Bird *et al.*, 1988).

Flow cytometry

CMT cells, their clones, and NCF cells were grown in 6 well plates (Corning) until 75% confluence. Medium was changed to 0.5% FBS medium at 75% confluence.

Cells were trypsinized and harvested every 24 hrs after serum-starvation. Harvested cells were washed twice in HBS, and fixed with 70% ethanol (You and Bird, 1995). Fixed cells were stained with propidium iodide, and cell cycle was analyzed by flow cytometry (DeInnocentes *et al.*, 2006; DeInnocentes *et al.*, 2009).

Statistical analysis

All the data comparisons between exponential and serum-starved cells were statistically analyzed using a two-sample student's *t* test. All of the experiments were performed in triplicates (n=3) and data was represented as mean ± standard deviation.

Section-3 Results
mRNA expression of p16 and associated genes

Well characterized (DeInnocentes *et al.*, 2009) parental canine mammary cancer cell lines (CMT28, CMT27, and CMT12) (Wolfe *et al.*, 1986), p16 transfected canine mammary tumor cell line single cell clones (CMT27A, CMT27H, CMT28A, and CMT28F) and normal canine fibroblasts (NCF) were used as a model to investigate the role of p16 in quiescence (DeInnocentes *et al.*, 2009; Bird *et al.*, 2011). Expression of mRNAs encoding p27, p16, CDK4, CDK6, cyclinD1, and L37 ribosomal protein were investigated using semi-quantitative rt-PCR. CMT27, CMT27A, CMT27H, CMT28, CMT28A, CMT28F, CMT12, and NCF cells all expressed mRNAs encoding p27, CDK4, CDK6, cyclinD1, and L37, while only NCF, CMT27A, CMT27H (weak expression), CMT28, CMT28A, and CMT28F expressed p16 mRNA (Fig.2a). This confirmed our previous report that rt-PCR of CMT28 RNAs detected expression of p16 mRNA while rt-PCR of RNAs derived from CMT27 or CMT12 cell lines did not, and also confirma that p16 expression defects appear to be common in CMTs. Surprisingly only NCF, CMT27A, CMT27H, CMT28A, and CMT28F translated p16 to detectable protein (Fig.2b), while CMT28 cells although express abundant p16 mRNA, did not synthesize detectable p16 protein. CMT28 cells may suffer a post-transcription defect that blocks p16 translation.

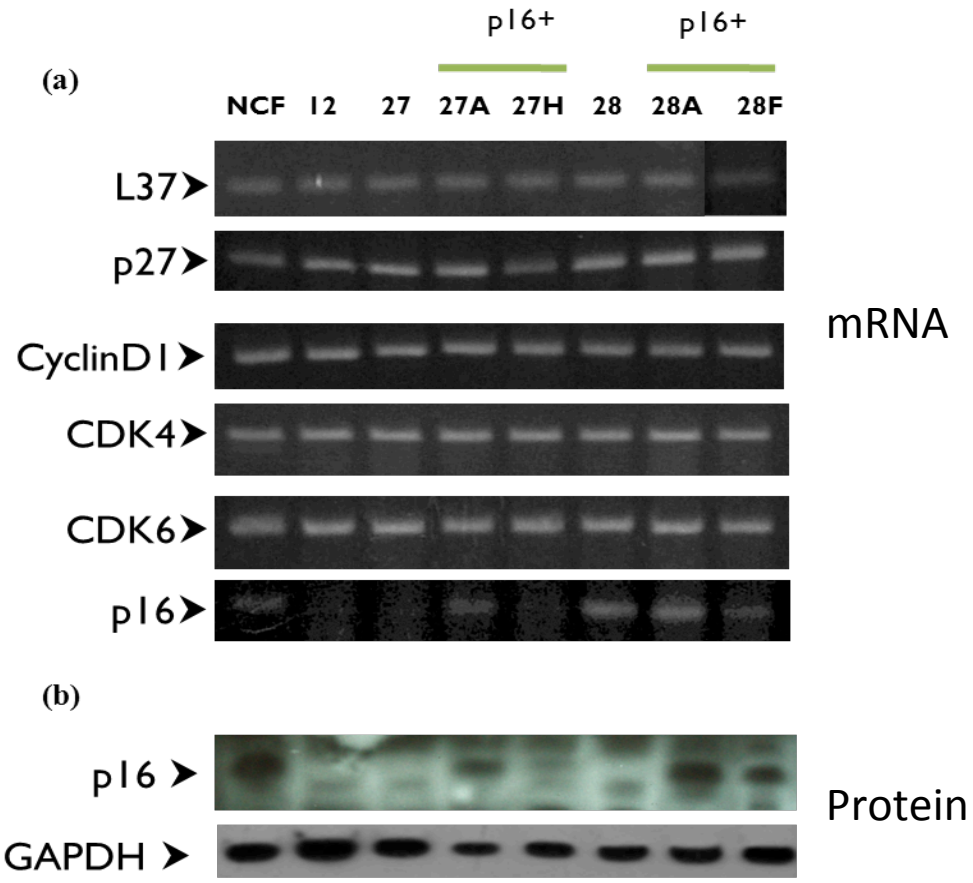

Figure 2: Semi-quantitative reverse transcriptase PCR of p16 and associated genes and western blot analysis of p16 and GAPDH.

(a) mRNA levels of p16, CDK4, CDK6, cyclinD1, p27, and L37 (as indicated) were evaluated in Canine Mammary Tumor (CMT) cell lines (12, 27, and 28), p16 transfected clones (27A, 27H, 28A, and 28F), and Normal canine fibroblasts (NCF) cells. Transcripts were amplified by rt-PCR and analyzed by agarose gel electrophoresis. (b) p16 protein expression level was analyzed by western blot using polyclonal rabbit anti-human-p16 antibody. Primary antibodies used were, p16 (rabbit anti-p16, Santa Cruz Biotechnology, Inc.) and GAPDH (mouse anti-GAPDH, AbD Serotec). Secondary antibodies used were, p16 (anti-rabbit IgG-HRP, Santa Cruz Biotechnology, Inc.) and GAPDH (anti-mouse IgG-HRP, Jackson Immunoresearch).

DNA replication and cell cycle exit following serum-starvation

To study the role of p16 expression in cell cycle exit and re-entry, we developed a cell model using serum starvation (to stop cell growth) and re-feeding (for cell cycle re-entry) of canine breast cancer cell lines. The purpose was to deprive cells of required nutrients provided by FBS (fetal bovine serum) in culture medium to stop cell growth by inducing quiescence, and then provide cells with growth nutrients to induce cell cycle re-entry with relative synchrony (Coller *et al.*, 2006). Cells were cultured in L-15 medium with 10% FBS to approximately 75% confluence and then serum-starved (0.5% FBS medium) until they stopped proliferating but before cells started floating in suspension. Cells were monitored for morphology and confluence daily. Cells were analyzed for cell cycle phase distribution by flow cytometry and DNA synthesis by ^{3}H-thymidine incorporation into acid-precipitable molecules. DNA replication in NCF, CMT12, CMT27, CMT27A, CMT27H, CMT28, CMT28A, and CMT28F decreased by 72 hrs post-serum starvation in comparison with proliferating cells (Fig.3). Effect of serum-starvation was more profound in p16-transfected CMT27A and CMT27H cells in comparison to parental CMT27 cells. Serum-starvation had a clear and significant inhibitory effect on DNA replication in p16 transfected CMT27A and CMT27H cell lines (Fig.3).

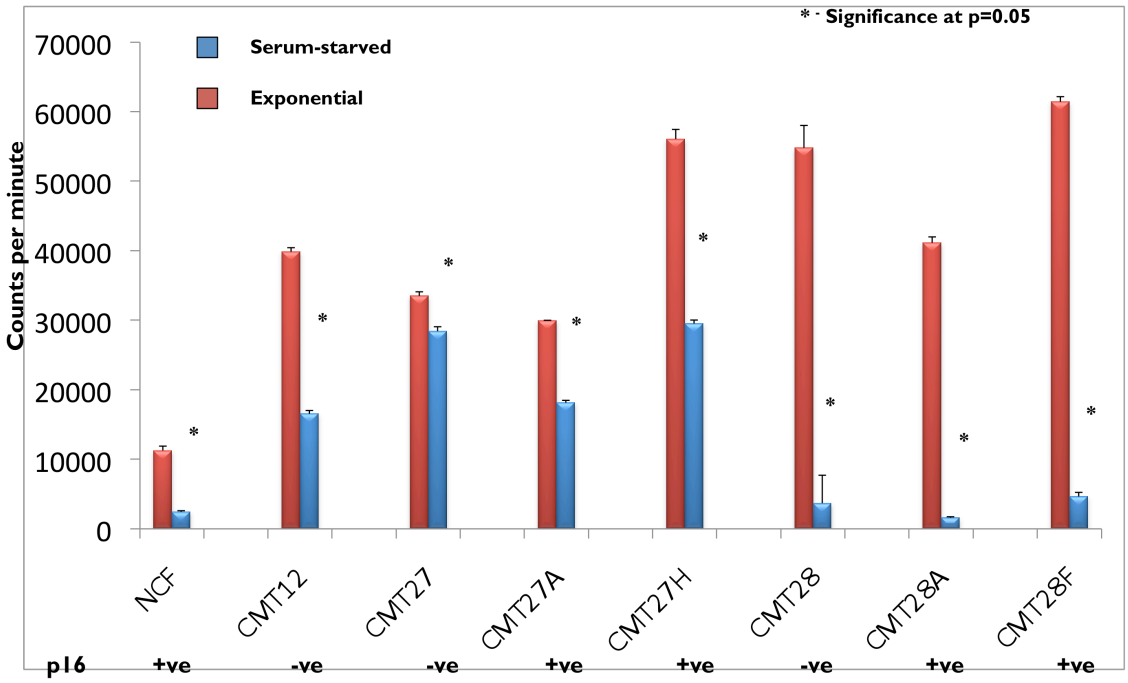

Figure 3: ^{3}H-thymidine incorporation assay.

Counts per minute (y axis) of acid precipitable ^{3}H-thymidine incorporation is compared between exponentially growing and serum-starved Canine Mammary Tumor (CMT) cell lines, p16 transfected clones, and Normal canine fibroblasts (NCF) cells Asterisks indicate statistically significant difference compared between serum-starved and exponential cells as determined by two-sample Student´s t test (*P < 0.05, n=3).

Reduction in DNA replication after serum-starvation suggested that CMT cell lines had stopped dividing. To ensure that reduction in DNA replication and/or cell numbers was due to cell cycle exit to quiescence and not due to apoptosis, we analyzed the cell cycle phase distribution of cells by flow cytometry. CMT12, CMT28, CMT28A, CMT28F, CMT27A, CMT27H, and NCF cells exited cell cycle post-starvation and accumulated in G1 phase by 72 hrs in comparison to proliferating cells (Fig.4). There was no enhanced cell accumulation in G1 phase in the case of CMT27 cells post serum-starvation (Fig.4b), while clones of p16 transfected CMT27, CMT27A and CMT27H re-acquired the ability to accumulate in G1 phase. CMT12, CMT27A, CMT27H, CMT28, CMT28A, CMT28F, and NCF cells have all exhibited clear effects of serum-starvation suggesting transition out of the cell cycle, while in comparison CMT27 demonstrated few effects of starvation (Fig.4). This clearly implicates p16 in cell cycle exit post-starvation.

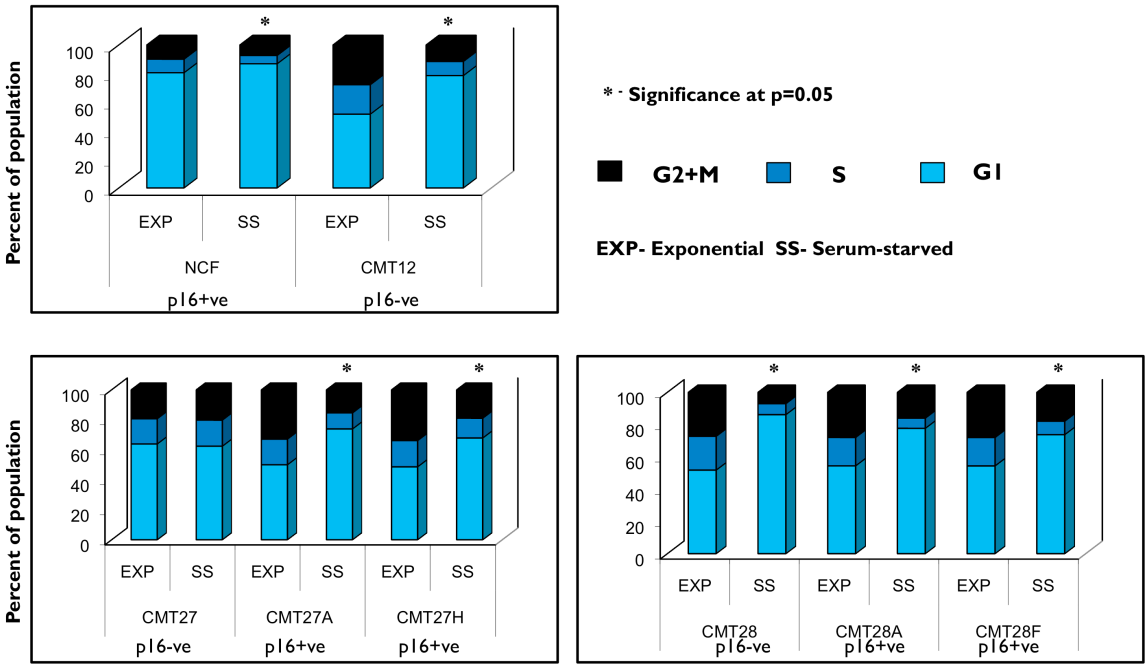

Figure 4: Cell cycle phase analysis (serum-starvation).

Cells were harvested every 24hrs post serum-starvation and stained with propidium iodide and analyzed for cell cycle phase by flow cytometry on a linear fluorescent scale. (a) CMT12 and NCF, (b) CMT27 and p16 transfected CMT27A and CMT27H clone cells, and (c) CMT28 and p16 transfected CMT28A and CMT28F clone cells. Asterisks indicate statistically significant difference of cells present in G1 phase compared between serum-starved and exponential cells as determined by two-sample Student´s t test (*P < 0.05, n=3).

Cell cycle phase analysis following serum re-feeding

To ensure that cells were entering the quiescent phase and not irreversible senescence due to serum-starvation, all the arrested cell cultures were refed with serum containing growth medium. Growth media containing FBS was added to NCF, CMT12, CMT28, CMT28A, CMT28F, CMT27, CMT27A, and CMT27H cell cultures after 72 hr of serum-starvation. Cells were harvested every 12 hr following serum re-feeding in all cell lines (Fig.5). Cells accumulated in S phase and G2/M phase after 24/36 hr of serum re-feeding for the following strains NCF, CMT27, CMT27A, CMT27H, CMT28, CMT28A, and CMT28F cells (Fig.5). CMT12 cells did not alter their cell cycle phase distribution following serum re-feeding which may suggest that CMT12 enter senescence post-serum-starvation although CMT12 cells don't express p16. In all the cell lines except CMT12 an increase in synchronous S phase re-entry was observed following serum re-feeding of starved cells suggesting they has re-entered cell cycle.

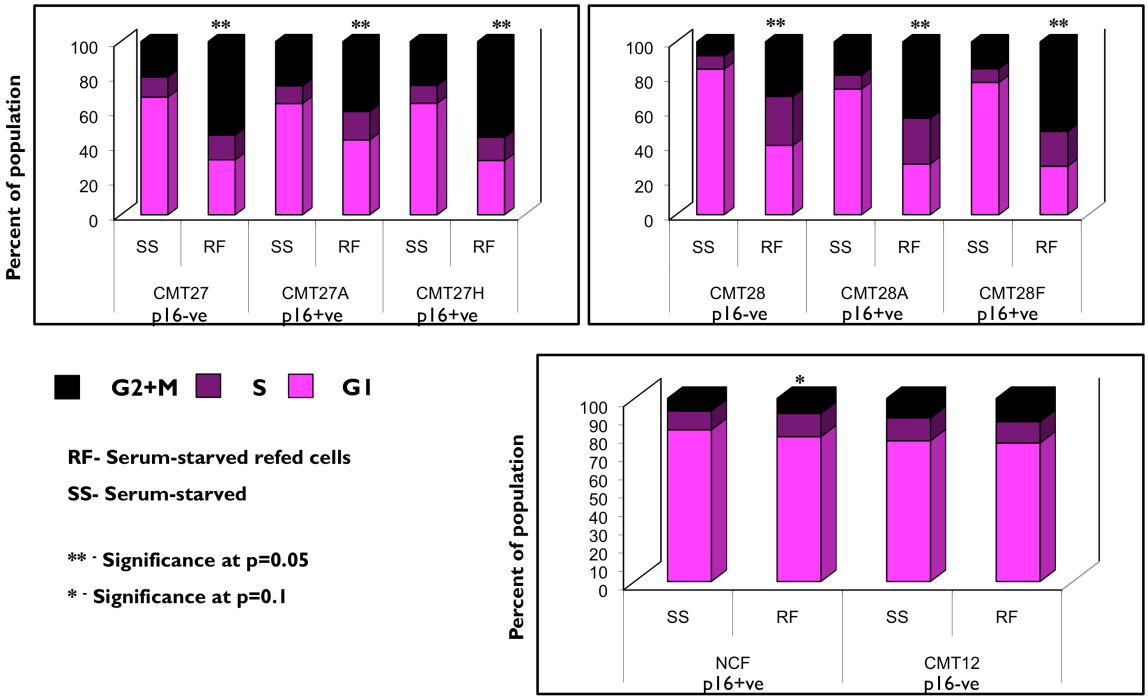

Figure 5: Cell cycle phase analysis (serum-refeeding).

Cells were harvested every 6/12 hrs post serum-refeeding and stained with propidium iodide and analyzed for cell cycle phase by flow cytometry on a linear fluorescent scale. (a) CMT27 and p16 transfected CMT27A and CMT27H clone cells, (b) CMT28 and p16 transfected CMT28A and CMT28F clone cells, and (c) CMT12 and NCF cells. Asterisks indicate statistically significant difference of cells present in G1 phase compared between serum-starved and exponential cells as determined by Student´s t test (*P < 0.05 **P < 0.1, respectively; n=3).

Quantitative rt-PCR analysis of quiescent cells

p27 expression has long been reported to be up-regulated during cellular quiescence (Sherr and Roberts, 1995) and therefore we have used p27 as a marker of cell quiescence. To further demonstrate that cells reach quiescence after cell cycle exit due to serum-starvation, we compared p27 mRNA expression of in exponentially proliferating and serum-starved cells using the comparative $\Delta\Delta C_t$ method. Ct (cycle threshold) values for p27 mRNA expression were compared between serum-starved and exponentially proliferating NCF, CMT12, CMT27, CMT27A, CMT27H, CMT28, CMT28A, and CMT28F cells and were normalized to expression of the L37 gene (Su and Bird, 1995). By observing the normalized expression of p27, it was clear that only serum-starved NCF, CMT27A, and CMT28F overexpressed p27 significantly, which demonstrated that only these cell lines achieve cell quiescence (Fig.6). Quantitative rt-PCR results showed that along with p27, the p16 gene was also overexpressed siginificantly in NCF and CMT28F quiescent cells and marginally but significantly in CMT27A cells, which suggests a possible role for p16 along with p27 in cell quiescence.

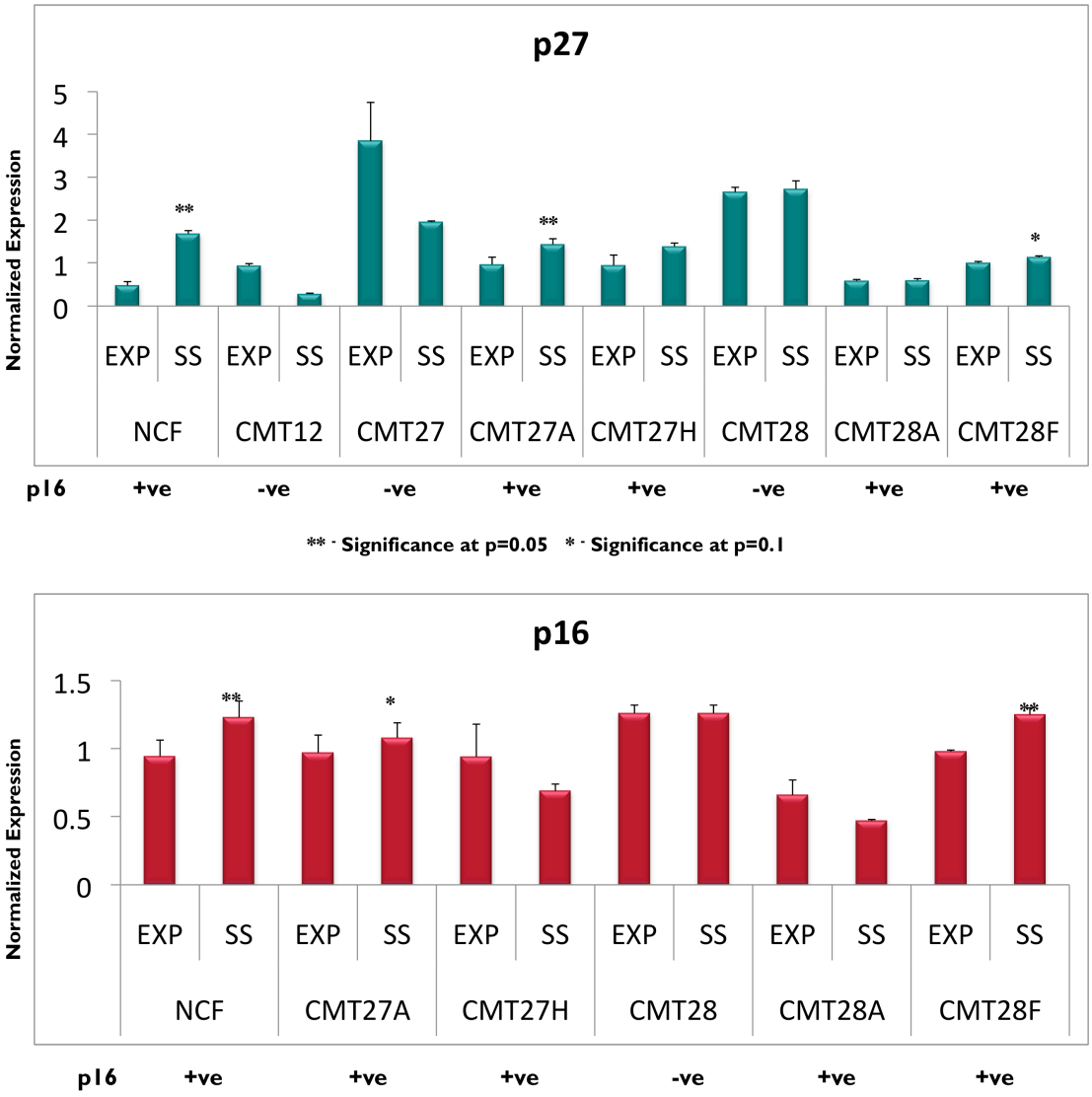

Figure 6: Quantitative rt-PCR analysis of p27 mRNA and p16 mRNA between exponentially growing and serum-starved cells.

(a) p27 mRNA transcripts and (b) p16 mRNA transcripts. Asterisks indicate statistically significant difference compared between serum-starved and exponential cells as determined by Student´s t test (*P < 0.05 **P < 0.1, respectively; n=3).

Comparative p16 protein expression in serum-starved and exponential cells

To further evaluate the role of p16 in cell quiescence, p16 protein expression was compared between exponential and serum-starved cells. p16 protein was found to accumulate to higher levels in NCF, CMT27A, CMT28A, and CMT28F cells (Fig.7). p16 protein accumulation in quiescent cells suggests that p16 has a role in cell quiescence in some normal and p16-transfected cancer cells.

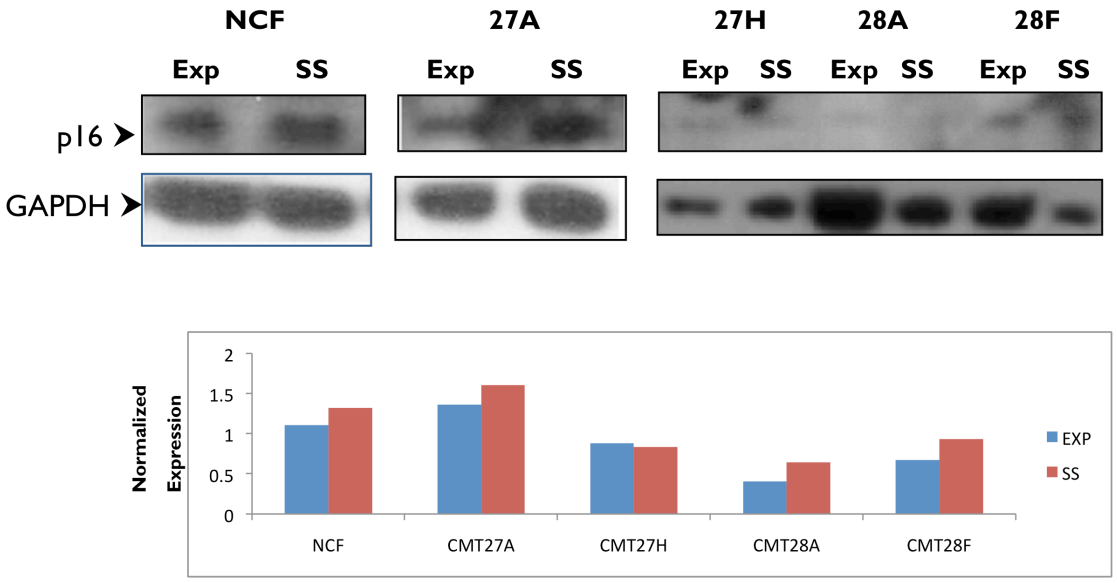

Figure 7: Western blot analysis of p16 and GAPDH protein.

Western blot analysis of p16 and GAPDH protein expression between exponential and serum-starved NCF, CMT27A, CMT27H, CMT28A, and CMT28F cells. Primary antibodies used were, p16 (rabbit anti-p16, Santa Cruz Biotechnology, Inc.) and GAPDH (mouse anti-GAPDH, AbD Serotec). Secondary antibodies used were, p16 (anti-rabbit IgG-HRP, Santa Cruz Biotechnology, Inc.) and GAPDH (anti-mouse IgG-HRP, Jackson Immunoresearch).

Section-4 Discussion

The cyclin-dependent kinase inhibitors act as multifunctional cell proliferation inhibitors that integrate signals to suppress cell growth by inhibiting CDK function (Morgan, 1997). The traditional role of p16 is inhibition of cell proliferation in G1 phase. Previous data suggests accumulation of p16 mRNA and protein occur as cells enter senescence (Zindy *et al.*, 1997). p21 acts as the mediator of cell cycle arrest prior to senescence while p16 induces, maintains, and promotes the senescent phenotype (Zindy *et al.*, 1997; Stein *et al.*, 1999). Under the stimulus of stress and senescence, levels of p16 increase with decreased levels of histone-lysine N-methyltransferase EZH2 enzyme (Bracken *et al.*, 2007). Accumulation of p16 in senescent cells, and its inhibitory role in CDK4/CDK6/cyclinD1 complex formation explains why expression of p16 could lead to arrest of cell cycle in G1 phase (Lea *et al.*, 2003) but little is known regarding the role of p16 once cells enter quiescence.

Cell quiescence is a temporary non-dividing cell stage, often occuring prior to terminal differentiation which is characterized by lower metabolism and no DNA synthesis (Yusuf and Fruman, 2003). Cell quiescence can be achieved experimentally by serum-starvation or contact-inhibition (Coller *et al.*, 2006). In the absence of growth factors, cells retire to G0 phase and are termed quiescent. Upon growth factor re-feeding, cells re-enter the cell cycle. We have used this phenomenon to observe the effects of p16 on entry and exit from cell quiescence.

We have used well-characterized CMT cell lines (Wolfe *et al.*, 1986) and normal canine fibroblasts (NCF) as our model of cell cycle exit and re-entry (DeInnocentes *et al.*,

2006; Bird *et al.*, 2009). The canine mammary tumor (CMT) cell lines used lack p16 expression either at the mRNA or protein expression level, while p27 and other proteins associated with regulation of cell cycle progression such as; CDK4, CDK6, cyclinD1, and Rb appear to be expressed at normal levels in all cell lines. In addition to these parental cell lines, we also employed p16-transfected cell line clones derived from them (DeInnocentes *et al.*, 2009). Many of these clones exhibited phenotype rescue of the neoplastic phenotype to varying degrees due to p16 transfection making these mammary cancer cell lines particularly interesting for evaluation following serum-starvation (DeInnocentes *et al.*, 2009).

Decreased levels of cyclinD1, increased level of p27, and accumulation of unphosphorylated pRb are the hallmarks of cell quiescence (Sherr and Roberts, 1995; Boonstra, 2003; Pajalunga *et al.*, 2007). CKIs p27 and p21 have been reported to play important roles in cell quiescence. p27 induces and maintains cell quiescence by sequestering the CDK/cyclinD1 complex (Carroll *et al.*, 2003; Georgia and Bhushan, 2006). p21 has been reported to be important for cell quiescence in development of mouse hematopoietic stem cells and influences quiescence in human foreskin fibroblasts in its absence (Cheng *et al.*, 2000; Pajalunga *et al.*, 2007). Other than p27 and p21, p16 has also been reported to induce a G0-like state in hematopoietic cells (Furukawa *et al.*, 2000). We observed that the p16 expressing (mRNA and protein) cell lines such as NCF, CMT28A, CMT28F, CMT27A and CMT27H, exited cell cycle post-serum-starvation while CMT27 cells, which lack p16 expression did not exit the cell cycle after serum-starvation. The continued growth of CMT27 cells after removal of growth factors from the medium suggested that the immortality in CMT27 could be due to some other internal

cellular mechanism defects, because these cells did not need any external growth factors to grow. CMT12 cells lacking p16 expression demonstrated cell cycle exit but did not show synchronous cell cycle re-entry. This suggested that CMT12 cells exited the cell cycle permanently and reached senescence post-serum-starvation. However, CMT28 cells, which also lacked p16 expression (protein level), exited the cell cycle post-serum-starvation, do not reach quiescence. We conclude that the lack of quiescence results from the lack of upregulation of p27 mRNA. The result suggested that factors other than p16 were responsible for cell cycle exit in CMT28 cells. From these observations, we concluded that cell lines expressing both p16 mRNA and protein do react to serum-starvation by exiting cell cycle with the exception of parent CMT28 cells. CMT28 cells have been reported to overexpress c-*erb*B-2 in comparison to CMT12 and CMT27 (Ahern *et al.*, 1996). It may be speculated that transformation in CMT28 cells is due to the up-regulated expression of c-*erb*B-2 receptors which makes them dependent upon growth factors. Both parental CMT28 cells and p16-transfected CMT28A and CMT28F cell clones were capable of exiting from the cell cycle in response to serum-starvation, although parent CMT28 cells don't express p16.

Lack of p16 expression has been associated with cancer (Gruis *et al.*, 1995; Swellam *et al.*, 2004), while p16 overexpression has been reported in cell senescence (Agherbi *et al.*, 2009) and cell cycle exit (Kivinen *et al.*, 1999; Wang *et al.*, 2008). It has also been reported that ectopic expression of p16 prevents re-entry into the cell cycle (Lea *et al.*, 2003). The effects of ectopic expression of p16 were also evident in our model of cell cycle exit and re-entry. p16-transfected CMT27A and CMT27H did exit cell cycle post serum-starvation in contrast to parental CMT27s. Rescue of cell cycle exit

in p16-transfected cells strongly suggests an important role for p16 in cell cycle exit comparable to the effect observed in normal canine fibroblasts post-serum-starvation. Because all of the p16 expressing cells and clones re-entered the cell cycle post-serum-refeeding, serum-starved CMT cells must have entered a temporary non-dividing phase post-serum-starvation. That is, they entered quiescence.

Suppression of proliferation is thought to be dependent on the upregulated levels of p16 and p27, which make these cells unable to activate cyclinD1, cyclinE and their associated cyclin-dependent kinases (Izzard *et al.*, 2002). Up-regulated levels of p27 mRNA have been reported in cell quiescence, which makes it an informative marker for cell quiescence. While each of the NCF, CMT27A, CMT27H, CMT28, CMT28A, and CMT28F cells demonstrated cell cycle exit post-serum-starvation, only NCF, CMT27A, and CMT28F expressed upregulated levels of p27 mRNA. This suggests that only these cell lines achieved quiescence and CMT27H, CMT28, and CMT28A cells may have progressed only to a pre-quiescent stage. Quiescent NCF, CMT27A, and CMT28F showed upregulated p16 mRNA expression after serum-starvation coordinately with elevated p27 mRNA expression, suggesting that p16 played a role in maintaining cell quiescence following cell cycle exit. p16 protein expression was elevated in NCF, CMT27A, CMT28A, and CMT28F cells and not in CMT27H cells. CMT28A cells did not show upregulated p16mRNA expression but did show elevated p16 protein expression post-serum-starvation. This further suggests that p16 plays a role in cell cycle exit. The effects of serum-starvation in quiescence were not evident in clones CMT27H. The difference between the two clones may be attributed to differential expression of p16 in CMT27H cell clones.

Cell cycle exit, due to serum-starvation and accumulation of p16 mRNA and protein in p16 positive cell lines, clearly showed the effects of p16 on cell quiescence and its role in cell cycle exit and cell quiescence. Until now, p16 has been primarily associated with cell senescence and cell cycle exit and not with cell quiescence. We have shown that p16 has a potential role in cell quiescence. Along with cell cycle exit, ectopic expression of p16 (CMT27A and CMT28F) also leads cells to quiescence.

Chapter 3: Alternative Binding Partners of p16

Section-1 Introduction

Tumor suppressor genes and oncogenes encode proteins that regulate transitions in and out of the cell cycle. Loss-of-function mutations in tumor suppressor genes and gain-of-function mutations in proto-oncogenes result in uncontrolled cell division, which leads to cancer (Tripathy and Benz, 1992). Tumor suppressor gene activity normally promote cell exit from the cell cycle and thus reduce the rate of cell proliferation.

p16 is an important tumor suppressor gene encoded on the 9p21 region of the human genome, on chromosome number 4 in mouse, and on chromosome 11 in dogs at the *INK4A/ARF/INK4B* gene locus (Serrano *et al.*, 1993; Kamb *et al.*, 1994; Asamoto *et al.*, 1998; Fosmire *et al.*, 2007). This gene locus is a 35 kb multigene region which encodes three distinct major tumor suppressor genes, *p15/INK4B*, *p14ARF*, and *p16/INK4A* (Sherr and Weber, 2000). The *INK4A/ARF/INK4B* gene locus is repressed in early passage and normal cells by polycomb-group proteins and histone H3 lysine 27 (H3K27) trimethylation (Kotake *et al.*, 2007; Kia *et al.*, 2008; Agger *et al.*, 2009) and its expression is induced during aging or by hyperproliferative oncogenic stimuli or stress. The *INK4A/ARF* gene locus has been speculated to have a global anti-aging effect by favoring cell quiescence and limiting cell proliferation (Matheu *et al.*, 2009).

The classic role of p16 is to check the cell cycle in early G1 phase and inhibit further transition out of the cell cycle from G1 to S phase as a component of a multi-protein regulatory complex. During G1, CDK4 and CDK6 form complexes with cyclinD1 which in turn phosphorylate the pRb protein (Weinberg, 1995). p16 inhibits binding of CDK4/6 with cyclinD1 which leaves pRb and pRb-related proteins like p107 and p103, non-phosphorylated and E2F bound and inactive (Serrano *et al.*, 1993; Walkley and Orkin, 2006). Binding of p16 results in changes in conformation of CDK proteins so that they can no longer bind cyclinD1 (Russo *et al.*, 1998).

14-3-3σ is different from other 14-3-3 proteins by having the unique amino acids Met202, Asp204, and His206 (Benzinger *et al.*, 2005). The 14-3-3 family of proteins has been linked with apoptosis, signal transduction, and cell cycle checkpoints but only 14-3-3σ has been reported in cancer cells (Hermeking, 2003). 14-3-3σ was first reported as human mammary epithelial receptor 1(HMER1) in human mammary epithelial cells and was downregulated in human mammary carcinoma cell lines leading to speculation that it was a potential marker of cellular differentiation (Prasad *et al.*, 1992). Today, 14-3-3σ is known as a negative regulator of the cell cycle which checks the cell cycle by inhibiting cyclin-CDK2 complex formation. 14-3-3σ works downstream from the p53 protein. In response to gamma irradiation and other DNA damaging agents, p53 transactivates the expression of 14-3-3σ, which leads to cell cycle arrest at the G2/M check point by sequestering CDC2-cyclinB complexes, (Hermeking *et al.*, 1997; Hermeking and Benzinger, 2006; Lee and Lozano, 2006) and 14-3-3σ stabilizes p53 in resonse to DNA damage by positive feedback. 14-3-3σ and three of its other family members 14-3-3τ, 14-

3-3ε, and 14-3-3γ have a role in stabilization and activation of p53 through different mechanisms (Lee and Lozano, 2006).

14-3-3 proteins are involved in the negative regulation of the G1/S phase transition. 14-3-3ε and 14-3-3β inhibits the CDC25 phosphatases, which are involved in the activation of CDK2-cyclin complex during G1/S phase transition (Chen *et al.*, 2003). Similarly, 14-3-3σ also has a role as a negative regulator of G1/S phase transition by binding to CDK2 and CDK4, as 14-3-3σ shares a sequence motif important for binding to cyclin-CDK2 with CKI and pRb-related tumor suppressors including p107, p130, p21, p27, and p57 (Laronga *et al.*, 2000). 14-3-3σ is considered to be an important marker in breast cancer prevention (Laronga *et al.*, 2000).

The 14-3-3 family plays a role in development by regulating the cell cycle. 14-3-3 proteins suppress CDC2 activity and facilitate cell cycle exit from mitosis (Su *et al.*, 2001). Absence of 14-3-3σ results in epithelial development and differentiation defects, and papillomas and squamous cell carcinomas in mutated mice (Lutzner *et al.*, 1985; Herron *et al.*, 2005)

14-3-3σ promoter methylation has been reported in sporadic female breast cancer (Luo *et al.*, 2010). Hyper-methylation of the 14-3-3σ promoter (Ferguson *et al.*, 2000) and 14-3-3σ expression downregulation (Vercoutter-Edouart *et al.*, 2001) in breast cancer patients has been reported, making them potent biomarkers for breast cancer development (Martinez-Galan *et al.*, 2008; Zurita *et al.*, 2010). 14-3-3σ downregulation has been observed in breast cancer (Umbricht *et al.*, 2001; Moreira *et al.*, 2005; Luo *et al.*, 2010) and other cancers such as; ovarian cancer (Akahira *et al.*, 2004; Ravi *et al.*, 2011), human

hepatocellular carcinoma (Iwata *et al.*, 2000), lung cancer (Osada *et al.*, 2002; Liu *et al.*, 2004), prostate cancer (Horie-Inoue and Inoue, 2006; Reibenwein *et al.*, 2007), gastric cancer (Suzuki *et al.*, 2000), and salivary gland adenoid cystic carcinoma (Uchida *et al.*, 2004), which suggests that it is an important cell cycle regulator that is often defective in a wide range of cancer cells.

p16-associated proteins are suppressed when cells exit cell cycle since levels of cyclinD, CDK4, and CDK6 decrease during cell differentiation (Molenaar *et al.*, 2008; Laine *et al.*, 2010), levels of CDK2 and CDK4 decrease in senescent fibroblasts (Lucibello *et al.*, 1993), and cyclinD1 expression is suppressed in quiescent cells (Laine *et al.*, 2010). In contrast p16 expression either remains constant or increases during post-proliferative cell fates which suggests the existence of possible alternate binding partners of p16 following cell cycle exit. We hypothesize that 14-3-3σ might be an alternate binding partner for p16.

Section-2 Materials and Methods

Cell culture

Human p16 transfected CMT27A and CMT27 cells were cultured in L-15 medium (Gibco) with antibiotics (ampicillin) (Sigma), and 10% FBS (Hyclone) in tissue culture flasks (Corning) at 37^{o}C (air, 95%; CO_2, 5%) (DeInnocentes *et al.*, 2006) except that geniticin (100 μg/ml, Invitrogen) was added only to p16 transfected CMT27A cells (DeInnocentes *et al.*, 2009).

Isolation of RNA, primer design and semi-quantitative and quantitative rt-PCR

Cell cultures were grown to 75-80% confluence and the medium was changed to 0.5% FBS medium at 75% confluence to initiate quiescence. Total cellular RNA was isolated using RNA Stat 60 (Tel-Test, Inc.) at '0' days of serum-starvation and '3' days of serum-starvation. Concentration of RNA was determined by absorbance at 260 nm (You and Bird, 1995). 14-3-3 σ (sense 5'-GTCTTCTACCTGAAGATGAAGGGC-3', antisense 5'-GAAGGTGGTCTTGGCCAGTG-3') and L37 (sense 5'-AAGGGGACGTCATCGTTCGG-3', antisense 5'- AGGTGCCTCATTCGACCGGT-3') cDNA synthesis and amplification was done by reverse transcriptase semi-quantitative PCR using specific primers with limiting RNA dilution and minimum number of rounds of amplification (DeInnocentes *et al.*, 2009). Amplicons were gel purified, cloned into vector pCR2.1 (Invitrogen) and sequenced (DNA core facility, Massachusetts General Hospital, Cambridge, MA). The process of RNA extraction, semi-quantative analysis by rt-PCR, and sequencing was performed as previously described (DeInnocentes *et al.*, 2006).

Whole cell protein extraction

CMT27 and CMT27A cells were grown in L-15 medium with 10% FBS and antibiotics in tissue culture flasks (Corning) until 75% confluence. FBS concentration was changed from 10% to 0.5% at 75% confluence. Cells were trypsanized using 1X trypsin solution (1X trypsin, 1M EDTA, 1XHBS; Invitrogen) at 0-day and 3-day after serum-starvation. Cells were centrifuged at 1500xg at room temperature and washed twice with 1X PBS. Cells were re-suspended in lysis buffer (0.025 M Tris, 0.15 M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol; pH 7.4; Pierce). Cells suspended in lysis buffer were incubated on ice for 5 min with mixing at intervals. The cell lysate was centrifuged at 4^{o}C at 13000xg and the supernatant saved and stored at -85^{o}C.

Co-immunoprecipitation

CMT27 and CMT27A whole cell protein from exponential and serum-starved cell cultures was used for co-immunoprecipitation according to the manufacturer's guidelines (Pierce). Anti-human polyclonal p16 primary antibody (sc-759; Santa Cruz Biotechnology, Inc.) was immobilized on AminoLink plus coupling resins provided by the manufacturer. Resins were washed twice with 1X coupling buffer. 10 µg primary antibody was mixed with resins along with 3 µl of sodium cyanoborohydride solution and the solution was vortexed at room temperature for 2 hr. Resins were washed with 200 µl of 1X coupling buffer after antibody incubation. Resins were incubated in 200 µl of 1X quenching buffer and 3 µl of sodium cyanoborohydride for 15 minutes at room temperature. Resins were washed twice with 200 µl of 1X coupling buffer and 6 times with 100 µl of wash solution (Pierce). All the following co-immunoprecipitation steps

were performed at 4°C. Antibody immobilized resins were washed twice with 200 µl of lysis/wash buffer and 3ug of protein was added to the resins and incubated overnight at 4°C. The beads were collected after centrifugation through the column provided. Beads were washed twice with 1X PBS and were additionally washed with 1X conditioning buffer (Pierce). Co-immunoprecipitated protein was eluted using elution buffer (Pierce) to disrupt the antigen-antibody interaction. For antibody negative controls, samples were prepared in the same way other than the antibody immobilization step. Eluted protein was mixed with 5X reducing sample buffer and incubated at 100°C for 10 min. Reduced samples were run using running buffer (Pierce) on a 4-20% gradient polyacrylamide mini-gel (Pierce) at 100V. The gel was fixed twice for 30 min using fixation solution (50% methanol/7% acetic acid) and stained overnight in SYPRO Ruby (Invitrogen) at room temperature. The gel was de-stained for 2-3 hrs using wash buffer (10% methanol/7% acetic acid), photographed and analyzed by mass spectrometry for characterization of protein sequence/identity by the Targeted Metabolomics and Proteomics Lab (University of Alabama at Birmingham) using liquid chromatography – mass spectrometry (LC-MS/MS).

Section-3 Results

Alternate binding partners of p16 during quiescence

The well-characterized parental CMT cell line CMT27 and its p16 transfected clone, CMT27A, were used (DeInnocentes *et al.*, 2009). CMT27 cells were used as negative controls as they lacked p16 expression (DeInnocentes *et al.*, 2009) and p16 transfected CMT27A cells are capable of reaching quiescence following serum-starvation, due to ectopic p16 expression as mentioned in the previous chapter (Agarwal *et al.*, 2011 data not published). CMT27A cell protein lysates from exponential and quiescent cells co-immunoprecipitated using no primary antibody, parental CMT27 lysates from exponential and quiescent cells co-immunoprecipitated with antibody, and no cell lysate with antibody present with beads were included as control reactions (Fig.8). Entire lanes composed of exponential or serum-starved positive controls (CMT27A with antibody) or serum-starved negative controls (CMT27A without antibody) were analyzed by the LS-MS/MS technique. Proteins analyzed during data analysis which were common in all three groups were not considered (Appendix1-3). Likewise ubiquitous proteins and proteins associated with the cytoskeleton were not considered (Appendix 1-3). Proteins which were unique to each sample were analyzed for function. The list of identified proteins was shortlisted to two proteins which were only co-immunoprecipitated in quiescent CMT27A cell lysates and not in exponential or negative controls. 14-3-3σ and ubiquitin-40S ribosomal protein S27a were the only two proteins identified as co-immunoprecipitating uniquely in quiescent cells in comparison to exponential cells.

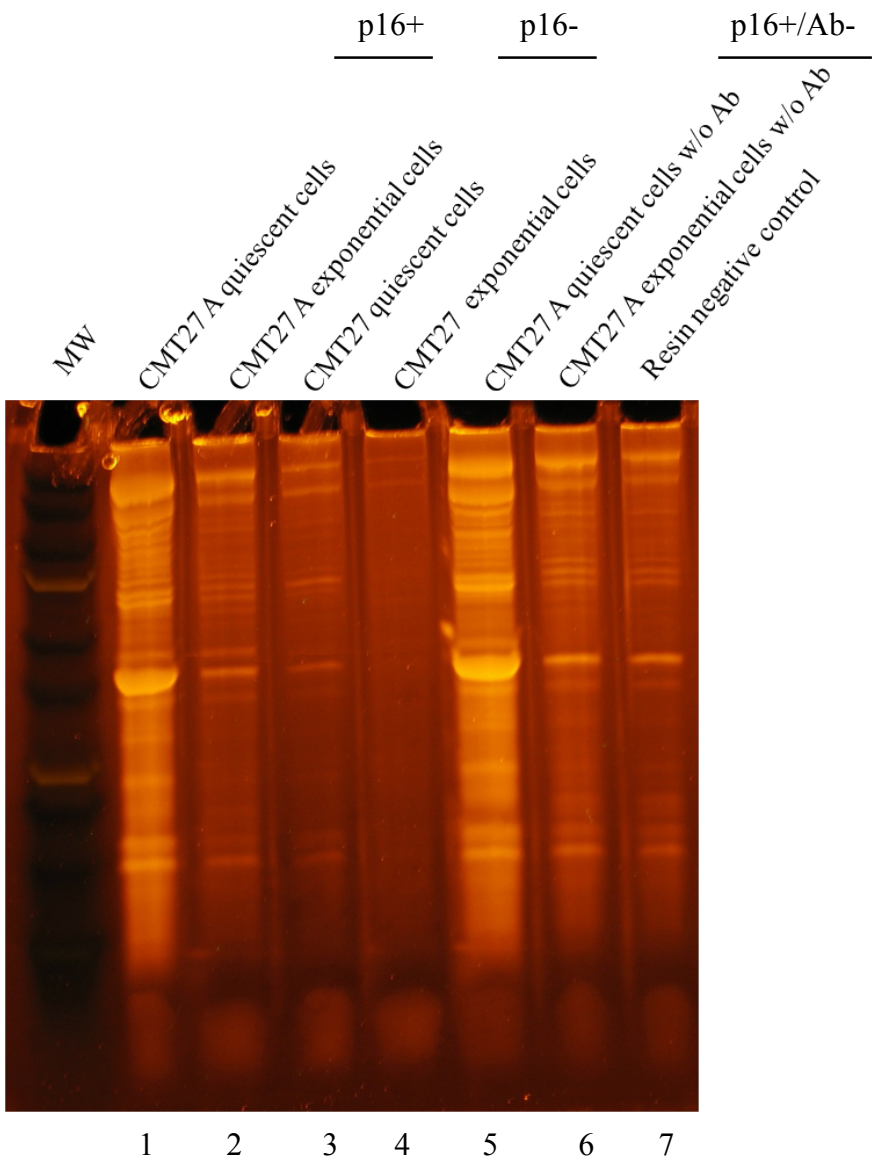

Figure 8: SDS-PAGE of p16 co-immunoprecipitated proteins.

Co-immunoprecipitation of cell lysates from serum-starved and exponential CMT27A and CMT27 parent cells using polyclonal rabbit anti-p16 antibody. Lanes 1 and 2 contain proteins eluted from CMT27A serum-starved and exponential cells. Lanes 3 and 4 negative controls contain proteins eluted from parental CMT27 (p16 negative) serum-starved and exponential cells. Lanes 5 and 6 are negative controls that contain proteins eluted from CMT27A serum-starved and exponential cells, but no antibody was used during co-immunoprecipitation. Lane 7 is a control lane, where no resin beads were added. The proteins were isolated from resin beads and run on SDS-PAGE. The gel was stained using SYPRO Ruby stain. MW are molecular weight standards.

Comparative semi-quantitative mRNA expression of 14-3-3σ

To verify that uniquely expressed proteins were present in p16-transfected cell lines, we compared 14-3-3σ and L37 constitutively expressed mRNA levels between exponential and quiescent parental p16-defective CMT cell lines (CMT12, CMT27, and CMT28), p16 transfected CMT clones (CMT27A and CMT28F), and NCFs using semi-quantitative rt-PCR. 14-3-3σ was expressed in all of the cell lines investigated. 14-3-3σ mRNA expression was normalized to L37 mRNA expression and normalized levels were compared between quiescent and exponential cells. The level of 14-3-3σ mRNA expression was high in quiescent NCF cells in comparison to exponential NCF cells (Fig.9). In contrast, although expression levels were generally higher, there was no difference in 14-3-3σ mRNA levels between any quiescent and exponential CMT cell line cells.

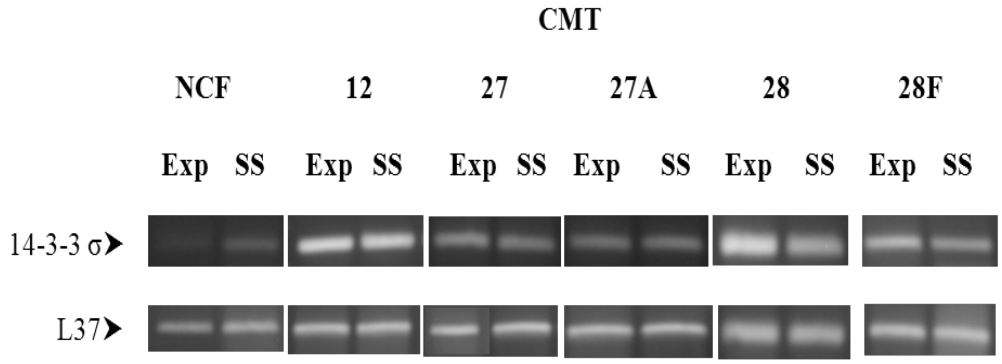

Figure 9: Semi-quantitative reverse transcriptase PCR of 14-3-3σ mRNA.

mRNA levels of 14-3-3σ and L37 were compared between serum-starved and exponential NCF, CMT12, CMT27, CMT27A, CMT28, and CMT28F cell lines. Transcripts were amplified by semi-quantitative rt-PCR and analyzed by agarose gel electrophoresis. Exp = exponentially growing cells and SS = Serum-starved cells.

Section-4 Discussion

p16/INK4A is an important tumor suppressor gene with multiple roles in cell cycle regulation, tumor suppression, cell senescence, cell quiescence, and cell differentiation. Typically p16 is thought to play a role in early G1 phase inhibiting the CDK4/6 and cyclinD1 complexes which makes p16 a natural binding partner of CDK4 and CDK6. It has been reported that p16 is overexpressed in cells reaching senescence, quiescence or differentiation, but in all these cellular phases, the levels of CDK4/6 and cyclinD1 expression, both at mRNA and protein levels, decreases suggesting the existence of a possible alternative binding partner for p16.

Some proteins, other than CDK4 and CDK6, have been reported to bind with p16, such as ISOC2, proliferating cell nuclear antigen (PCNA) and minichromosome maintenance protein 6 (MCM6) (Souza-Rodrigues *et al.*, 2007). ISOC2 has been found bound and co-localized with p16 using yeast two-hybrid cloning (Huang *et al.*, 2007). ISOC2 is a negative regulator of p16 and thus important for suppression of p16 in order to promote tumor proliferation and progression. PCNA and MCM6 are DNA replication-related proteins. PCNA is essential for DNA polymerase function during DNA replication and MCM6 is part of the MCM complex required for transient DNA double helix melting by DNA helicases. p16 binds to both PCNA and MCM6 during early G1 phase of the cell cycle and inhibits DNA replication. It has also been reported that p16 binds to protein involved in protein metabolism, cytoskeleton, RNA metabolism, and signal transduction (Souza-Rodrigues *et al.*, 2007). Another proposed binding partner of p16, thought to be important for cell growth inhibition by p16, is GRIM-19 (Gene associated with Retinoid-IFN-induced Mortality-19). GRIM-19 is a tumor suppressor

gene mutations of which have been found in primary human tumors. GRIM-19 and p16 synergistically inhibit cell cycle progression via the E2F pathway by promoting CDK4 inhibition by p16 (Sun *et al.*, 2010).

In our attempts to find alternative binding partners of p16, we identified proteins thought to be involved in protein and RNA metabolism, cytoskeletion function, and signal transduction as p16 binding partners. However, when compared to negative controls, we could verify that all these proteins eluting with p16 in co-immunoprecipitations, were most likely recovered as a result of non-specific interactions except for 14-3-3σ and Ubiquitin-40S ribosomal protein S27a complex. Both of these proteins were eluted only from the p16 positive quiescent cell lysate. Our results indicate that it is likely that 14-3-3σ is bound to p16 in p16 positive cells and only in the quiescent phase.

As discussed earlier, like p16, 14-3-3σ is a negative regulator of cell cycle. 14-3-3σ checks the cell cycle at G1/S and G2/M phase transitions by sequestering CDK2/cyclinE complex and CDC2/cyclinB complex respectively. It has been reported very recently that p16 can also down-regulate CDK1 protein expression by upregulating the miR-410 and miR-650 (Chien *et al.*, 2011), thereby inhibiting the cell cycle during the G2/M phase along with inhibiting the cell cycle at the G1/S phase by blocking the E2F pathway. Both p16 and 14-3-3σ are negative regulators of cell cycle through different pathways. In light of our data we propose that 14-3-3σ may be a possible binding partner of p16 or p16 associated complex, or a co-factor of p16 suggesting another pathway of inhibition of the cell cycle during G2/M phase by p16 in conjunction with 14-3-3σ.

p16 is an important tumor suppressor gene which regulates gene expression at different levels by modifying functional equilibrium of post-transcription factors, and consequently of miRNAs, and also by binding to post-transcriptional regulators (hnRNP C1/C2 and hnRNP A2/B1) (Souza-Rodrigues *et al.*, 2007). We suggest that p16 has a role in cell cycle exit followed by cell quiescence and differentiation by binding with 14-3-3σ.

Co-abrogation of p16 and 14-3-3σ functions as a consequence of promoter-methylation, has been reported in squamous cell carcinoma (Gasco *et al.*, 2002) and vulval squamous neoplasia (Gasco *et al.*, 2002). In order to explore the relationship between 14-3-3σ and p16 and the possibility of alternative binding partner for p16 in cell quiescence, semi-quantitative rt-PCR was employed to compare the expression of 14-3-3σ in exponential and quiescent cells. Our results show that, along with p16, mRNA expression of 14-3-3σ was also up-regulated in quiescent NCF cells. There was no difference in expression of 14-3-3σ evident in any of the CMT cell lines; even in those that achieved quiescence.

The only regulatory partners of 14-3-3σ protein previously identified were p53 and the associated proteins such as MDM2, Akt, and CDKs. 14-3-3σ potentiates the function of MDM2 negative regulators such as ARF, L5, L11, and L23, and antagonizes the MDM2 positive regulators such as Gankyrin, YY1, KAP1, in order to stabilize p53 (Lee and Lozano, 2006) which results in suppression of the cell cycle and also potentiates cell apoptosis. Our result suggests that 14-3-3σ may associate with p16 following exit from the cell cycle perhaps suppressing transcriptional activation of p53 and promoting

reversibility of quiescence. Suppressing p53 activation would also promote resistance to apoptosis.

Chapter 4: Role of p16 in Cell Differentiation

Section-1 Introduction

Embryonic development begins with cell division and growth of the fertilized zygote. Cells continue to proliferate and specialize as they differentiate. In general differentiated cells are non-replicating cells which express specific phenotypes. The switch from dividing cells to a differentiated phenotype is thought to be triggered by induction of an initial set of transcription factors or regulatory genes in response to a differentiation signal (Ben-Tabou de-Leon, 2007). The activation of initial regulatory genes in turn activates down-stream regulatory genes controlling the next regulatory state until the cells reach terminal differentiation.

Cyclin dependent kinases such as p57 (Tury *et al.*, 2011), p27 (Quaroni *et al.*, 2000), p21 (Munoz-Alonso *et al.*, 2011), p53 (Sola *et al.*, 2011), and p16 (Loercher *et al.*, 2005) can play an important role in cell differentiation. p27 and p21 are among the important regulatory factors which signal the end of cell replication and trigger cell differentiation (Quaroni *et al.*, 2000; Tamamori-Adachi *et al.*, 2004). p21 is associated with growth inhibition and differentiation in various carcinomas including head and neck carcinoma and ovarian carcinomas (de Jong *et al.*, 1999). It has been reported that p27 and p21 induce partial lactational differentiation in breast epithelial cells (Coppock *et al.*, 2007). Megakaryocyte differentiation (Munoz-Alonso *et al.*, 2011), muscle, cartilage, skin, and nasal epithelium differentiation (Parker *et al.*, 1995), and keratinocyte

differentiation (Wong *et al.*, 2010) are some examples where p21 plays an active role in differentiation.

Since CDKs and cyclins are frequentely suppressed in differentiated cells, we hypothesize that other CKIs such as p16 also play an important role in cell differentiation by binding to an alternative binding partner.

Section-2 Materials and Methods

Cell culture and differentiation

3T3-L1 fibroblasts (obtained from Dr. Robert L. Judd, College of Veterinary Medicine, Auburn University) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (ampicillin) (Sigma), and 10% FBS (Hyclone) in 60 mm dishes (Corning) at 37^{o}C (air, 95%; CO2, 5%). At 100% confluence cells were induced to differentiate to adipocytes by incubating them in DMEM supplemented with antibiotics, 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 250 nM dexamethasone, 0.28 mM rosiglitazone, and 4 μg/ml insulin for 3 days. After 3 days the media was replaced with DMEM containing antibiotics, 10% FBS, 0.28 mM rosiglitazone, and 4 μg/ml insulin for an additional three days. Finally, differentiated cells were grown in DMEM with antibiotics and 10% FBS (Bedi *et al.*, 2006).

Preparation of RNA, primer design, rt-PCR and DNA sequencing

Total RNA was isolated from differentiated adipocytes using RNA Stat 60 (Tel-Test, Inc.). The concentration of RNA was determined by absorbance at 260 nm (You and Bird, 1995). Reverse transcriptase semi-quantitative PCR of p16, CDK4, CDK6, L37, cyclinD1, p27, adiponectin, and 14-3-3σ was performed using gene specific primers (table 1 and table 2).

Table 2: List of primers sequence used for reverse-transcriptase PCR for cell differentiation model.

Gene	Primer (5'-3')	Genbank Accession*	Amplicon Size (bp)
Adiponectin sense	5'-GGAGAGCCTGGAGAAGCC-3'	NM_009605	188
Adiponectin anti-sense	5'-ATGTGGTAAGAGAAGTAGTA GAGTC-3'		
14-3-3σ sense	5'-GTCTTCTACCTGAAGATGA AGGGC-3'	XM_544477	243
14-3-3σ anti-sense	5'-GAAGGTGGTCTTGGCCAGTG-3'		

* Primers were designed based upon the gene sequence present in the Genbank database.

Section-3 Results

To explore the role of p16 in cell differentiation, we have used 3T3-L1 cells as a differentiation model. 3T3-L1 cells were successfully differentiated into adipocytes after culturing for 10 days in differentiation media (Fig.10). Semi-quantitative reverse transcriptase PCR was performed to compare the mRNA expression of p16, CDK4, CDK6, p27, adiponectin, L37 and 14-3-3σ between the undifferentiated 3T3-L1 fibroblasts and adipocytes (Fig.11a). mRNA expression of p16, CDK4, CDK6, p27, adiponectin, and 14-3-3σ were normalized to expression from the L37 gene (Fig. 11b). Adiponenctin mRNA expression was only found in differentiated adepocytes and not in 3T3-L1 cells, and served as a marker of differentiated adipocytes (Fig. 11a). CDK4, p27, and p16 mRNA expression was downregulated in differentiated adipocytes. Adiponectin and 14-3-3σ mRNA expression was upregulated in differentiated adipocytes. There was no difference in the mRNA expression of CDK6 in adipocytes.

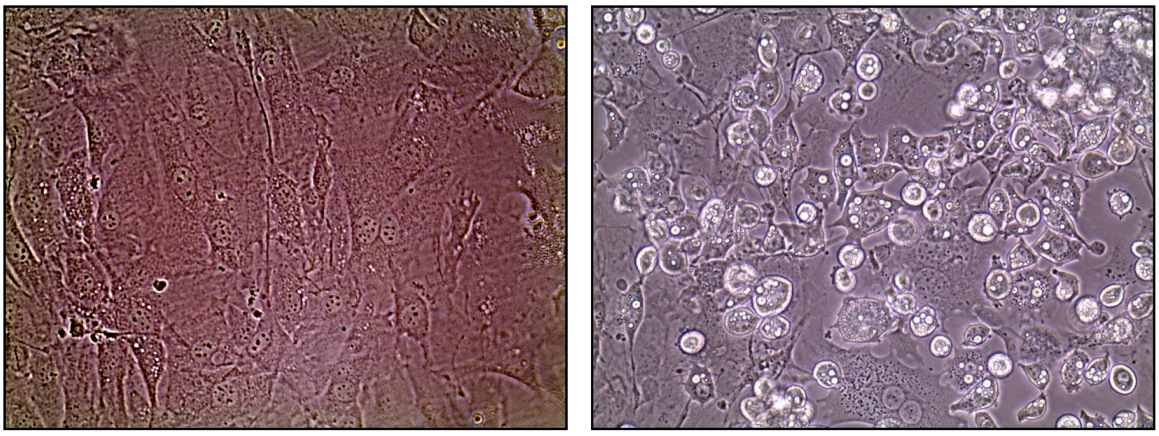

Figure 10: Microscopic image of pre-adipocyte 3T3L-1 Cells and differentiated adipocytes.

Phase contrast microscopy of live cells at 40X magnification. The *Left* image shows 3T3-L1 fibroblasts which when differentiated become lipid laden and mature differentiated adipocytes (*right* image).

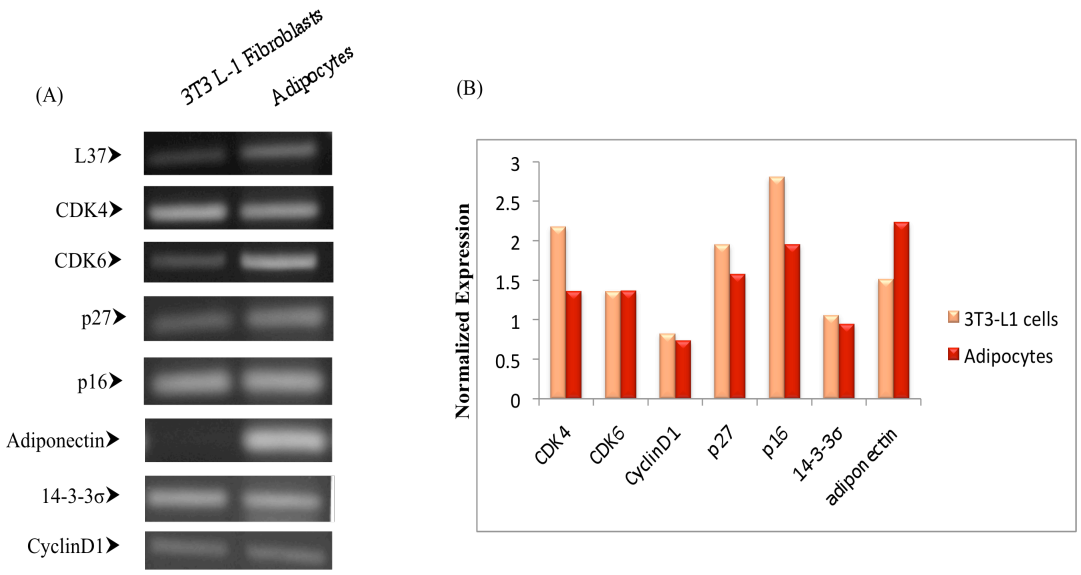

Figure 11: Semi-quantitative reverse transcriptase PCR of p16 and associated genes.

(a) mRNA levels of 14-3-3σ, p16, CDK4, CDK6, cyclinD1, p27, adiponenctin and L37 were compared between undifferentiated pre-adipocytes (3T3L-1-blue bars) cells and differentiated adipocytes (red bars). Transcripts were amplified by semi-quantitative rt-PCR and analyzed by agarose gel electrophoresis. (b) Relative expression of mRNAs was compared between differentiated and undifferentiated cells using NIH image scan software and was normalized to L37 mRNA expression. Adiponectin is only expressed in adipocytes and not in un-differentiated 3T3L-1 cells, and so serves as a positive control.

Section-4 Discussion

Cyclin-dependent kinase inhibitors play important roles in cell differentiation along with other differentiation factors. For example, p27 is a inducer/regulator of epithelial cell differentiation (Quaroni, 2000), Schwann cell differentiation (Li, 2011), human gastric epithelial cells differentiation (Wei, 2011), pituitary progenitor cells differentiation (Bilodeau *et al.*, 2009) gliogenesis and neurogenesis in *Xenopus* retinogenesis (Ohnuma *et al.*, 1999), and differentiation of erythroid precursors (Denicourt and Dowdy, 2004). Similarly, increased p16 expression is associated with the terminally differentiated post-mitotic phenotype. p16 expression is up-regulated in terminally differentiated human adult brain tissue (Lois *et al.*, 1995), in human embryonic teratocarcinoma cells (NT2) as they differentiate into post-mitotic neurons, (Lois *et al.*, 1995), during keratinocyte differentiation (Lee *et al.*, 2000), and during melanocyte differentiation (Loercher *et al.*, 2005). While, p16 and p21 CKIs augment expression of the skeletal muscle-specific gene myoD, cyclin-CDK complexes suppress its expression, thus inhibiting the differentiation of myoblast (Skapek *et al.*, 1995). CKIs, p16 and p21 inhibit cyclinD1-CDK4/6 complex. Knockdown of cyclinD and associated CDK4/6 is required for neuronal differentiation (Molenaar *et al.*, 2008) as well as myoblast cell cycle exit and differentiation (Saab *et al.*, 2006). CDK6 downregulation is important for differentiation (Grossel and Hinds, 2006; Fujimoto *et al.*, 2007) and p27 up-regulation along with down-regulation of CDK2, CDK4, and CDK6 is required for neutrophil and granulocyte differentiation (Klausen *et al.*, 2004).

CKIs, p27 and p16 expression was downregulated during pre-adipocyte proliferation (Auld and Morrison, 2006; Hasan *et al.*, 2011) and CKI p21 is required and

upregulated during adipocyte differentiation (Inoue *et al.*, 2008). In this study, we confirm that the expression of cyclinD1 and CDK4 is down-regulated during adipocytes differentiation. However, in contrast there was a coordinated-downregulation in the expression of p16 and 14-3-3σ in differentiated adipocyte cells. However this data is preliminary and we need to perform quantitative PCR and Western blots to analyze quantitative differences in expression at both mRNA and protein levels. We also need to use other differentiation models to investigate the role of p16 in cell differentiation.

Chapter 5: Characterization and Gene Expression Defects of p14ARF in Canine Mammary Cancer Cell Lines

Section-1 Introduction

Cell division occurs in a cyclic manner known as the cell cycle which is composed of four phases; G1, S, G2, and M phase (Enoch and Nurse, 1991). DNA duplication takes place in S phase and cytokinesis in M phase. G1 and G2 are gap phases, which provide the time for cells to ensure suitability of the external and internal environment and preparation for DNA duplication and division. Progression from one cell cycle phase to another is regulated by association of cyclin-dependent kinases (CDKs) and cyclins, CDKs are activated after forming heterodimers with associated phase-specific cyclins (Satyanarayana and Kaldis, 2009). G1 phase is the first phase of the cell cycle and the length of G1 depends on external environmental and extracellular signals. In response to adequate external and internal stimuli, cells progress from G1 phase to S phase where DNA replication takes place (Sherr and Roberts, 1995). If cells fail to enter S phase they enter a reversible quiescent state (G0) instead. But, if during the G1-S phase transition, the cell passes through the restriction (R) point, then the cell is committed to enter S phase even after removal of growth factors (Zetterberg *et al.*, 1995).

The cell cycle is tightly regulated by cyclin-dependent kinase inhibitors (CKIs) (Vidal and Koff, 2000). Progression of the cell cycle from G1 phase to S phase is

inhibited by two important CKIs; p16 and p21. p16 is a 16 kDa protein encoded by the *INK4A* gene locus (Serrano *et al.*, 1993; Kamb *et al.*, 1994). The same gene locus encodes three more genes. One of the genes translates into a 14 kDa protein known as p14ARF in humans, with no amino acid homology with p16. p16 and p14ARF share the last two common exons but differ in their first exons (Fig. 12) leading to the use of different translation start sites and reading frames. *p16* uses exon1α of the *INK4A* gene locus, while *p14ARF* uses exon1β which located further upstream on the same gene locus. This structural arrangement has been described in humans and mouse (Li *et al.*, 1995; Quelle *et al.*, 1995). Both *p16* and *p14ARF* are tumor suppressor genes but they have different functions (Kim and Sharpless, 2006). p16 inhibits the G1/S phase transition by inhibiting CDK4/6-CyclinD1 complexes (Russo *et al.*, 1998). p14ARF, on the other hand, stabilizes p53 expression in association with mdm2 (Boehme and Blattner, 2009). p53 up-regulates CKI p21 transcription causing inhibition of G1/S phase transition.

p14ARF inhibits MDM2, which results in stabilization of the important tumor suppressor p53. p53 is a transcription factor, which activates expression of proteins required for cell-cycle inhibition and apoptosis (Boehme and Blattner, 2009). One of the downstream regulatory protein activations mediated by p53 is CKI p21 up-regulation which checks the cell cycle late in the G1/S phase transition. p53 also acts as a transcription repressor of other genes (Gomez-Lazaro *et al.*, 2004). p53 is more stable in mammary epithelial cells in comparison to fibroblasts in humans, which suggests the importance of p53 in mammary epithelial cell growth (Delmolino *et al.*, 1993). Under normal conditions, p53 is rapidly degraded via E3 ubiquitin ligase MDM2. Under

conditions of stress or any other dysfunction, p14ARF binds and blocks MDM2, thereby releasing and stabilizing p53 by blocking MDM2. Wild type p53-induced phosphatase 1 (Wip1/Ppm1d) stabilizes MDM2 and downregulates p53 expression (Lin *et al.*, 2007). In contrast, it has also been reported that p14ARF and p53 have an inverse correlation with each other with respect to their activities in human cancer cell lines (Stott *et al.*, 1998). Disruption of Wip1 activates p53, p16, and p14ARF pathways, through p38MAPK signaling, and suppresses mouse embryo fibroblast transformation by oncogenes *in-vivo* (Bulavin *et al.*, 2004).

Functional effects/targets of p14ARF are not limited to p53 as p14ARF has other independent roles in cellular systems such as vascular regression of the developing eye (McKeller *et al.*, 2002) and arrest of cell cycle in murine embryo fibroblasts in the absence of p53 (Weber *et al.*, 2000). In addition to MDM2, p14ARF also binds to E2F-1, MDMX, HIF1-α, topoisomerase I, c-myc, and nucleophosmine (NPM) (Boehme and Blattner, 2009). p19ARF (the mouse ortholog of human p14ARF) is able to induce cell cycle arrest in mammalian fibroblasts in a manner analogous to p16 (Quelle *et al.*, 1995). E2F induces cell proliferation by activating S phase regulatory proteins but, according to one report, E2F can induce senescence in human diploid fibroblasts by inducing p14ARF expression, which is required for p53 stabilization (Dimri *et al.*, 2000). Inactivation of the p14ARF-p53-p21 pathway can also reverse senescence induced by ras signaling in mouse mammary carcinoma (Swarbrick *et al.*, 2008).

In summary, the *INK4A/ARF* gene locus is a key regulator of cell cycle because mutations at this site can adeversely affect both the G1-cyclin/CDK-pRb-E2F pathway through p16 and the MDM2-p53-p21 pathwasy through p14ARF. Thus, defects in the

INK4A/ARF gene locus provide very effective mechanisms for the promotion of neoplasm.

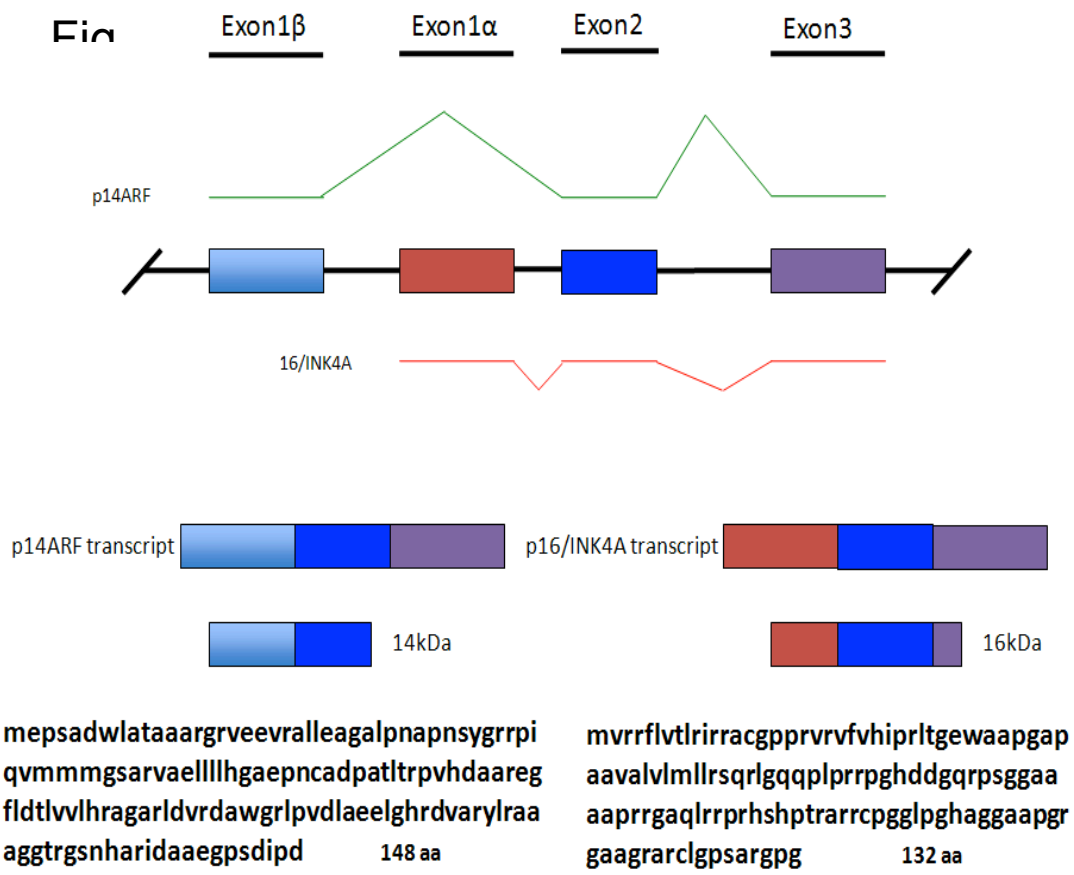

Figure 12: Schematic representation of two alternate transcripts from the INK4A/ARF locus in mammals.

p14ARF mRNA is encoded from exon1β, exon2, and exon3, while p16 mRNA is encoded from exon1α, exon2, and exon3. Both p14ARF and p16 share exon 2 and exon 3 but differ in exon1. Both sequences are encoded from the same gene but have alternate reading frames. Therefore, each transcript translates into two different sized proteins in humans (148 aa and 132 aa) with no similarity at all in amino acid sequence or function.

Cancer is caused by the progressive accumulation of multiple gene mutations (Bartek and Lukas, 2001), epigenetic dysregulation of gene mechanisms and protein pathways (Neumeister *et al.*, 2002), and abnormal function and regulation of cell cycle proteins (Nigg, 1995). The American Cancer Society reports that one out of three cancers diagnosed in women in the US will be breast cancer. There were estimated to be 230,480 new invasive cases in 2011 and an estimated 39,520 deaths due to breast cancer just in the US every year.

Breast carcinoma is also the most common spontaneous malignancy in unspayed female dogs, which comprise approximately 52% of all neoplasms in female dogs (MacEwen and Kurzman, 1996). Breast cancer in both women and female dogs have the same etiology (origin of disease) and in both species a majority of tumor cases are estrogen receptor or progesterone receptor (ER/PR) dependent (Bird *et al.*, 2008). Dogs have no known retroviruses and there is no viral etiology in canine mammary tumors (Bird *et al.*, 2008). Ovariohysterectomy prior to the fourth estrus cycle reduces risk to 0.05% from 25% (MacEwen, 1990). Canine breast cancer involves the same tissue types as human breast cancer. Both canine and human patients have poor clinical outcomes particularly once metastases have developed. This makes development of novel strategies to better treat and manage mammary cancer a high priority in both human and canine patients.

Because humans and dogs share the same environment, and many disease characteristics, dogs can be used as an intermediate model to better understand disease (Smith and Bird, 2010). Characterizing the tumor suppressor defects in canine cancer is a critical step in developing this intermediate model. We have characterized p14ARF

defects in canine breast carcinoma cell lines including the characterization of p14ARF mRNA and protein sequences. This is the first report validating canine p14ARF mRNA expression and includes mRNA and protein sequences beyond those predicted from the canine genome.

Section-2 Materials and Methods

Cell culture

Canine mammary tumor cell lines CMT12, CMT27, and CMT28, obtained from Dr. L. Wolfe (Wolfe *et al.*, 1986), and NCF (normal canine fibroblast) cells were used (DeInnocentes *et al.*, 2009). CMT12, CMT27, CMT28, and NCF were cultured in L-15 medium (Gibco) with antibiotics (ampicillin; Sigma), and 10% fetal bovine serum (Hyclone) in 25 cm^2 and 75 cm^2 tissue culture flasks (Corning) at 37^oC (air, 95%; CO2, 5%) as previously described (DeInnocentes *et al.*, 2006).

Preparation of RNA, primer designing, rt-PCR and DNA sequencing

Cells were grown until 75-80% of the cell surface was confluent and total RNA was isolated using RNA Stat 60 (Tel-Test, Inc.). RNA was also isolated from peripheral blood mononuclear cells (PBMC) of *Canis Lupus* greywolf (Pine Mountain Wildlife Park). Concentration of RNA was determined by absorbance at 260 nm (You and Bird, 1995). p16, p14ARF, and L37 cDNA synthesis and amplification was performed by reverse transcriptase semi-quantitative PCR using specific primers for p16 (sense 5'-AGCTGCTGCTGCTCCACGG-3'; antisense 5'-ACCAGCGTGTCCAGGAAGCC-3') (Koenig *et al.*, 2002), p14ARF (sense 5'-CGAGTGAGGGCTTTCGTGGTG-3'; antisense 5'-ACCACCAGCGTGTCCAGGAAG-3'), and L37 (sense 5'-AAGGGGACGTCATCGTTCGG-3'; antisense 5'- AAGGGGACGTCATCGTTCGG-3'). Amplicons were gel purified, cloned into vector pCR2.1 (Invitrogen) and sequenced (Auburn University Genomics and Sequencing Laboratory). The process of RNA extraction, semi-quantative analysis by rt-PCR, and sequencing was performed as

previously described (DeInnocentes *et al.*, 2006). p14ARF sequence was predicted using Vector NTI and was compared with p14ARF cDNA sequences from multiple species present in the Genbank database.

Section-3 Results

mRNA expression of p14ARF, p16, and L37

Well characterized canine mammary cancer cell lines (CMT12, CMT27, and CMT28) and normal thoracic canine fibroblasts (NCF) were used as a model to investigate the expression of p14ARF in canine cells. mRNA levels of ribosome protein L37, p14ARF, and p16 genes was compared between CMT cell lines and NCF using semi-quantitative rt-PCR (DeInnocentes *et al.*, 2009). Both NCF and CMT28 cells express p14ARF but p14ARF expression was defective in CMT12 and CMT27 cells (Fig.13). We have previously shown that rt-PCR of RNA extracted from NCF and CMT28 cells demonstrate expression of p16 (Fig.2). In contrast p16 expression was not detected by rt-PCR of RNA derived from CMT27 or CMT12 cell lines (Fig.13). Levels of p14ARF and p16 were higher in CMT 28 in comparison to NCF confirming previously published data on p16 (DeInnocentes *et al.*, 2009). Because p14ARF and p16 expression are defective in two canine mammary cell lines (CMT12 and CMT27), it is very likely that the defect in the bicistronic INK4/ARF locus is in a region shared by the two cistrons such as exons 2 and/or 3.

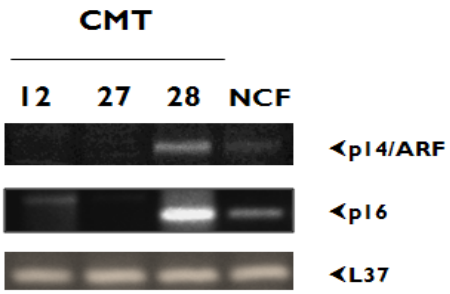

Figure 13: Semi-quantitative reverse transcriptase PCR of p16, p14ARF, and L37.

mRNA levels of p16, p14ARF, and L37 were evaluated in canine mammary tumor (CMT) cell lines and normal canine fibroblasts (NCF) cells by semi-quantitative PCR. Amplicons were analyzed by rt-PCR and agarose gel electrophoresis.

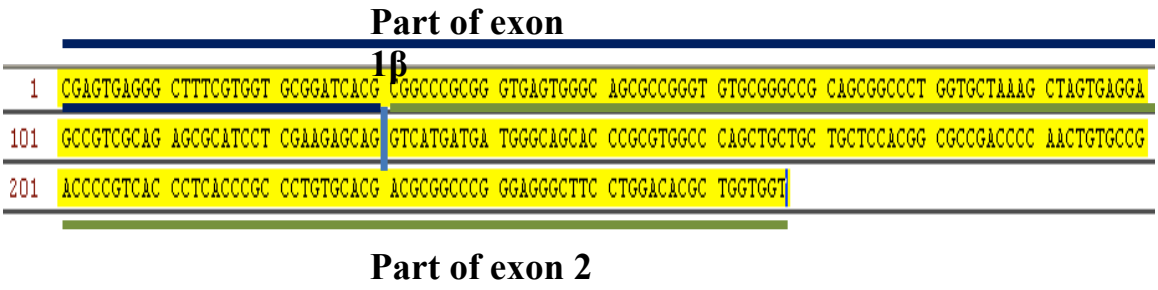

Figure 14: Partial p14ARF mRNA nucleotide sequence from NCF cells.

The partial nucleotide sequence of p14ARF from NCF was amplified and sequenced. Alignment with the published canine p14ARF gene sequence (Genbank accession: FM883643) predicted that the boundary of exon1β and exon 2 should be between positions 131 and 132 (blue vertical line).

p14ARF amplicons of 267 bp from RNAs isolated from CMT28, NCF, and wolf cells were cloned and sequenced twice for each cell line and wolf cells. NCF and CMT28 amplified nucleotide sequences were different by one base pair which results in one amino acid difference in the encoded protein. The amplified NCF (267bp), CMT28 (267bp), and wolf (*Canis lupus* grey wolf 221bp) partial p14ARF nucleotide sequences were aligned with *Canis lupus* familiaris CDKN2A p14ARF gene (Genbank No. FM883643) (Fig.16). By aligning the partial sequence amplified in our laboratory with published p14ARF gene sequence, we predicted the location of the exon 1β/exon 2 boundary (Fig.14).

The nucleotide sequence upstream and downstream of the exon 1beta exon 2 boundary was aligned with p14ARF mRNA sequences of human (Genbank No. NM_058195), pig (Genbank No. NM_213735), mouse (Genbank No. NM_009877), opossum (Genbank No. NM_001032973), and dog p14ARF mRNAs and the open reading frame was predicted (Fig.15a). The canine p14ARF protein sequence was predicted by translating the predicted mRNA sequence using Vector NTI (Fig.15b; Invitrogen).

(a)

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  1  TGCGCGGGTC CCCGTCCTCG GTCGGGGGGC GGGCGCCGCG CTGCCTACCT CTGATGCCTA AAGGCGGCGC AGCGATCGAG GAGCACAGCA GAGGTGGCGG
101  CGGCGAGATT ATGGTGCGCG CGTTTTTGAT CACAGTGCGG ATTCGGCGCG CTGGGGGCCC GCCGCGAGTG AGGGCTTTCG TGGTGCGGAT CACGCGGCCC
201  GCGGGTGAGT GGGCAGCGCC GGGTGTGCGG GCCGCAGCGG CCCTGGTGCT AAAGCTAGTG AGGAGCCGTC GCAGAGCGCA TCCTCGAAGA GCAGGTCATG
301  ATGATGGGCA GCACCCGCGT GGCCCAGCTG CTGCTGCTCC ACGGCGCCAA CCCCAACTGT GCCGACCCCG TCACCCTCAC CCGCCCTGTG CACGACGCGG
401  CCCGGGAGGG CTTCCTGGAC ACGCTGGTGG TGCTGCACCG AGCCGGGGCG CGGCTGGACG TGCGCGATGC CTGGGGCCGC CTGCCCGTGG ACCTGGCTGA
501  GGAGCGGGGC CACGGCGCTG TCGCTGCGTA CCTGCGCGCA GCCGCGGGGG GCACCGAAAG TGGTAGCCAC GCCCGTACGG AAGGTGCGGA AGGTCACGCA
601  GGTGAGGTGC CACATCTGAG TTGGAACTCG GAGAGCTTCC GCAGCCCAGA GCACTCCTTT TTCAGAAGAG AAAGCTGATT CTAGGAGAGC AAGGTCTTGT
701  GTCTCGCTGC AGCTCTCACT GCCAA
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(b)

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  1  MVRAFLITVR IRRAGGPPRV RAFVVRITRP AGEWAAPGVR AAAALVLKLV
 51  RSRRRAHPRR AGHDDGQHPR GPAAAAPRRQ PQLCRPRHPH PPCARRGPGG
101  LPGHAGGAAP SRGAAGRARC LGPPARGPG
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Figure 15: Predicted mRNA sequence, oral reading frame and protein sequence of canine p14ARF.

(a) Predicted mRNA sequence from p14ARF exon 1β and exon 2 and open reading frame located in these two exons, based upon the alignment with the canine p14ARF gene in the Genbank (FM883643) and other species, p14ARF mRNA sequences. (b) Predicted amino acid sequence of canine p14ARF based upon the predicted open reading frame using Vector NTI.

The open reading frame of the canine p14ARF mRNA sequence was compared with open reading frames from the same gene in mammalian species (Fig.16). The CMT28 sequence is identical to the published canine p14ARF gene sequence. There is one nucleotide difference between NCF and CMT28 amplified sequence which results in one amino acid change in the partial protein sequences of both canine cell lines. This amino acid change is non-conservative Gln (polar) to Arg (basic) and is likely an allelic variant in normal animals. This variant shows nucleotide similarity between three unrelated dogs of different breeds as well as wolf. When canine p14ARF sequence was compared to the human p14ARF sequence, there were 12 silent nucleotide changes in the predicted canine p14ARF nucleotide sequence. Nine of the differences were shared in both NCF and CMT28 partial amplified sequences. In the exon1β sequence, there is an absence of 9 nucleotide base pairs compared to the human and 6 nucleotide base pairs compared to the mouse and pig p14ARF sequences making the canine p14ARF protein sequence 3 amino acids shorter than the comparable human p14ARF protein sequence. Furthermore, there are 31 amino acid differences between the entire human and predicted canine p14ARF protein sequences. Out of these amino acid differences, 18 amino-acid differences are non-conservative while the remaining 13 are conservative amino-acid differences. There is 85.7% similarity in nucleotide sequences between canine and human p14ARF and 75.9% similarity in amino acid sequence. The difference in similarity between nucleotide and amino acid sequences can be attributed to the native genetic code. Since 3 nucleotides code for 1 amino acid, there is a greater chance for significant percent difference at the amino acid vs. the nucleic acid base pair level.

Dendograms comparing clusters of p14ARF mRNA and protein sequences from dog with those from human and other mammalian species did show clustering of dog and human together in comparison to mouse sequence (Fig.17).

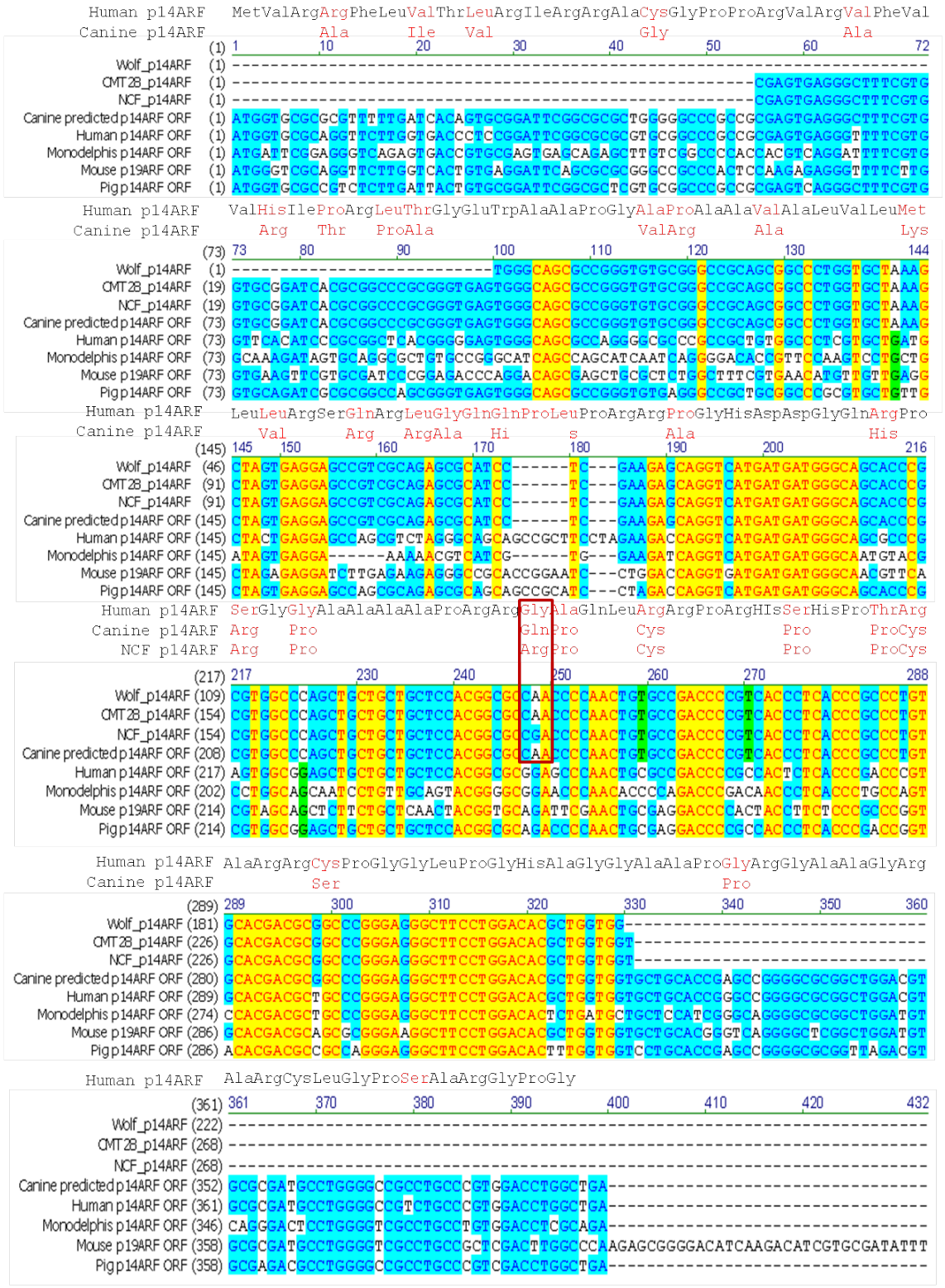
Human p14ARF MetValArgArgPheLeuValThrLeuArgIleArgArgAlaCysGlyProProArgValArgValPheVal
Canine p14ARF Ala Ile Val Gly Ala
Wolf_p14ARF
CMT28_p14ARF
NCF_p14ARF
Canine predicted p14ARF ORF
Human p14ARF ORF
Monodelphis p14ARF ORF
Mouse p19ARF ORF
Pig p14ARF ORF
Human p14ARF ValHisIleProArgLeuThrGlyGluTrpAlaAlaProGlyAlaProAlaAlaValAlaLeuValLeuMet
Canine p14ARF Arg Thr ProAla ValArg Ala Lys
Human p14ARF LeuLeuArgSerGlnArgLeuGlyGlnGlnProLeuProArgArgProGlyHisAspAspGlyGlnArgPro
Canine p14ARF Val Arg ArgAla Hi s Ala His
Human p14ARF SerGlyGlyAlaAlaAlaAlaProArgArgGlyAlaGlnLeuArgArgProArgHIsSerHisProThrArg
Canine p14ARF Arg Pro Gln Pro Cys Pro ProCys
NCF p14ARF Arg Pro Arg Pro Cys Pro ProCys
Human p14ARF AlaArgArgCysProGlyGlyLeuProGlyHisAlaGlyGlyAlaAlaProGlyArgGlyAlaAlaGlyArg
Canine p14ARF Ser Pro
Human p14ARF AlaArgCysLeuGlyProSerAlaArgGlyProGly

Figure 16: Canine and mammalian p14ARF mRNA sequence alignments.

DNA sequences representing the transcript cDNAs derived from CMT28 cells, wolf PBMCs, and NCFs were aligned using AlignX (CLUSTAL-W/Vector NTI) software and compared to other mammalian p14ARF open reading frames. Canine p14ARF amplicons from cell lines CMT28 and NCF were 267 bp long and the amplicon from wolf PBMCs of 221 bp long representing nucleotides 56-331 of the canine p14ARF ORF encoding amino acids 19-89, with respect to the human sequence. Amplicons were cloned, sequenced and aligned to p14ARF cDNA sequences from pig (NM213735), *Monodelhis* (Opossum) (NM001032973), mouse (NM009877), human (NM058195), and the predicted mRNA sequence extrapolated from a canine genomic fragment (FM883643) in Genbank. Differences in the canine compared to the human p16/INK4A protein sequence are noted. There is one nucleotide difference between NCF and all other canine sequences that results in a single allelic difference in the amino acid sequence (red box).

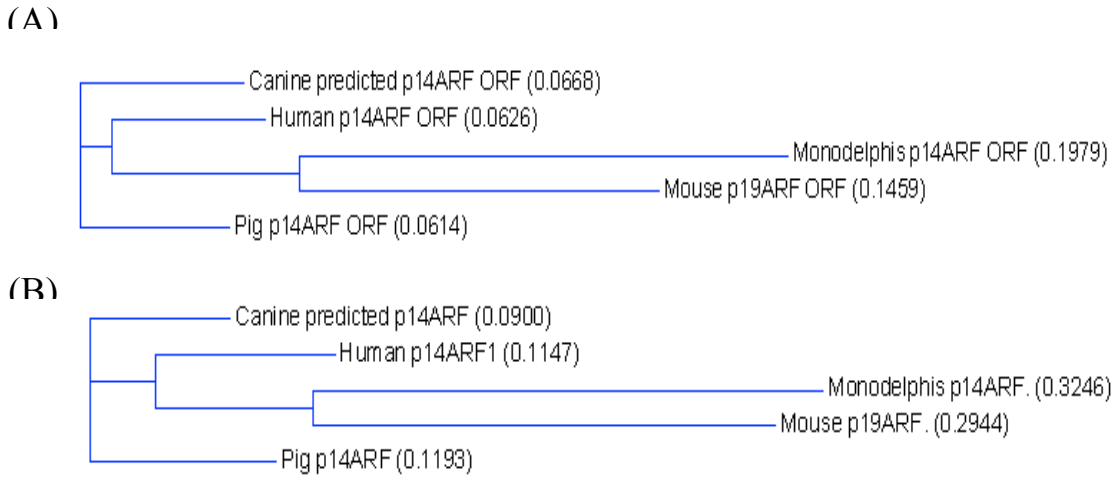

Figure 17: Dendrograms of mammalian p14ARF mRNA and protein sequences.

A neighbor-joining algorithm (ClustalW—Vector NTI) was used to calculate a rooted relationship dendrogram for related mammalian sequences encoding p14ARF. Published canine, human, rat, mouse, *Monodelphis*, pig and all of the canine amino acid sequences were compared. This includes the CMT28 and NCF cell lines and wolf PBMC-derived p14ARF sequences. (a) Dendogram of p14ARF mRNA sequences of human, pig, *Monodelphis* (opossum), and the predicted mRNA sequence extrapolated from a canine genomic fragment from Genbank, (b) dendogram of p14ARF protein sequences of human, pig, *Monodelphis* (opossum), and the predicted mRNA sequence extrapolated from a canine genomic fragment from Genbank. Value in parentheses shows the length of each tree branch proportional to the number of nucleotide base pair or amino acid differences between related mammalian sequences.

Section-4 Discussion

The *INK4A/ARF* gene locus is a very important tumor suppressor gene locus, and is unique in eukaryotes. The *INK4/ARF* gene locus can be globally inactivated by genetic or epigenetic alteration in various kinds of cancer. Loss of heterozygosity in the *INK4* gene locus on chromosome 11 has been reported in canine fibrosarcoma (Aguirre-Hernandez *et al.*, 2009). Homozygous deletion and loss of heterozygosity in INK4 family members are frequently observed in nervous system tumors (Almeida *et al.*, 2008). p14ARF/p16 inactivation has been reported in malignant peripheral nerve sheath tumors (Endo *et al.*, 2011), and oral squamous cell carcinoma (Ohta *et al.*, 2009). p14ARF and other p53 pathway proteins are down-regulated in human colorectal cancer (Tachibana *et al.*, 2004) and head and neck cancer (Hoque *et al.*, 2002). Ectopic expression of p14ARF stabilized p53 accumulation which is required for cell cycle arrest and apoptosis (Huang *et al.*, 2003). Ectopic expression of p14ARF has also been reported to restore p53 function and induce growth arrest in human lung cancer cell lines (Gao *et al.*, 2001) which makes it a gene of interest for gene therapy in cancer therapeutics. In our research, we have found that p14ARF mRNA is not expressed in two well-established canine mammary cancer cell lines (CMT12 and CMT27) and p14ARF mRNA is relatively over-expressed in CMT28 in comparison to NCF.

The dog is an excellent model for the study of human cancers because dogs have very close etiology to human cancers and dogs share the same environment with their human owners. Dogs with spontaneous cancers can be used as intermediate models to bridge the biological distance between induced mouse tumors and human cancers (Bird *et al.*, 2008). CMT cells are also very good *in vitro* models for human cancers and have

been characterized for gene expression defects in tumor suppressor genes such as *p16*, *p21*, and *p53* and for gene activation defects in oncogenes such as c-*erb*B-2 and c-*yes* (DeInnocentes *et al.*, 2006; Bird *et al.*, 2008; DeInnocentes *et al.*, 2009). It is important to characterize and evaluate the gene expression defects in canine tumor models as they are similar to defects found in human tumors. Continued research on CMT models builds validation and reinforces the strength of the model. In this report, we have amplified a partial sequence of the *p14ARF* open reading frame and predicted the p14ARF mRNA and protein sequence for dogs. We observed 85.7% nucleotide sequence similarity and 75.9% amino acid similarity between dogs and humans p14ARF sequences. When nucleotide and proteins sequences of dogs and other mammalian species were analyzed and compared, the sequences of dogs and humans clustered more closely than sequence comparisons of humans and mice. The observed similarity between dogs and humans further validates the strength of the canine tumor model.

This is the first report regarding the canine p14ARF mRNA and protein sequence beyond predictions from the canine genome. A single nucleotide polymorphism found in the shared second exon of *p14ARF/p16* sequence in dogs (A-G/Q-R) confirms a previously published report of p16 sequence in dogs (Aguirre-Hernandez *et al.*, 2009).

Chapter 6: Conclusions

In normal cells, tumor suppressors and oncogenes are criticalfor cell cycle regulation (Tripathy and Benz, 1992). Tumor suppressor genes function to check cell growth by suppressing cell cycle progression, promoting entry into a post-proliferative state and thus inhibiting unwanted cell proliferation. As a consequence the activity of tumor suppressor genes is not only limited to cell cycle regulation, since this set of genes also has important roles in cell fate and development.

My research was based on the tumor suppressor gene *p16* (also named as *INK4A* and *CDKN2A*) and *p14ARF* (Serrano, 1997). p16 checks cell cycle in early G1/S phase, where it inhibits binding of cyclinD1 to associated kinases CDK4/6 thus inhibiting release of the S phase transcription factor E2F from its inhibitory binding partner pRb. p16 expression has been found defective in a variety of cancers, second in number only to p53 defects in tumor cells (Weinberg, 1995). p16 is also defective in established immortalized cell lines such as L6 mouse myoblasts and NIH3T3 mouse fibroblasts (Kamb *et al.*, 1994) that were not derived from neoplastic tissues.

It has previously been reported that *p16* is a tumor suppressor gene and its expression increases by many fold in senescent cells (Zindy *et al.*, 1997). Although p16 promotes cell cycle exit, it is unclear whether it is required for cells to enter quiescence, or to become terminally differentiated.

We have previously reported phenotype rescue of canine mammary cancer cell lines due to ectopic expression of p16 (DeInnocentes *et al.*, 2009). In the current research we have demonstrated that p16 expression not only results in cell cycle exit but also

maintains cells in the quiescent phase. Well characterized canine mammary cancer cell lines such as CMT12, CMT27, and CMT28 were used along with normal canine fibroblasts (NCF) as a positive control. p16 transfected clones of CMT27 and CMT28 were used as phenotype-rescued experimental models. All of the cell lines expressed cyclinD1, CDK4, CDK6, p27, and L37, but only NCF and the p16 transfected CMT clones CMT27A, CMT27H, CMT28A, and CMT28F expressed *p16* gene at both the mRNA and protein levels. CMT28 parental cells expressed p16 at the mRNA level only but did not express p16 protein.

Serum-starvation of all CMT cell lines, their clones, and NCF cells was performed to induce cell cycle exit. Serum-starvation resulted in significantly decreased DNA replication in all of the cell lines except CMT27. p16 transfected CMT27A and CMT27H cells clones showed enhanced starvation effects in comparison to parental cells. To connect the decrease in DNA replication with cell cycle exit, not cell death, we analyzed cell cycle phase cell distribution post-serum-starvation using fluorescence-activated cell sorting.

CMT27 cells did not exit cell cycle after serum-starvation, while p16-transfected CMT27 clones CMT27A and CMT27H exited the cell cycle, indicating p16 expression promoted cell cycle exit. NCF, CMT28, CMT28A, CMT28F, and CMT12 cells did exit cell cycle, although CMT28 and CMT12 cells did not express p16. As has been mentioned before, p16 is thought to induce cell senescence which is a permanent non-dividing cell stage (Zindy *et al.*, 1997). To demonstrate that our experimental model cells did not leave the cell cycle permanently, we refed cells with serum rich medium. Synchronous cell-cycle re-entry was observed upon refeeding in all of the cell lines

except CMT12, proving that serum-starvation in most canine mammary tumor cell lines resulted in induction of a temporary non-dividing stage. CMT12 cells appear to exit the cell cycle permanently after serum-starvation and may have reached cell senescence.

Quiescent phase cells, post cell cycle exit, enter a temporary non-dividing stage characterized by over-expression of p27 (Sherr and Roberts, 1995). Quantitative PCR analyses was used to compare p27 and p16 mRNA expression in serum-starved and exponentially growing cells normalized to ribosomal subunit protein L37 mRNA expression. Only p16 expressing serum-starved cell lines NCF, CMT27A, and CMT28F over-expressed p27 in comparison to exponential cells, demonstrating that serum-starvation led to cell cycle exit and quiescence. Along with p27 mRNA, these cells overexpresse p16 mRNA and also overexpress p16 protein in comparison to exponentially growing cells. This suggests a potential role for p16 in quiescent cells and that p16 may be an important factor in cell quiescence.

We have shown that p16 is overexpressed during quiescence and as previously reported p16 is overexpressed in cell differentiation (Lois *et al.*, 1995). Usual partners of p16 i.e. cyclinD1 or CDK4/6 are underexpressed in both quiescence and differentiation state which could suggest the presence of a possible alternative partner for p16. Co-immunoprecipitation of p16 with 14-3-3σ and ubiquitin-40S ribosomal protein S27a demonstrated that p16 indeed does bind alternate targets in quiescent CMT27A cells.

14-3-3σ is an inhibitor of cell proliferation and is inactivated co-incidentally along with p16 in squamous cell carcinoma (Gasco *et al.*, 2002; Lee and Lozano, 2006). We show here that 14-3-3σ mRNA is upregulated in quiescent NCF cells along with p16

upregulation, suggesting coordinate expression of p16 and 14-3-3σ in NCF cells, although this is not the case in CMT27A and CMT28F quiescent cells. We therefore conclude that 14-3-3σ is a very likely possible alternative partner of p16 in cell quiescence.

We have also developed a cell development model to observe the role of p16 in cell differentiation. We have differentiated pre-adipocyte 3T3L-1 cells into adipocytes and have compared mRNA expression of p16, 14-3-3σ, and p16 associated proteins. All of the proteins investigated were expressed in both nondifferentiated and differentiated cells, however, we found *coordinated* downregulation in expression of only p16 and 14-3-3σ. Probing the subtilities of this model will require more research and perhaps different development models to verify the role of p16 in cell differentiation.

p16 is encoded at an important gene locus which encodes four transcripts from the same locus. p14ARF is one of these encoded proteins and is also an important tumor suppressor gene, but works by an entirely independent mechanism.

Like p16, p14ARF expression is also absent in CMT12 and CMT27 cells although it is expressed in NCF and CMT28 cells. We have sequenced and cloned a partial canine p14ARF sequences from NCF, CMT28, and wolf PBMCs and have predicted the full p14ARF sequence for dogs. We observed 85.7% nucleotide sequence and 75.9% amino acid sequence similarity between dog and human p14ARF. Dog and human mRNA and protein sequences cluster together in the hierarchical clustering analaysis. Our research re-validates the fact that canine tumor cells can be used as an efective model for human cancer studies. A single nucleotide polymorphism was found in

the shared exon2 of the p14ARF sequence in dogs (A-G/Q-R), confirming a previously published report of p16 sequence in dogs (Aguirre-Hernandez *et al.*, 2009). To the best of our knowledge this constitutes the first report regarding the canine p14ARF mRNA sequence and protein sequence prediction.

It is important to characterize and evaluate gene expression defects in canine tumor models as they are similar to defects found in human tumors. Using intermediate models such as dogs, who shares same etiology and same environment with humans, is an excellent way to bridge the biological distance between induced mouse tumors and human cancers (Bird *et al.*, 2008). Such canine intermediate models make it easier to investigate human cancer biology and develop effective therapies based on these investigations.

In my current research, I have explored an alternate role for the tumor suppressor gene p16 in cell quiescence and cell differentiation using dogs as models. This report is a step towards the development of novel therapeutic strategies where we can use tumor suppressor genes to direct the cells away from abnormal proliferation towards cell quiescence and differentiation perhaps by targeting these newly discovered alternative p16 binding partners.

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Appendix 1: Proteins identified using LC-MS/MS in gel lanes excised from a SDS-PAGE used to seperate proteins precipitated from serum-starved CMT27A cells lysate using anti-human p16 Ab.

The entire gel lane was excised and sent for protein identification using the LS-MS/MS technique. Each gel lane was divided into 8 samples. In the following table, the first column contains the sample number, the second column contains protein identified from that sample, the third column contains the corresponding MOWSE score, and the fourth column contains the accession number.

Sample Number	Protein Identification	Mowse score	Accesion number
Sample-1	Myosin-9	34893	MYH9_HUMAN
	Myosin-10	6786	MYH10_HUMAN
	Myosin-14	4448	MYH14_HUMAN
	Myosin-11	3233	MYH11_HUMAN
	Semenogelin-1	751	SEMG1_HUMAN
	Semenogelin-2	535	SEMG2_HUMAN
	Actin, cytoplasmic 1	486	ACTB_HUMAN
	Fibronectin	291	FINC_HUMAN
	Protein S	178	S10A8_HUMAN
	Desmoplakin	165	DESP_HUMAN
	Prolactin-inducible protein	142	PIP_HUMAN
	Myosin light polypeptide 6	141	MYL6_HUMAN
	Serpin B3 OS=3DHomo sapiens	135	SPB3_HUMAN
	Annexin A1 OS=3DHomo sapiens	134	ANXA1_HUMAN
	Galectin-7 OS=3DHomo sapiens	131	LEG7_HUMAN
	Beta-actin-like protein 2	123	ACTBL_HUMAN
	Myosin-VI	123	MYO6_HUMAN
	Junction plakoglobin	105	PLAK_HUMAN
	Leucine zipper protein 1	104	LUZP1_HUMAN
	Dermcidin	98	DCD_HUMAN
	Myosin-Ic	89	MYO1C_HUMAN
	14-3-3 protein sigma	85	1433S_HUMAN
	Heat shock protein	84	HS90B_HUMAN

	Heat shock protein	84	TRAP1_HUMAN
	Calmodulin-like protein 5	83	CALL5_HUMAN
	14-3-3 protein zeta/delta	80	1433Z_HUMAN
	Glial fibrillary acidic protein	77	GFAP_HUMAN
	Annexin A2 OS=3DHomo sapiens	75	ANXA2_HUMAN
	Protein S100-A7	74	S10A7_HUMAN
	Elongation factor 1-alpha 1	71	EF1A1_HUMAN
Sample-2	Myosin-9	38246	MYH9_HUMAN
	Myosin-10	11074	MYH10_HUMAN
	Myosin-14	4549	MYH14_HUMAN
	Myosin-11	2691	MYH11_HUMAN
	Alpha-actinin-4	2470	ACTN4_HUMAN
	Alpha-actinin-1	1443	ACTN1_HUMAN
	Myosin-Ic	977	MYO1C_HUMAN
	Myosin-VI	893	MYO6_HUMAN
	Myosin-Id	780	MYO1D_HUMAN
	Actin, cytoplasmic 1	620	ACTB_HUMAN
	Alpha-actinin-2	564	ACTN2_HUMAN
	Myosin-Ib	452	MYO1B_HUMAN
	Alpha-actinin-3	442	ACTN3_HUMAN
	Fibronectin	408	FINC_HUMAN
	Beta-actin-like protein 2	181	ACTBL_HUMAN
	Myosin light polypeptide 6	147	MYL6_HUMAN
	Spectrin beta chain, brain 3	140	SPTN4_HUMAN
	Myosin-3 OS=3DHomo sapiens	123	MYH3_HUMAN
	Myosin-Ia OS=3DHomo sapiens	113	MYO1A_HUMAN
	Dermcidin OS=3DHomo sapiens	102	DCD_HUMAN
	Myosin-7 OS=3DHomo sapiens	100	MYH7_HUMAN
	Myosin-6 OS=3DHomo sapiens	95	MYH6_HUMAN
	Myosin-4 OS=3DHomo sapiens	92	MYH4_HUMAN
	Myosin-7B OS=3DHomo sapiens	92	MYH7B_HUMAN
	Myosin-2 OS=3DHomo sapiens	92	MYH2_HUMAN
	Myosin-13 OS=3DHomo sapiens	92	MYH13_HUMAN
	Myosin-8 OS=3DHomo sapiens	92	MYH8_HUMAN
	Myosin-1 OS=3DHomo sapiens	92	MYH1_HUMAN
	Semenogelin-1	83	SEMG1_HUMAN
	Neurofilament heavy polypeptide	79	NFH_HUMAN
Sample-3	Myosin-9	17522	MYH9_HUMAN
	Myosin-10	5407	MYH10_HUMAN
	Myosin-14	3588	MYH14_HUMAN
	Myosin-11	1835	MYH11_HUMAN
	Actin, cytoplasmic 1	1142	ACTB_HUMAN
	Alpha-actinin-4	1098	ACTN4_HUMAN
	Myosin-VI	902	MYO6_HUMAN
	Myosin-Ib	649	MYO1B_HUMAN
	Myosin-Ic	554	MYO1C_HUMAN

	Myosin-Id	534	MYO1D_HUMAN
	Alpha-actinin-1	481	ACTN1_HUMAN
	Beta-actin-like protein 2	382	ACTBL_HUMAN
	Coatomer subunit alpha	361	COPA_HUMAN
	Fibronectin	359	FINC_HUMAN
	78 kDa glucose-regulated protein	313	GRP78_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	299	PLOD1_HUMAN
	Protein disulfide-isomerase	297	PDIA1_HUMAN
	Myosin light polypeptide 6	285	MYL6_HUMAN
	Tropomyosin alpha-4 chain	283	TPM4_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	274	PLOD3_HUMAN
	Tropomyosin alpha-1 chain OS=3DHomo sapiens	242	TPM1_HUMAN
	Tropomyosin alpha-3 chain OS=3DHomo sapiens	215	TPM3_HUMAN
	Alpha-actinin-2 OS=3DHomo sapiens	184	ACTN2_HUMAN
	Myosin-Ia OS=3DHomo sapiens	181	MYO1A_HUMAN
	Succinate dehydrogenase [ubiquinone] flavoprotein =subunit,=20	177	DHSA_HUMAN
	Heat shock cognate 71 kDa protein	173	HSP7C_HUMAN
	Heat shock protein HSP 90-beta	163	HS90B_HUMAN
	Myosin-Va OS=3DHomo sapiens	154	MYO5A_HUMAN
	Heat shock-related 70 kDa protein 2	142	HSP72_HUMAN
	Heat shock protein 75 kDa, mitochondrial	134	TRAP1_HUMAN
	Heat shock 70 kDa protein 6	124	HSP76_HUMAN
	Protein S100-A8 OS=3DHomo sapiens	115	S10A8_HUMAN
	Heat shock 70 kDa protein 1A/1B	111	HSP71_HUMAN
	Alpha-actinin-3 OS=3DHomo sapiens	107	ACTN3_HUMAN
	Prolyl 4-hydroxylase subunit alpha-1	94	P4HA1_HUMAN
	Myosin regulatory light chain 12A	91	ML12A_HUMAN
	Ubiquitin-40S ribosomal protein S27a	90	RS27A_HUMAN
	Myosin-Vc	80	MYO5C_HUMAN
	Myosin-3	79	MYH3_HUMAN
	Protein flightless-1 homolog	79	FLII_HUMAN
	Procollagen galactosyltransferase 1	70	GT251_HUMAN
	Myosin-7	70	MYH7_HUMAN
Sample-4	Actin, cytoplasmic 2	20217	ACTG_HUMAN
	Actin, cytoplasmic 1	20162	ACTB_HUMAN
	Myosin-9	11264	MYH9_HUMAN
	POTE ankyrin domain family member E	10287	POTEE_HUMAN
	Actin, alpha skeletal muscle	5456	ACTS_HUMAN
	Myosin-10	3888	MYH10_HUMAN
	POTE ankyrin domain family member I	2750	POTEI_HUMAN
	Beta-actin-like protein 2	2377	ACTBL_HUMAN

	POTE ankyrin domain family member J	2257	POTEJ_HUMAN
	Putative beta-actin-like protein 3	2168	ACTBM_HUMAN
	Myosin-14	1474	MYH14_HUMAN
	Myosin-11	1405	MYH11_HUMAN
	Tropomyosin alpha-1 chain	1021	TPM1_HUMAN
	Alpha-actinin-4	970	ACTN4_HUMAN
	Myosin-VI	621	MYO6_HUMAN
	Myosin-Ic	540	MYO1C_HUMAN
	Tropomyosin alpha-3 chain	537	TPM3_HUMAN
	Tropomyosin beta chain	519	TPM2_HUMAN
	Serpin H1	418	SERPH_HUMAN
	Alpha-actinin-1	391	ACTN1_HUMAN
	Tropomyosin alpha-4 chain	372	TPM4_HUMAN
	Myosin-Id	314	MYO1D_HUMAN
	Myosin-Ib	313	MYO1B_HUMAN
	Coatomer subunit alpha	278	COPA_HUMAN
	Tropomodulin-3	268	TMOD3_HUMAN
	Myosin light polypeptide 6	236	MYL6_HUMAN
	F-actin-capping protein subunit alpha-1	184	CAZA1_HUMAN
	Alpha-actinin-2	183	ACTN2_HUMAN
	Gelsolin	128	GELS_HUMAN
	Alpha-actinin-3	110	ACTN3_HUMAN
	Myosin-Ia	110	MYO1A_HUMAN
	Fibronectin	104	FINC_HUMAN
	Actin-related protein 3	93	ARP3_HUMAN
	Elongation factor 2	89	EF2_HUMAN
	ATP-dependent RNA helicase DDX3X	81	DDX3X_HUMAN
	Multifunctional protein ADE2	79	PUR6_HUMAN
	Neurofilament heavy polypeptide	78	NFH_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	78	PLOD1_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	77	PLOD3_HUMAN
	Pyruvate kinase isozymes M1/M2	76	KPYM_HUMAN
	Myosin-3	72	MYH3_HUMAN
	Myosin-13	72	MYH13_HUMAN
	Myosin-8	72	MYH8_HUMAN
	Myosin-4	72	MYH4_HUMAN
	Myosin-7B	72	MYH7B_HUMAN
	Prolyl 3-hydroxylase 1	71	P3H1_HUMAN
Sample-5	Myosin-9	4630	MYH9_HUMAN
	Actin, cytoplasmic 2	3512	ACTG_HUMAN
	Actin, alpha cardiac muscle 1	1928	ACTC_HUMAN
	Tropomyosin alpha-1 chain	1866	TPM1_HUMAN
	Myosin-10	1814	MYH10_HUMAN
	POTE ankyrin domain family member E	1688	POTEE_HUMAN

	Myosin-14	1251	MYH14_HUMAN
	Tropomyosin alpha-4 chain	1185	TPM4_HUMAN
	Tropomyosin alpha-3 chain	1053	TPM3_HUMAN
	Tropomyosin beta chain	935	TPM2_HUMAN
	Beta-actin-like protein 2	880	ACTBL_HUMAN
	Myosin-11	804	MYH11_HUMAN
	Myosin-VI	767	MYO6_HUMAN
	Putative beta-actin-like protein 3	709	ACTBM_HUMAN
	Annexin A2	640	ANXA2_HUMAN
	POTE ankyrin domain family member J	599	POTEJ_HUMAN
	Alpha-actinin-4	467	ACTN4_HUMAN
	Myosin light polypeptide 6	448	MYL6_HUMAN
	F-actin-capping protein subunit beta	413	CAPZB_HUMAN
	F-actin-capping protein subunit alpha-1	408	CAZA1_HUMAN
	Prolyl 4-hydroxylase subunit alpha-1	402	P4HA1_HUMAN
	Myosin-Ib	326	MYO1B_HUMAN
	Myosin-Ic	310	MYO1C_HUMAN
	Putative tropomyosin alpha-3 chain-like protein	309	TPM3L_HUMAN
	F-actin-capping protein subunit alpha-2	281	CAZA2_HUMAN
	Myosin regulatory light chain 12A	188	ML12A_HUMAN
	T-complex protein 1 subunit theta	177	TCPQ_HUMAN
	Protein disulfide-isomerase	161	PDIA1_HUMAN
	Peroxiredoxin-1	151	PRDX1_HUMAN
	Myosin light chain 3	144	MYL3_HUMAN
	ATP synthase subunit beta, mitochondrial	141	ATPB_HUMAN
	Serpin H1	140	SERPH_HUMAN
	Coatomer subunit alpha	138	COPA_HUMAN
	Alpha-actinin-1	137	ACTN1_HUMAN
	Myosin-Ia	132	MYO1A_HUMAN
	Tubulin beta chain	127	TBB5_HUMAN
	Tropomodulin-3	125	TMOD3_HUMAN
	Myosin-Id	123	MYO1D_HUMAN
	Prohibitin	119	PHB_HUMAN
	78 kDa glucose-regulated protein	116	GRP78_HUMAN
	Heat shock protein HSP 90-beta	115	HS90B_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	113	PLOD1_HUMAN
	Casein kinase II subunit alpha	103	CSK21_HUMAN
	Alpha-actinin-2	102	ACTN2_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	95	PLOD3_HUMAN
	T-complex protein 1 subunit delta	94	TCPD_HUMAN
	Alpha-actinin-3	90	ACTN3_HUMAN
	Actin-related protein 2/3 complex subunit 2	86	ARPC2_HUMAN
	Macrophage-capping protein	85	CAPG_HUMAN

	Procollagen galactosyltransferase 1	84	GT251_HUMAN
	Heat shock protein HSP 90-alpha	83	HS90A_HUMAN
	Protein flightless-1 homolog	77	FLII_HUMAN
	Tubulin alpha-1A chain	75	TBA1A_HUMAN
	T-complex protein 1 subunit gamma	72	TCPG_HUMAN
	Protein S100-A8	72	S10A8_HUMAN
Sample-6	Myosin-9	4417	MYH9_HUMAN
	Actin, cytoplasmic 1	2379	ACTB_HUMAN
	Myosin-10	2107	MYH10_HUMAN
	POTE ankyrin domain family member E	1334	POTEE_HUMAN
	Actin, alpha cardiac muscle 1	1234	ACTC_HUMAN
	Actin, alpha skeletal muscle V=3D1	1220	ACTS_HUMAN
	Tropomyosin alpha-1 chain	1106	TPM1_HUMAN
	Myosin regulatory light chain 12A	1047	ML12A_HUMAN
	Myosin-14	1041	MYH14_HUMAN
	Myosin-11	999	MYH11_HUMAN
	Alpha-actinin-4	698	ACTN4_HUMAN
	Myosin light polypeptide 6	636	MYL6_HUMAN
	Tropomyosin alpha-4 chain	576	TPM4_HUMAN
	Beta-actin-like protein 2	558	ACTBL_HUMAN
	Tropomyosin beta chain	556	TPM2_HUMAN
	Tropomyosin alpha-3 chain	457	TPM3_HUMAN
	Keratin, type I cytoskeletal 14	446	K1C14_HUMAN
	Myosin-Ib	397	MYO1B_HUMAN
	Myosin-Ic	394	MYO1C_HUMAN
	Myosin light chain 4	352	MYL4_HUMAN
	F-actin-capping protein subunit beta	340	CAPZB_HUMAN
	Alpha-actinin-1	293	ACTN1_HUMAN
	Myosin-Ia	257	MYO1A_HUMAN
	40S ribosomal protein S3	234	RS3_HUMAN
	Myosin light chain 6B	229	MYL6B_HUMAN
	Annexin A2	226	ANXA2_HUMAN
	40S ribosomal protein S4, X isoform	217	RS4X_HUMAN
	F-actin-capping protein subunit alpha-2	207	CAZA2_HUMAN
	Myosin-VI	203	MYO6_HUMAN
	Protein-L-isoaspartate(D-aspartate) =O-methyltransferase=20	197	PIMT_HUMAN
	Coatomer subunit alpha	196	COPA_HUMAN
	Myosin light chain 3	177	MYL3_HUMAN
	Prolyl 4-hydroxylase subunit alpha-1	162	P4HA1_HUMAN
	Peroxiredoxin-1	154	PRDX1_HUMAN
	Myosin light chain 1/3, skeletal muscle isoform	150	MYL1_HUMAN
	F-actin-capping protein subunit alpha-1	149	CAZA1_HUMAN
	Serpin H1	145	SERPH_HUMAN
	Elongation factor 1-alpha 1	140	EF1A1_HUMAN

	Multifunctional protein ADE2	135	PUR6_HUMAN
	Peptidyl-prolyl cis-trans isomerase B	132	PPIB_HUMAN
	40S ribosomal protein S9	130	RS9_HUMAN
	Calmodulin	128	CALM_HUMAN
	Actin-related protein 2/3 complex subunit 4	124	ARPC4_HUMAN
	60S ribosomal protein L18	121	RL18_HUMAN
	Plectin	115	PLEC_HUMAN
	T-complex protein 1 subunit theta	113	TCPQ_HUMAN
	Alpha-actinin-2	111	ACTN2_HUMAN
	Alpha-actinin-3	110	ACTN3_HUMAN
	ADP-ribosylation factor 4	106	ARF4_HUMAN
	Tubulin beta chain	101	TBB5_HUMAN
	Putative tropomyosin alpha-3 chain-like protein	101	TPM3L_HUMAN
	Actin-related protein 2/3 complex subunit 3	99	ARPC3_HUMAN
	Tubulin alpha-1A chain	91	TBA1A_HUMAN
	Macrophage-capping protein	87	CAPG_HUMAN
	40S ribosomal protein SA	87	RSSA_HUMAN
	Phosphatidylinositol-5-phosphate 4-kinase type-2 =gamma=20	85	PI42C_HUMAN
	Procollagen galactosyltransferase 1	81	GT251_HUMAN
	ADP-ribosylation factor 1	80	ARF1_HUMAN
	40S ribosomal protein S3a	78	RS3A_HUMAN
	S-phase kinase-associated protein 1	77	SKP1_HUMAN
	Leucine-rich repeat flightless-interacting protein 2=20	76	LRRF2_HUMAN
	Leucine-rich repeat flightless-interacting protein 1=20	76	LRRF1_HUMAN
	Heat shock 70 kDa protein 1-like	75	HS71L_HUMAN
	Heat shock 70 kDa protein 1A/1B	75	HSP71_HUMAN
	Heat shock 70 kDa protein 6	75	HSP76_HUMAN
	78 kDa glucose-regulated protein	75	GRP78_HUMAN
	Protein S100-A8	75	S10A8_HUMAN
	Myosin-Id	74	MYO1D_HUMAN
	Neurofilament heavy polypeptide	73	NFH_HUMAN
	4-trimethylaminobutyraldehyde dehydrogenase	72	AL9A1_HUMAN
	Fibronectin	72	FINC_HUMAN
	Vimentin	72	VIME_HUMAN
	Peroxiredoxin-4	71	PRDX4_HUMAN
Sample-7	Myosin light polypeptide 6	4436	MYL6_HUMAN
	Myosin regulatory light chain 12A	4253	ML12A_HUMAN
	Myosin-9	3672	MYH9_HUMAN
	Actin, cytoplasmic 2	2816	ACTG_HUMAN
	Myosin regulatory light polypeptide 9	1943	MYL9_HUMAN
	POTE ankyrin domain family member E	1938	POTEE_HUMAN

	Myosin-10	990	MYH10_HUMAN
	Calmodulin	858	CALM_HUMAN
	Actin, aortic smooth muscle	801	ACTA_HUMAN
	Myosin light chain 6B	459	MYL6B_HUMAN
	Tropomyosin alpha-1 chain	411	TPM1_HUMAN
	Tropomyosin alpha-4 chain	396	TPM4_HUMAN
	Myosin light chain 3	379	MYL3_HUMAN
	Myosin-11	366	MYH11_HUMAN
	Beta-actin-like protein 2	353	ACTBL_HUMAN
	Alpha-actinin-4	312	ACTN4_HUMAN
	Myosin-14	293	MYH14_HUMAN
	Tropomyosin beta chain	272	TPM2_HUMAN
	40S ribosomal protein S3	248	RS3_HUMAN
	F-actin-capping protein subunit alpha-1	230	CAZA1_HUMAN
	F-actin-capping protein subunit beta	227	CAPZB_HUMAN
	Annexin A2	226	ANXA2_HUMAN
	Peptidyl-prolyl cis-trans isomerase A	225	PPIA_HUMAN
	Tubulin beta-2A chain	215	TBB2A_HUMAN
	Tropomyosin alpha-3 chain	202	TPM3_HUMAN
	Histone H2B type 1-B	191	H2B1B_HUMAN
	Histone H2A type 1-B/E	191	H2A1B_HUMAN
	Histone H2B type 1-N	171	H2B1N_HUMAN
	Serpin H1	169	SERPH_HUMAN
	Histone H2A type 2-C	169	H2A2C_HUMAN
	Alpha-actinin-1	161	ACTN1_HUMAN
	Peroxiredoxin-1	155	PRDX1_HUMAN
	Histone H4	154	H4_HUMAN
	Desmoplakin	143	DESP_HUMAN
	40S ribosomal protein S13	143	RS13_HUMAN
	F-actin-capping protein subunit alpha-2	119	CAZA2_HUMAN
	60S ribosomal protein L22	112	RL22_HUMAN
	Elongation factor 1-alpha 1	99	EF1A1_HUMAN
	Alpha-actinin-3	99	ACTN3_HUMAN
	Alpha-actinin-2	99	ACTN2_HUMAN
	S-phase kinase-associated protein 1	97	SKP1_HUMAN
	Calmodulin-like protein 3	96	CALL3_HUMAN
	40S ribosomal protein S9	93	RS9_HUMAN
	Histone H2B type 1-A	93	H2B1A_HUMAN
	Filaggrin-2	90	FILA2_HUMAN
	Histone H2A.V	89	H2AV_HUMAN
	Actin-related protein 2/3 complex subunit 3	89	ARPC3_HUMAN
	40S ribosomal protein S4, X isoform	88	RS4X_HUMAN
	Hornerin	81	HORN_HUMAN
	Histone H3.1t	79	H31T_HUMAN
	Tropomodulin-3	78	TMOD3_HUMAN
	Vinculin	78	VINC_HUMAN

	Glyceraldehyde-3-phosphate dehydrogenase	77	G3P_HUMAN
	Coatomer subunit alpha	77	COPA_HUMAN
	40S ribosomal protein S3a	77	RS3A_HUMAN
	Ras-related protein Rab-5B	77	RAB5B_HUMAN
	Protein S100-A7	75	S10A7_HUMAN
	Serine/threonine-protein kinase DCLK1	73	DCLK1_HUMAN
	Macrophage-capping protein	70	CAPG_HUMAN
	Elongation factor 2	70	EF2_HUMAN
Sample-8	Myosin-9	2172	MYH9_HUMAN
	Actin, cytoplasmic 1	2075	ACTB_HUMAN
	Myosin light polypeptide 6	2072	MYL6_HUMAN
	Myosin regulatory light chain 12A	1544	ML12A_HUMAN
	POTE ankyrin domain family member E	1445	POTEE_HUMAN
	Myosin-10	750	MYH10_HUMAN
	Myosin regulatory light polypeptide 9	699	MYL9_HUMAN
	Alpha-actinin-4	661	ACTN4_HUMAN
	Calmodulin	584	CALM_HUMAN
	POTE ankyrin domain family member F	506	POTEF_HUMAN
	Histone H4	431	H4_HUMAN
	Alpha-actinin-1	385	ACTN1_HUMAN
	Myosin-11	381	MYH11_HUMAN
	POTE ankyrin domain family member I	356	POTEI_HUMAN
	Myosin light chain 6B	325	MYL6B_HUMAN
	Myosin light chain 3	318	MYL3_HUMAN
	40S ribosomal protein S3	283	RS3_HUMAN
	Beta-actin-like protein 2	242	ACTBL_HUMAN
	Myosin-14	225	MYH14_HUMAN
	Histone H2B type 1-B	222	H2B1B_HUMAN
	Tubulin beta chain	206	TBB5_HUMAN
	Tubulin beta-2C chain	201	TBB2C_HUMAN
	Histone H2B type 1-C/E/F/G/I	193	H2B1C_HUMAN
	Serpin H1	176	SERPH_HUMAN
	Heat shock protein HSP 90-beta	149	HS90B_HUMAN
	Peroxiredoxin-1	146	PRDX1_HUMAN
	60S ribosomal protein L18	143	RL18_HUMAN
	Elongation factor 1-alpha 1	143	EF1A1_HUMAN
	SH3 domain-binding glutamic acid-rich-like protein 3=20	142	SH3L3_HUMAN
	60S ribosomal protein L22	140	RL22_HUMAN
	F-actin-capping protein subunit beta	139	CAPZB_HUMAN
	F-actin-capping protein subunit alpha-1	139	CAZA1_HUMAN
	Heat shock protein HSP 90-alpha	133	HS90A_HUMAN
	Tropomyosin alpha-1 chain	132	TPM1_HUMAN
	Histone H2A.V	129	H2AV_HUMAN
	Histone H2A type 1-B/E	128	H2A1B_HUMAN

	40S ribosomal protein SA	126	RSSA_HUMAN
	Histone H2B type 1-A	116	H2B1A_HUMAN
	40S ribosomal protein S4, X isoform	111	RS4X_HUMAN
	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	111	PLOD3_HUMAN
	Histone H2A type 1-D	111	H2A1D_HUMAN
	40S ribosomal protein S17	107	RS17_HUMAN
	Protein S100-A8	104	S10A8_HUMAN
	Tricarboxylate transport protein, mitochondrial	104	TXTP_HUMAN
	60S ribosomal protein L30	100	RL30_HUMAN
	40S ribosomal protein S7	99	RS7_HUMAN
	60S ribosomal protein L14	98	RL14_HUMAN
	Protein S100-A4	97	S10A4_HUMAN
	Spatacsin	95	SPTCS_HUMAN
	Myosin-Ic	94	MYO1C_HUMAN
	40S ribosomal protein S18	90	RS18_HUMAN
	Putative heat shock protein HSP 90-beta 2	90	H90B2_HUMAN
	Ras-related protein Rab-5B	89	RAB5B_HUMAN
	Tubulin alpha-1A chain	89	TBA1A_HUMAN
	Destrin	88	DEST_HUMAN
	40S ribosomal protein S3a	87	RS3A_HUMAN
	Alpha-enolase	87	ENOA_HUMAN
	Lysozyme C	86	LYSC_HUMAN
	EH domain-containing protein 2	82	EHD2_HUMAN
	Peptidyl-prolyl cis-trans isomerase B	81	PPIB_HUMAN
	40S ribosomal protein S24	81	RS24_HUMAN
	Protein S100-A7	80	S10A7_HUMAN
	Histone H3.1t	79	H31T_HUMAN
	60S ribosomal protein L13	79	RL13_HUMAN
	Tubulin beta-1 chain	78	TBB1_HUMAN
	Macrophage-capping protein	76	CAPG_HUMAN
	Cysteine and glycine-rich protein 1	75	CSRP1_HUMAN
	Nascent polypeptide-associated complex subunit alpha=20	75	NACA_HUMAN
	Histone H1.3	74	H13_HUMAN
	Histone H1.2	74	H12_HUMAN
	Inosine-5'-monophosphate dehydrogenase 2	73	IMDH2_HUMAN
	Cleavage and polyadenylation specificity factor =subunit 6=20	72	CPSF6_HUMAN
	60S ribosomal protein L4	72	RL4_HUMAN
	Peptidyl-prolyl cis-trans isomerase A	72	PPIA_HUMAN
	Pyruvate kinase isozymes M1/M2	70	KPYM_HUMAN

Appendix 2: Proteins identified using LC-MS/MS in gel lanes excised from a SDS-PAGE gel used to seperate proteins precipitated from exponentially growing CMT27A cells lysate using anti-human p16 Ab.

The entire gel lane was excised and sent for protein identification using the LS-MS/MS technique. Each gel lane was divided into 8 samples. In the following table, the first column contains the sample number, the second column contains protein identified from that sample, the third column contains the corresponding MOWSE score, and the fourth column contains the accession number.

Sample Number	Protein Identification	Mowse score	Accesion number
Sample 1	Myosin-9	26766	MYH9_HUMAN
	Myosin-10	5303	MYH10_HUMAN
	Myosin-14	3091	MYH14_HUMAN
	Myosin-11	1911	MYH11_HUMAN
	Actin, cytoplasmic 1	475	ACTB_HUMAN
	POTE ankyrin domain family member E	185	POTEE_HUMAN
	Myosin light polypeptide 6	160	MYL6_HUMAN
	POTE ankyrin domain family member F	141	POTEF_HUMAN
	Myosin-VI	140	MYO6_HUMAN
	Myosin-Ic	139	MYO1C_HUMAN
	Alpha-actinin-4	133	ACTN4_HUMAN
	Beta-actin-like protein 2	126	ACTBL_HUMAN
	Leucine zipper protein 1	80	LUZP1_HUMAN
	Plectin	73	PLEC_HUMAN
	Myosin-7B	72	MYH7B_HUMAN
	Myosin-7	72	MYH7_HUMAN
	Myosin-3	72	MYH3_HUMAN
	Myosin-4	72	MYH4_HUMAN
	Myosin-6	72	MYH6_HUMAN
	Myosin-13	72	MYH13_HUMAN
Sample 2	Myosin-9	17827	MYH9_HUMAN
	Myosin-10	7971	MYH10_HUMAN
	Alpha-actinin-4	2389	ACTN4_HUMAN
	Myosin-14	2216	MYH14_HUMAN

	Myosin-11	2008	MYH11_HUMAN
	Alpha-actinin-1	1886	ACTN1_HUMAN
	Myosin-Id	1327	MYO1D_HUMAN
	Myosin-VI	1294	MYO6_HUMAN
	Myosin-Ic	951	MYO1C_HUMAN
	Myosin-Ib	743	MYO1B_HUMAN
	Alpha-actinin-2	629	ACTN2_HUMAN
	Actin, cytoplasmic 1	588	ACTB_HUMAN
	Alpha-actinin-3	377	ACTN3_HUMAN
	POTE ankyrin domain family member E	296	POTEE_HUMAN
	Beta-actin-like protein 2	206	ACTBL_HUMAN
	Plectin	199	PLEC_HUMAN
	Myosin-Ia	198	MYO1A_HUMAN
	Myosin light polypeptide 6	161	MYL6_HUMAN
	Myosin-6	123	MYH6_HUMAN
	Myosin-XVIIIa	123	MY18A_HUMAN
	Ribosome-binding protein 1	118	RRBP1_HUMAN
	Myosin-3	114	MYH3_HUMAN
	Ras GTPase-activating-like protein IQGAP1	109	IQGA1_HUMAN
	Myosin-4	106	MYH4_HUMAN
	Myosin-7	106	MYH7_HUMAN
	Myosin-1	106	MYH1_HUMAN
	Myosin-7B	106	MYH7B_HUMAN
	Myosin-2	106	MYH2_HUMAN
	Myosin-13	106	MYH13_HUMAN
	Leucine zipper protein 1	105	LUZP1_HUMAN
	Myosin-Ig	102	MYO1G_HUMAN
	Filamin-A	94	FLNA_HUMAN
	Triple functional domain protein	93	TRIO_HUMAN
	Protein FAM184B	76	F184B_HUMAN
	Filamin-B	72	FLNB_HUMAN
	Clathrin heavy chain 1	72	CLH1_HUMAN
Sample 3	Myosin-9	10205	MYH9_HUMAN
	Myosin-10	5074	MYH10_HUMAN
	Myosin-14	2395	MYH14_HUMAN
	Myosin-11	1659	MYH11_HUMAN
	Actin, cytoplasmic 1	1564	ACTB_HUMAN
	Alpha-actinin-4	1442	ACTN4_HUMAN
	Myosin-VI	1163	MYO6_HUMAN
	POTE ankyrin domain family member E	662	POTEE_HUMAN
	POTE ankyrin domain family member F	559	POTEF_HUMAN
	Alpha-actinin-1	555	ACTN1_HUMAN
	Myosin-Ib	543	MYO1B_HUMAN
	Myosin-Ic	541	MYO1C_HUMAN
	Myosin-Id	528	MYO1D_HUMAN

	Pyruvate kinase isozymes M1/M2	425	KPYM_HUMAN
	Heat shock cognate 71 kDa protein	417	HSP7C_HUMAN
	Protein arginine N-methyltransferase 5	385	ANM5_HUMAN
	Beta-actin-like protein 2	382	ACTBL_HUMAN
	ATP synthase subunit beta, mitochondrial	319	ATPB_HUMAN
	78 kDa glucose-regulated protein	317	GRP78_HUMAN
	Heat shock-related 70 kDa protein 2	303	HSP72_HUMAN
	Coatomer subunit alpha	299	COPA_HUMAN
	Glutamate dehydrogenase 1, mitochondrial	280	DHE3_HUMAN
	POTE ankyrin domain family member J	270	POTEJ_HUMAN
	Succinate dehydrogenase [ubiquinone] flavoprotein =subunit,=20 mitochondrial	267	DHSA_HUMAN
	Tropomyosin alpha-1 chain	251	TPM1_HUMAN
	Myosin light polypeptide 6	250	MYL6_HUMAN
	Heat shock 70 kDa protein 1-like	237	HS71L_HUMAN
	Heat shock protein HSP 90-beta	236	HS90B_HUMAN
	Heat shock 70 kDa protein 1A/1B	235	HSP71_HUMAN
	Glutamate dehydrogenase 2, mitochondrial	228	DHE4_HUMAN
	Alpha-actinin-2	214	ACTN2_HUMAN
	Heat shock protein HSP 90-alpha	193	HS90A_HUMAN
	Myosin-Va	172	MYO5A_HUMAN
	ATP synthase subunit alpha, mitochondrial	171	ATPA_HUMAN
	Myosin-Ia	167	MYO1A_HUMAN
	Heat shock 70 kDa protein 6	159	HSP76_HUMAN
	Cytoplasmic dynein 1 intermediate chain 2	144	DC1I2_HUMAN
	Plectin	135	PLEC_HUMAN
	Tropomyosin alpha-4 chain	126	TPM4_HUMAN
	Tropomyosin beta chain	123	TPM2_HUMAN
	ATP-citrate synthase	116	ACLY_HUMAN
	Heat shock protein 75 kDa, mitochondrial	115	TRAP1_HUMAN
	Tropomyosin alpha-3 chain	102	TPM3_HUMAN
	T-complex protein 1 subunit gamma	98	TCPG_HUMAN
	60 kDa heat shock protein, mitochondrial	94	CH60_HUMAN
	Myosin-Vb	88	MYO5B_HUMAN
	Myosin regulatory light chain 12A	78	ML12A_HUMAN
	Cytoskeleton-associated protein 4	77	CKAP4_HUMAN
	Myosin light chain 3	76	MYL3_HUMAN
	Actin-related protein 3	72	ARP3_HUMAN
	Tubulin alpha-1A chain	71	TBA1A_HUMAN
Sample 4	Actin, cytoplasmic 2	13610	ACTG_HUMAN
	Actin, cytoplasmic 1	13602	ACTB_HUMAN
	POTE ankyrin domain family member E	7035	POTEE_HUMAN
	Actin, alpha cardiac muscle 1	5299	ACTC_HUMAN
	Actin, alpha skeletal muscle	5233	ACTS_HUMAN
	Myosin-9	4654	MYH9_HUMAN
	POTE ankyrin domain family member F	3912	POTEF_HUMAN

	Beta-actin-like protein 2	2675	ACTBL_HUMAN
	POTE ankyrin domain family member I	2307	POTEI_HUMAN
	POTE ankyrin domain family member J	1947	POTEJ_HUMAN
	Myosin-10	1860	MYH10_HUMAN
	Myosin-11	967	MYH11_HUMAN
	Tropomyosin alpha-1 chain	931	TPM1_HUMAN
	Alpha-actinin-4	810	ACTN4_HUMAN
	Annexin A2	696	ANXA2_HUMAN
	Myosin-VI	673	MYO6_HUMAN
	Myosin-14	608	MYH14_HUMAN
	Tropomyosin alpha-3 chain	557	TPM3_HUMAN
	Myosin-Ic	372	MYO1C_HUMAN
	Tropomyosin alpha-4 chain	356	TPM4_HUMAN
	Alpha-actinin-1	350	ACTN1_HUMAN
	Tropomyosin beta chain	343	TPM2_HUMAN
	Multifunctional protein ADE2	260	PUR6_HUMAN
	Myosin-Ib	255	MYO1B_HUMAN
	Myosin-Id	247	MYO1D_HUMAN
	F-actin-capping protein subunit beta	237	CAPZB_HUMAN
	F-actin-capping protein subunit alpha-1	220	CAZA1_HUMAN
	F-actin-capping protein subunit alpha-2	209	CAZA2_HUMAN
	Coatomer subunit alpha	191	COPA_HUMAN
	ATP synthase subunit beta, mitochondrial	191	ATPB_HUMAN
	Protein arginine N-methyltransferase 5	153	ANM5_HUMAN
	Alpha-actinin-2	131	ACTN2_HUMAN
	Gelsolin	131	GELS_HUMAN
	Tropomodulin-3	128	TMOD3_HUMAN
	Heat shock protein HSP 90-beta	111	HS90B_HUMAN
	Succinate dehydrogenase [ubiquinone] flavoprotein =subunit,=20 mitochondrial	91	DHSA_HUMAN
	Pyruvate kinase isozymes M1/M2	89	KPYM_HUMAN
	Myosin light polypeptide 6	83	MYL6_HUMAN
	Keratin, type I cytoskeletal 13	76	K1C13_HUMAN
	Fructose-bisphosphate aldolase A	73	ALDOA_HUMAN
	Tripeptidyl-peptidase 2	73	TPP2_HUMAN
	Heat shock protein HSP 90-alpha	71	HS90A_HUMAN
Sample 5	Myosin-9	3013	MYH9_HUMAN
	Actin, cytoplasmic 2	2398	ACTG_HUMAN
	Actin, alpha skeletal muscle	1327	ACTS_HUMAN
	Actin, aortic smooth muscle	1316	ACTA_HUMAN
	Myosin-10	1209	MYH10_HUMAN
	POTE ankyrin domain family member E	1186	POTEE_HUMAN
	Tropomyosin alpha-1 chain	813	TPM1_HUMAN
	POTE ankyrin domain family member I	668	POTEI_HUMAN
	Alpha-actinin-4	635	ACTN4_HUMAN
	Beta-actin-like protein 2	612	ACTBL_HUMAN

	Putative beta-actin-like protein 3	611	ACTBM_HUMAN
	Myosin-11	575	MYH11_HUMAN
	Tropomyosin beta chain	507	TPM2_HUMAN
	Tropomyosin alpha-4 chain	505	TPM4_HUMAN
	Tropomyosin alpha-3 chain	494	TPM3_HUMAN
	Myosin-VI O	436	MYO6_HUMAN
	Annexin A2	424	ANXA2_HUMAN
	Myosin-Ic	354	MYO1C_HUMAN
	Myosin-14	339	MYH14_HUMAN
	Myosin light polypeptide 6	336	MYL6_HUMAN
	Alpha-actinin-1	326	ACTN1_HUMAN
	T-complex protein 1 subunit theta	256	TCPQ_HUMAN
	F-actin-capping protein subunit beta	251	CAPZB_HUMAN
	Multifunctional protein ADE2	251	PUR6_HUMAN
	Peroxiredoxin-1	192	PRDX1_HUMAN
	Myosin regulatory light chain 12A	161	ML12A_HUMAN
	Myosin-Ib	152	MYO1B_HUMAN
	Putative tropomyosin alpha-3 chain-like protein	147	TPM3L_HUMAN
	Alpha-actinin-3	143	ACTN3_HUMAN
	F-actin-capping protein subunit alpha-1	140	CAZA1_HUMAN
	F-actin-capping protein subunit alpha-2	136	CAZA2_HUMAN
	Glutamate dehydrogenase 1, mitochondrial	132	DHE3_HUMAN
	Myosin-Id	132	MYO1D_HUMAN
	Plectin	130	PLEC_HUMAN
	Alpha-actinin-2	126	ACTN2_HUMAN
	Myosin light chain 3	112	MYL3_HUMAN
	Myosin light chain 1/3, skeletal muscle isoform	112	MYL1_HUMAN
	T-complex protein 1 subunit beta	102	TCPB_HUMAN
	Myosin regulatory light polypeptide 9	100	MYL9_HUMAN
	Coatomer subunit alpha	96	COPA_HUMAN
	Myosin-Ia	93	MYO1A_HUMAN
	ATP synthase subunit beta, mitochondrial	90	ATPB_HUMAN
	Macrophage-capping protein	89	CAPG_HUMAN
	Translin	88	TSN_HUMAN
	Heat shock protein HSP 90-beta	88	HS90B_HUMAN
	T-complex protein 1 subunit delta	87	TCPD_HUMAN
	Heat shock protein HSP 90-alpha	84	HS90A_HUMAN
	40S ribosomal protein S4, X isoform	82	RS4X_HUMAN
	Protein arginine N-methyltransferase 5	77	ANM5_HUMAN
	Transcription intermediary factor 1-beta	77	TIF1B_HUMAN
	Filamin-B	75	FLNB_HUMAN
	Filamin-A	75	FLNA_HUMAN
	Filamin-C	75	FLNC_HUMAN
	Tropomodulin-3	75	TMOD3_HUMAN

	Elongation factor 1-gamma	73	EF1G_HUMAN
	Coatomer subunit delta	71	COPD_HUMAN
	40S ribosomal protein S2	71	RS2_HUMAN
	T-complex protein 1 subunit epsilon	70	TCPE_HUMAN
Sample 6	Myosin-9	1865	MYH9_HUMAN
	Actin, cytoplasmic 1	1749	ACTB_HUMAN
	Myosin light polypeptide 6	1181	MYL6_HUMAN
	Actin, alpha cardiac muscle 1	1041	ACTC_HUMAN
	Myosin regulatory light chain 12A	1012	ML12A_HUMAN
	POTE ankyrin domain family member E	940	POTEE_HUMAN
	Myosin-10	783	MYH10_HUMAN
	Beta-actin-like protein 2	577	ACTBL_HUMAN
	Alpha-actinin-4	515	ACTN4_HUMAN
	Tropomyosin alpha-1 chain	498	TPM1_HUMAN
	Annexin A2	445	ANXA2_HUMAN
	Calmodulin	387	CALM_HUMAN
	Myosin-11	357	MYH11_HUMAN
	Tropomyosin beta chain	311	TPM2_HUMAN
	Myosin light chain 6B	289	MYL6B_HUMAN
	Myosin-14	285	MYH14_HUMAN
	Multifunctional protein ADE2	255	PUR6_HUMAN
	Alpha-actinin-1	231	ACTN1_HUMAN
	Myosin light chain 3	217	MYL3_HUMAN
	Tropomyosin alpha-3 chain	196	TPM3_HUMAN
	Tropomyosin alpha-4 chain	190	TPM4_HUMAN
	Myosin-VI	166	MYO6_HUMAN
	Alpha-actinin-3	156	ACTN3_HUMAN
	F-actin-capping protein subunit beta	148	CAPZB_HUMAN
	Alpha-actinin-2	140	ACTN2_HUMAN
	Vinculin	139	VINC_HUMAN
	Myosin-Ic	137	MYO1C_HUMAN
	Histone H2B type 1-B	119	H2B1B_HUMAN
	Elongation factor 1-alpha 1	114	EF1A1_HUMAN
	S-phase kinase-associated protein 1	108	SKP1_HUMAN
	Serine/threonine-protein phosphatase 2A 55 kDa =regulatory=20 subunit B alpha isoform	106	2ABA_HUMAN
	ADP-ribosylation factor 1	99	ARF1_HUMAN
	40S ribosomal protein S4, X isoform	98	RS4X_HUMAN
	Peroxiredoxin-1	97	PRDX1_HUMAN
	Actin-related protein 2/3 complex subunit 4	96	ARPC4_HUMAN
	40S ribosomal protein S2	95	RS2_HUMAN
	Myosin-Ib	93	MYO1B_HUMAN
	Myosin-Ia	93	MYO1A_HUMAN
	Coatomer subunit alpha	89	COPA_HUMAN

	ADP-ribosylation factor 4	86	ARF4_HUMAN
	Phosphatidylinositol-5-phosphate 4-kinase type-2 =gamma=20	82	PI42C_HUMAN
	Heat shock protein HSP 90-beta	80	HS90B_HUMAN
	Transcription intermediary factor 1-beta	77	TIF1B_HUMAN
	60S ribosomal protein L22	72	RL22_HUMAN
	Myosin-13	72	MYH13_HUMAN
	Myosin-6	72	MYH6_HUMAN
	Myosin-2	72	MYH2_HUMAN
	Myosin-7B	72	MYH7B_HUMAN
	Myosin-4	72	MYH4_HUMAN
	Myosin-7	72	MYH7_HUMAN
	Pyruvate kinase isozymes M1/M2	71	KPYM_HUMAN
	F-actin-capping protein subunit alpha-1	71	CAZA1_HUMAN
Sample 7	Myosin light polypeptide 6	990	MYL6_HUMAN
	Myosin-9	909	MYH9_HUMAN
	Actin, cytoplasmic 1	888	ACTB_HUMAN
	Myosin regulatory light chain 12A	711	ML12A_HUMAN
	POTE ankyrin domain family member E	452	POTEE_HUMAN
	Alpha-actinin-4	414	ACTN4_HUMAN
	Histone H4	345	H4_HUMAN
	Myosin-10	321	MYH10_HUMAN
	POTE ankyrin domain family member F	310	POTEF_HUMAN
	Calmodulin	306	CALM_HUMAN
	Myosin light chain 6B	255	MYL6B_HUMAN
	40S ribosomal protein S3	238	RS3_HUMAN
	POTE ankyrin domain family member I	236	POTEI_HUMAN
	Histone H2B type 1-C/E/F/G/I	235	H2B1C_HUMAN
	Ras-related protein Rab-5B	215	RAB5B_HUMAN
	Myosin-14	211	MYH14_HUMAN
	Beta-actin-like protein 2	200	ACTBL_HUMAN
	Histone H2B type 1-B	200	H2B1B_HUMAN
	Putative beta-actin-like protein 3	194	ACTBM_HUMAN
	Myosin light chain 3	187	MYL3_HUMAN
	Myosin-11	187	MYH11_HUMAN
	40S ribosomal protein SA	182	RSSA_HUMAN
	Alpha-actinin-1	180	ACTN1_HUMAN
	Annexin A2	176	ANXA2_HUMAN
	Myosin-Ic	144	MYO1C_HUMAN
	F-actin-capping protein subunit beta	143	CAPZB_HUMAN
	Ras-related protein Rab-5C	139	RAB5C_HUMAN
	Histone H2A type 1-B/E	136	H2A1B_HUMAN
	Histone H2A type 1-D	135	H2A1D_HUMAN
	Multifunctional protein ADE2	132	PUR6_HUMAN
	Peroxiredoxin-1	130	PRDX1_HUMAN
	Histone H2A.V	125	H2AV_HUMAN

	Heterogeneous nuclear ribonucleoprotein K	116	HNRPK_HUMAN
	Pyruvate kinase isozymes M1/M2	113	KPYM_HUMAN
	Serpin H1	113	SERPH_HUMAN
	40S ribosomal protein S9	109	RS9_HUMAN
	Small ubiquitin-related modifier 3	103	SUMO3_HUMAN
	Single-stranded DNA-binding protein 2	96	SSBP2_HUMAN
	Vinculin	96	VINC_HUMAN
	Alpha-enolase	95	ENOA_HUMAN
	Macrophage-capping protein	91	CAPG_HUMAN
	Heterogeneous nuclear ribonucleoprotein D0	89	HNRPD_HUMAN
	SH3 domain-binding glutamic acid-rich-like protein 3=20	89	SH3L3_HUMAN
	Keratin, type I cytoskeletal 28	88	K1C28_HUMAN
	60S ribosomal protein L30	88	RL30_HUMAN
	Heat shock protein HSP 90-beta	87	HS90B_HUMAN
	Alpha-actinin-3	85	ACTN3_HUMAN
	40S ribosomal protein S2	84	RS2_HUMAN
	Tropomyosin alpha-1 chain	83	TPM1_HUMAN
	60S ribosomal protein L14	80	RL14_HUMAN
	Spatacsin	80	SPTCS_HUMAN
	Heat shock protein HSP 90-alpha	77	HS90A_HUMAN
	Tubulin alpha-1A chain	76	TBA1A_HUMAN
	ADP-ribosylation factor 5	76	ARF5_HUMAN
	60S ribosomal protein L31	73	RL31_HUMAN
	Cleavage and polyadenylation specificity factor =subunit 6=20	72	CPSF6_HUMAN
	Beta-enolase	72	ENOB_HUMAN
	Ras-related protein Rab-34	71	RAB34_HUMAN
Sample 8	Myosin-9	721	MYH9_HUMAN
	Actin, cytoplasmic 1	606	ACTB_HUMAN
	Myosin light polypeptide 6	502	MYL6_HUMAN
	Myosin regulatory light chain 12A	380	ML12A_HUMAN
	POTE ankyrin domain family member E	366	POTEE_HUMAN
	Myosin-10	327	MYH10_HUMAN
	Alpha-actinin-4	326	ACTN4_HUMAN
	Actin, aortic smooth muscle	279	ACTA_HUMAN
	POTE ankyrin domain family member F	274	POTEF_HUMAN
	Histone H4	258	H4_HUMAN
	Myosin-11	224	MYH11_HUMAN
	Myosin-14	216	MYH14_HUMAN
	Heat shock protein HSP 90-alpha	201	HS90A_HUMAN
	Heat shock protein HSP 90-beta	180	HS90B_HUMAN
	60S ribosomal protein L14	155	RL14_HUMAN
	40S ribosomal protein S3	152	RS3_HUMAN

	Histone H2B type 1-C/E/F/G/I	139	H2B1C_HUMAN
	Myosin light chain 3	130	MYL3_HUMAN
	40S ribosomal protein SA	125	RSSA_HUMAN
	Elongation factor 2	119	EF2_HUMAN
	Multifunctional protein ADE2	116	PUR6_HUMAN
	Alpha-enolase	116	ENOA_HUMAN
	Beta-actin-like protein 2	116	ACTBL_HUMAN
	Inosine-5'-monophosphate dehydrogenase 2	104	IMDH2_HUMAN
	Peroxiredoxin-1	96	PRDX1_HUMAN
	Heat shock protein 75 kDa, mitochondrial	95	TRAP1_HUMAN
	Tubulin beta-2A chain	93	TBB2A_HUMAN
	ADP-ribosylation factor 1	92	ARF1_HUMAN
	Serpin H1	91	SERPH_HUMAN
	40S ribosomal protein S9	88	RS9_HUMAN
	Elongation factor 1-delta	88	EF1D_HUMAN
	Histone H2B type 1-J	87	H2B1J_HUMAN
	Tubulin alpha-1A chain	84	TBA1A_HUMAN
	F-actin-capping protein subunit beta	84	CAPZB_HUMAN
	Beta-enolase	83	ENOB_HUMAN
	SH3 domain-binding glutamic acid-rich-like protein 3=20	80	SH3L3_HUMAN
	F-actin-capping protein subunit alpha-1	80	CAZA1_HUMAN
	ADP/ATP translocase 3	79	ADT3_HUMAN
	Ras-related protein Rab-35	79	RAB35_HUMAN
	Ras-related protein Rab-15	79	RAB15_HUMAN
	Ras-related protein Rab-8A	79	RAB8A_HUMAN
	Pyruvate kinase isozymes M1/M2	77	KPYM_HUMAN
	Protein S100-A4	76	S10A4_HUMAN
	40S ribosomal protein S18	75	RS18_HUMAN
	Elongation factor 1-alpha 1	74	EF1A1_HUMAN
	40S ribosomal protein S4, X isoform	73	RS4X_HUMAN
	Dermcidin	70	DCD_HUMAN
	Chloride intracellular channel protein 1	70	CLIC1_HUMAN

Appendix 3: Proteins identified using LC-MS/MS in gel lanes excised from a SDS-PAGE gel used to seperate proteins precipitated from serum-starved CMT27A cells lysate with no anti-human p16 Ab.

The entire gel lane was excised and sent for protein identification using the LS-MS/MS technique. Each gel lane was divided into 8 samples. In the following table, the first column contains the sample number, the second column contains protein identified from that sample, the third column contains the corresponding MOWSE score, and the fourth column contains the accession number.

Sample Number	Protein Identification	Mowse score	Accesion number
Sample-1	Myosin-9	8734	MYH9_HUMAN
	Myosin-10	3509	MYH10_HUMAN
	Myosin-11	1286	MYH11_HUMAN
	Myosin-14	1277	MYH14_HUMAN
	Actin, cytoplasmic 1	716	ACTB_HUMAN
	POTE ankyrin domain family member E	549	POTEE_HUMAN
	Fibronectin	446	FINC_HUMAN
	Myosin light polypeptide 6	325	MYL6_HUMAN
	Myosin regulatory light chain 12A	272	ML12A_HUMAN
	Keratin, type II cytoskeletal 6A	220	K2C6A_HUMAN
	Beta-actin-like protein 2	140	ACTBL_HUMAN
	Leucine zipper protein 1	101	LUZP1_HUMAN
	POTE ankyrin domain family member I	99	POTEI_HUMAN
	Myosin-3	83	MYH3_HUMAN
	Myosin-7	83	MYH7_HUMAN
	Alpha-actinin-4	83	ACTN4_HUMAN
	Myosin-7B	79	MYH7B_HUMAN
	Myosin-4	79	MYH4_HUMAN
	Myosin-6	79	MYH6_HUMAN
	Protein S100-A8	78	S10A8_HUMAN
	Plectin	74	PLEC_HUMAN
	Collagen alpha-1(I) chain	71	CO1A1_HUMAN

Sample-2	Myosin-9	5465	MYH9_HUMAN
	Myosin-10	3003	MYH10_HUMAN
	Myosin-11	1166	MYH11_HUMAN
	Myosin-14	1070	MYH14_HUMAN
	Actin, cytoplasmic 1	656	ACTB_HUMAN
	POTE ankyrin domain family member E	464	POTEE_HUMAN
	Myosin-Id	373	MYO1D_HUMAN
	Fibronectin	332	FINC_HUMAN
	Myosin regulatory light chain 12A	292	ML12A_HUMAN
	Myosin-Ic	286	MYO1C_HUMAN
	Myosin-Ib	274	MYO1B_HUMAN
	Myosin light polypeptide 6	271	MYL6_HUMAN
	Myosin-VI	221	MYO6_HUMAN
	Filamin-A	149	FLNA_HUMAN
	Beta-actin-like protein 2	132	ACTBL_HUMAN
	Coatomer subunit alpha	109	COPA_HUMAN
	Heat shock protein HSP 90-beta	107	HS90B_HUMAN
	Heat shock protein HSP 90-alpha	96	HS90A_HUMAN
	Serpin H1	85	SERPH_HUMAN
	Alpha-actinin-4	79	ACTN4_HUMAN
	Myosin-3	78	MYH3_HUMAN
	Calmodulin	71	CALM_HUMAN
	Myosin-7B	70	MYH7B_HUMAN
	Myosin-7	70	MYH7_HUMAN
	Myosin-2	70	MYH2_HUMAN
	Myosin-6	70	MYH6_HUMAN
	Myosin-4	70	MYH4_HUMAN
	Myosin-13	70	MYH13_HUMAN
Sample-3	Myosin-9	3107	MYH9_HUMAN
	Myosin-10	1117	MYH10_HUMAN
	Myosin-14	584	MYH14_HUMAN
	Actin, cytoplasmic 1	571	ACTB_HUMAN
	Myosin-11	543	MYH11_HUMAN
	78 kDa glucose-regulated protein	390	GRP78_HUMAN
	POTE ankyrin domain family member E	368	POTEE_HUMAN
	Protein disulfide-isomerase	347	PDIA1_HUMAN
	Myosin regulatory light chain 12A	308	ML12A_HUMAN
	Heat shock 70 kDa protein 1A/1B	305	HSP71_HUMAN
	Heat shock cognate 71 kDa protein	289	HSP7C_HUMAN
	Myosin light polypeptide 6	245	MYL6_HUMAN
	Pyruvate kinase isozymes M1/M2	215	KPYM_HUMAN
	Serpin H1	209	SERPH_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	203	PLOD3_HUMAN

	Heat shock 70 kDa protein 6	202	HSP76_HUMAN
	Myosin-Ic	197	MYO1C_HUMAN
	Heat shock 70 kDa protein 1-like	189	HS71L_HUMAN
	Fibronectin	181	FINC_HUMAN
	Prolyl 4-hydroxylase subunit alpha-1	181	P4HA1_HUMAN
	Heat shock protein HSP 90-beta	164	HS90B_HUMAN
	ATP synthase subunit alpha, mitochondrial	139	ATPA_HUMAN
	Procollagen galactosyltransferase 1	131	GT251_HUMAN
	ATP synthase subunit beta, mitochondrial	130	ATPB_HUMAN
	Tropomyosin alpha-1 chain	129	TPM1_HUMAN
	POTE ankyrin domain family member F	117	POTEF_HUMAN
	Succinate dehydrogenase [ubiquinone] flavoprotein =subunit,=20 mitochondrial	116	DHSA_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	110	PLOD2_HUMAN
	Dermcidin	109	DCD_HUMAN
	Tropomyosin beta chain	108	TPM2_HUMAN
	Coatomer subunit alpha	91	COPA_HUMAN
	Endoplasmin	80	ENPL_HUMAN
	Prolyl 4-hydroxylase subunit alpha-2	79	P4HA2_HUMAN
	Alpha-actinin-4	76	ACTN4_HUMAN
	Stress-70 protein, mitochondrial	75	GRP75_HUMAN
	Beta-actin-like protein 2	71	ACTBL_HUMAN
	Myosin-Ib	71	MYO1B_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	71	PLOD1_HUMAN
	T-complex protein 1 subunit gamma	71	TCPG_HUMAN
Sample-4	Myosin-9	2557	MYH9_HUMAN
	Actin, cytoplasmic 2	1519	ACTG_HUMAN
	Serpin H1	1041	SERPH_HUMAN
	Tropomyosin alpha-1 chain	905	TPM1_HUMAN
	Actin, aortic smooth muscle	861	ACTA_HUMAN
	POTE ankyrin domain family member E	849	POTEE_HUMAN
	Keratin, type I cytoskeletal 9	828	K1C9_HUMAN
	Myosin-10	768	MYH10_HUMAN
	Tropomyosin alpha-3 chain	488	TPM3_HUMAN
	Beta-actin-like protein 2	455	ACTBL_HUMAN
	Myosin-11	453	MYH11_HUMAN
	Tropomyosin beta chain	422	TPM2_HUMAN
	Myosin-14	346	MYH14_HUMAN
	Annexin A2	333	ANXA2_HUMAN

	78 kDa glucose-regulated protein	311	GRP78_HUMAN
	Myosin light polypeptide 6	271	MYL6_HUMAN
	Tropomyosin alpha-4 chain	223	TPM4_HUMAN
	Myosin regulatory light chain 12A	219	ML12A_HUMAN
	Heat shock cognate 71 kDa protein	196	HSP7C_HUMAN
	40S ribosomal protein SA	194	RSSA_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	189	PLOD3_HUMAN
	Myosin-Id	187	MYO1D_HUMAN
	Myosin-Ic	179	MYO1C_HUMAN
	Prolyl 4-hydroxylase subunit alpha-1	163	P4HA1_HUMAN
	Fructose-bisphosphate aldolase A	160	ALDOA_HUMAN
	Tubulin beta chain	146	TBB5_HUMAN
	Protein disulfide-isomerase	139	PDIA1_HUMAN
	Tubulin beta-2A chain	137	TBB2A_HUMAN
	Fibronectin	133	FINC_HUMAN
	Peroxiredoxin-1	131	PRDX1_HUMAN
	Heat shock 70 kDa protein 1-like	127	HS71L_HUMAN
	Polyadenylate-binding protein 1	123	PABP1_HUMAN
	Tubulin beta-2C chain	121	TBB2C_HUMAN
	ATP synthase subunit beta, mitochondrial	113	ATPB_HUMAN
	Heat shock protein HSP 90-beta	109	HS90B_HUMAN
	Dermcidin	106	DCD_HUMAN
	Heat shock protein HSP 90-alpha	106	HS90A_HUMAN
	Tubulin beta-3 chain	102	TBB3_HUMAN
	Heat shock 70 kDa protein 6	101	HSP76_HUMAN
	Multifunctional protein ADE2	100	PUR6_HUMAN
	Zinc-binding alcohol dehydrogenase domain-containing=20 protein 2	99	ZADH2_HUMAN
	Heat shock 70 kDa protein 1A/1B	93	HSP71_HUMAN
	Protein S100-A7	92	S10A7_HUMAN
	Protein S100-A8	90	S10A8_HUMAN
	Tropomodulin-3	90	TMOD3_HUMAN
	Myosin light chain 6B	89	MYL6B_HUMAN
	Nascent polypeptide-associated complex subunit alpha=20	88	NACA_HUMAN
	Putative tubulin beta-4q chain	86	TBB4Q_HUMAN
	Pyruvate kinase isozymes M1/M2	86	KPYM_HUMAN
	Protein disulfide-isomerase A6	85	PDIA6_HUMAN
	Myosin-VI	84	MYO6_HUMAN
	Heat shock protein 75 kDa, mitochondrial	84	TRAP1_HUMAN
	Tubulin alpha-1A chain	82	TBA1A_HUMAN
	Neurofilament heavy polypeptide	77	NFH_HUMAN
	Coatomer subunit alpha	77	COPA_HUMAN

	Filaggrin-2	72	FILA2_HUMAN
	Nucleolin	72	NUCL_HUMAN
Sample-5	Myosin-9	1647	MYH9_HUMAN
	Actin, cytoplasmic 1	818	ACTB_HUMAN
	Tropomyosin alpha-1 chain	689	TPM1_HUMAN
	Serpin H1	674	SERPH_HUMAN
	Tropomyosin alpha-4 chain	620	TPM4_HUMAN
	Tropomyosin beta chain	544	TPM2_HUMAN
	Myosin-10	481	MYH10_HUMAN
	Tropomyosin alpha-3 chain	469	TPM3_HUMAN
	Actin, aortic smooth muscle	422	ACTA_HUMAN
	POTE ankyrin domain family member E	412	POTEE_HUMAN
	Prolyl 4-hydroxylase subunit alpha-1	403	P4HA1_HUMAN
	Myosin-11	290	MYH11_HUMAN
	Myosin light polypeptide 6	287	MYL6_HUMAN
	Myosin-14	248	MYH14_HUMAN
	40S ribosomal protein S3	222	RS3_HUMAN
	Protein disulfide-isomerase	217	PDIA1_HUMAN
	Myosin regulatory light chain 12A	216	ML12A_HUMAN
	Beta-actin-like protein 2	212	ACTBL_HUMAN
	POTE ankyrin domain family member I	205	POTEI_HUMAN
	Tubulin beta-2C chain	196	TBB2C_HUMAN
	Desmoplakin	193	DESP_HUMAN
	Junction plakoglobin	185	PLAK_HUMAN
	F-actin-capping protein subunit beta	177	CAPZB_HUMAN
	Tubulin beta chain	172	TBB5_HUMAN
	Annexin A2	160	ANXA2_HUMAN
	40S ribosomal protein S18	157	RS18_HUMAN
	Myosin light chain 6B	152	MYL6B_HUMAN
	Putative tropomyosin alpha-3 chain-like protein	149	TPM3L_HUMAN
	Elongation factor 1-alpha 1	135	EF1A1_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	135	PLOD3_HUMAN
	Prohibitin-2	133	PHB2_HUMAN
	Filaggrin-2	109	FILA2_HUMAN
	Elongation factor 1-alpha 2	109	EF1A2_HUMAN
	Tubulin alpha-1A chain	109	TBA1A_HUMAN
	Peroxiredoxin-1	108	PRDX1_HUMAN
	Alpha-enolase	105	ENOA_HUMAN
	78 kDa glucose-regulated protein	101	GRP78_HUMAN
	Dermcidin	101	DCD_HUMAN
	Protein S100-A7	100	S10A7_HUMAN
	40S ribosomal protein S3a	97	RS3A_HUMAN

	40S ribosomal protein S4, X isoform	96	RS4X_HUMAN
	Glyceraldehyde-3-phosphate dehydrogenase	96	G3P_HUMAN
	Heat shock cognate 71 kDa protein	95	HSP7C_HUMAN
	14-3-3 protein zeta/delta	90	1433Z_HUMAN
	Heat shock 70 kDa protein 1A/1B	85	HSP71_HUMAN
	T-complex protein 1 subunit theta	84	TCPQ_HUMAN
	Macrophage-capping protein	82	CAPG_HUMAN
	Nuclease-sensitive element-binding protein 1	81	YBOX1_HUMAN
	Lysozyme C	81	LYSC_HUMAN
	Plectin	81	PLEC_HUMAN
	Nascent polypeptide-associated complex subunit alpha=20	80	NACA_HUMAN
	F-actin-capping protein subunit alpha-1	79	CAZA1_HUMAN
	40S ribosomal protein S25	78	RS25_HUMAN
	Hornerin	75	HORN_HUMAN
	Heat shock protein HSP 90-beta	73	HS90B_HUMAN
	Protein S100-A9	73	S10A9_HUMAN
	Proteasome subunit alpha type-4	72	PSA4_HUMAN
	Eukaryotic initiation factor 4A-I	71	IF4A1_HUMAN
	Eukaryotic initiation factor 4A-II	71	IF4A2_HUMAN
	Keratinocyte proline-rich protein	70	KPRP_HUMAN
Sample-6	Myosin-9	1665	MYH9_HUMAN
	Myosin regulatory light chain 12A	1085	ML12A_HUMAN
	Actin, cytoplasmic 1	985	ACTB_HUMAN
	Serpin H1	612	SERPH_HUMAN
	Myosin-10	540	MYH10_HUMAN
	Actin, alpha cardiac muscle 1	505	ACTC_HUMAN
	Tropomyosin alpha-1 chain	466	TPM1_HUMAN
	POTE ankyrin domain family member E	465	POTEE_HUMAN
	POTE ankyrin domain family member F	370	POTEF_HUMAN
	Myosin light polypeptide 6	358	MYL6_HUMAN
	40S ribosomal protein S18	294	RS18_HUMAN
	Tubulin beta chain	290	TBB5_HUMAN
	Myosin-11	280	MYH11_HUMAN
	Tropomyosin beta chain	271	TPM2_HUMAN
	Tropomyosin alpha-4 chain	262	TPM4_HUMAN
	Tubulin beta-2C chain	260	TBB2C_HUMAN
	Tropomyosin alpha-3 chain	249	TPM3_HUMAN
	Beta-actin-like protein 2	229	ACTBL_HUMAN
	Myosin-14	213	MYH14_HUMAN
	Tubulin beta-2A chain	209	TBB2A_HUMAN

	Prolyl 4-hydroxylase subunit alpha-1	208	P4HA1_HUMAN
	Peroxiredoxin-1	191	PRDX1_HUMAN
	40S ribosomal protein S3	177	RS3_HUMAN
	Protein disulfide-isomerase	173	PDIA1_HUMAN
	Tubulin beta-3 chain	143	TBB3_HUMAN
	Protein-L-isoaspartate(D-aspartate) =O-methyltransferase=20	143	PIMT_HUMAN
	40S ribosomal protein SA	137	RSSA_HUMAN
	60S ribosomal protein L12	136	RL12_HUMAN
	Elongation factor 1-alpha 1	128	EF1A1_HUMAN
	60S ribosomal protein L11	128	RL11_HUMAN
	Calmodulin	126	CALM_HUMAN
	Annexin A2	123	ANXA2_HUMAN
	Peptidyl-prolyl cis-trans isomerase B	121	PPIB_HUMAN
	F-actin-capping protein subunit beta	121	CAPZB_HUMAN
	Histone H1.3	112	H13_HUMAN
	Multifunctional protein ADE2	109	PUR6_HUMAN
	Elongation factor 1-gamma	107	EF1G_HUMAN
	40S ribosomal protein S3a	103	RS3A_HUMAN
	S-phase kinase-associated protein 1	101	SKP1_HUMAN
	60S ribosomal protein L18	100	RL18_HUMAN
	60S ribosomal protein L28	99	RL28_HUMAN
	Glyceraldehyde-3-phosphate dehydrogenase	97	G3P_HUMAN
	ATP synthase subunit alpha, mitochondrial	97	ATPA_HUMAN
	40S ribosomal protein S17	95	RS17_HUMAN
	Myosin light chain 1/3, skeletal muscle isoform	93	MYL1_HUMAN
	Plectin	93	PLEC_HUMAN
	40S ribosomal protein S13	91	RS13_HUMAN
	Tubulin alpha-1A chain	87	TBA1A_HUMAN
	Glutathione S-transferase P	83	GSTP1_HUMAN
	Elongation factor 1-beta	82	EF1B_HUMAN
	Spatacsin	82	SPTCS_HUMAN
	Ras-related protein Ral-A	81	RALA_HUMAN
	60S ribosomal protein L19	80	RL19_HUMAN
	60S ribosomal protein L23a	80	RL23A_HUMAN
	60S ribosomal protein L7a	79	RL7A_HUMAN
	Macrophage-capping protein	79	CAPG_HUMAN
	Hornerin	79	HORN_HUMAN
	Putative tropomyosin alpha-3 chain-like protein	79	TPM3L_HUMAN
	Pyruvate kinase isozymes M1/M2	78	KPYM_HUMAN
	Nascent polypeptide-associated complex subunit alpha=20	78	NACA_HUMAN

	60S ribosomal protein L14	77	RL14_HUMAN
	Actin-related protein 2/3 complex subunit 4	77	ARPC4_HUMAN
	Prolyl 4-hydroxylase subunit alpha-2	77	P4HA2_HUMAN
	Myosin light chain 3	76	MYL3_HUMAN
	40S ribosomal protein S4, X isoform	75	RS4X_HUMAN
	Heat shock 70 kDa protein 1-like	75	HS71L_HUMAN
	Peptidyl-prolyl cis-trans isomerase A	74	PPIA_HUMAN
	ATP synthase subunit O, mitochondrial	72	ATPO_HUMAN
	Ras-related protein Rab-5B	72	RAB5B_HUMAN
	78 kDa glucose-regulated protein	72	GRP78_HUMAN
	Filaggrin-2	70	FILA2_HUMAN
Sample-7	Myosin light polypeptide 6	1168	MYL6_HUMAN
	Myosin-9	997	MYH9_HUMAN
	Myosin regulatory light chain 12A	975	ML12A_HUMAN
	Actin, cytoplasmic 1	585	ACTB_HUMAN
	40S ribosomal protein S18	414	RS18_HUMAN
	POTE ankyrin domain family member E	388	POTEE_HUMAN
	Calmodulin	357	CALM_HUMAN
	Serpin H1	346	SERPH_HUMAN
	Actin, aortic smooth muscle	288	ACTA_HUMAN
	Tubulin beta chain	231	TBB5_HUMAN
	Myosin-10	227	MYH10_HUMAN
	Peptidyl-prolyl cis-trans isomerase A	189	PPIA_HUMAN
	Putative beta-actin-like protein 3	183	ACTBM_HUMAN
	40S ribosomal protein S3	183	RS3_HUMAN
	40S ribosomal protein S25	177	RS25_HUMAN
	40S ribosomal protein S13	168	RS13_HUMAN
	Tropomyosin alpha-1 chain	147	TPM1_HUMAN
	Myosin light chain 3	139	MYL3_HUMAN
	60S ribosomal protein L22	139	RL22_HUMAN
	40S ribosomal protein S19	135	RS19_HUMAN
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	133	PLOD3_HUMAN
	Protein disulfide-isomerase	131	PDIA1_HUMAN
	Glyceraldehyde-3-phosphate dehydrogenase	130	G3P_HUMAN
	S-phase kinase-associated protein 1	124	SKP1_HUMAN
	Myosin-14	123	MYH14_HUMAN
	Beta-actin-like protein 2	122	ACTBL_HUMAN
	Elongation factor 1-alpha 1	122	EF1A1_HUMAN
	40S ribosomal protein S6	121	RS6_HUMAN
	Histone H4	116	H4_HUMAN
	40S ribosomal protein S17	111	RS17_HUMAN

	40S ribosomal protein S14	111	RS14_HUMAN
	60S ribosomal protein L30	109	RL30_HUMAN
	Prolyl 4-hydroxylase subunit alpha-1	107	P4HA1_HUMAN
	Peptidyl-prolyl cis-trans isomerase B	107	PPIB_HUMAN
	Myosin-11	105	MYH11_HUMAN
	40S ribosomal protein S24	102	RS24_HUMAN
	Tropomyosin alpha-3 chain	100	TPM3_HUMAN
	Thioredoxin	97	THIO_HUMAN
	40S ribosomal protein S23	96	RS23_HUMAN
	Spatacsin	93	SPTCS_HUMAN
	60S ribosomal protein L11	92	RL11_HUMAN
	Dermcidin	92	DCD_HUMAN
	78 kDa glucose-regulated protein	91	GRP78_HUMAN
	PDZ and LIM domain protein 1	90	PDLI1_HUMAN
	Serine/threonine-protein kinase OSR1	87	OXSR1_HUMAN
	60S ribosomal protein L13 x	86	RL13_HUMAN
	Neurofilament heavy polypeptide	86	NFH_HUMAN
	Protein S100-A8	85	S10A8_HUMAN
	40S ribosomal protein S16	84	RS16_HUMAN
	General transcription factor II-I	84	GTF2I_HUMAN
	Destrin	84	DEST_HUMAN
	40S ribosomal protein S4, X isoform	83	RS4X_HUMAN
	Filaggrin-2	82	FILA2_HUMAN
	Ras-related protein Ral-A	80	RALA_HUMAN
	60S ribosomal protein L24	80	RL24_HUMAN
	ADP/ATP translocase 2	80	ADT2_HUMAN
	Peptidylprolyl cis-trans isomerase A-like 4A/B/C	79	PAL4A_HUMAN
	60S ribosomal protein L12	78	RL12_HUMAN
	40S ribosomal protein S3a	77	RS3A_HUMAN
	60S ribosomal protein L13a	77	RL13A_HUMAN
	60S acidic ribosomal protein P2	76	RLA2_HUMAN
	Elongation factor 1-delta	76	EF1D_HUMAN
	Macrophage-capping protein	76	CAPG_HUMAN
	F-actin-capping protein subunit alpha-1	74	CAZA1_HUMAN
	Transcription elongation factor B polypeptide 2	74	ELOB_HUMAN
	60S ribosomal protein L27a	74	RL27A_HUMAN
	Tubulin alpha-1A chain	72	TBA1A_HUMAN
	Serine/threonine-protein kinase DCLK1	71	DCLK1_HUMAN
	Wolframin	71	WFS1_HUMAN
	Tropomyosin alpha-4 chain	71	TPM4_HUMAN
	60S ribosomal protein L23a	70	RL23A_HUMAN

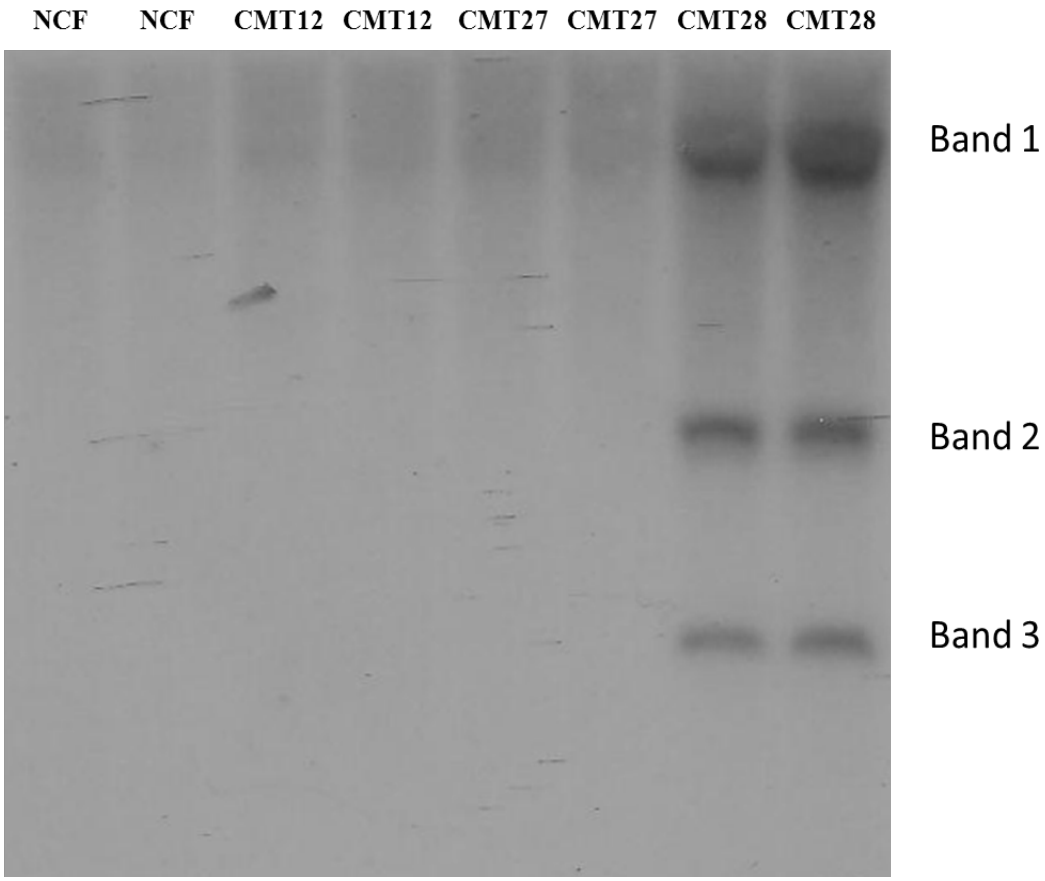
	Tubulin beta-1 chain	70	TBB1_HUMAN
Sample-8	Myosin light polypeptide 6	609	MYL6_HUMAN
	Myosin-9	526	MYH9_HUMAN
	Myosin regulatory light chain 12A	491	ML12A_HUMAN
	Actin, cytoplasmic 1	456	ACTB_HUMAN
	40S ribosomal protein S18	337	RS18_HUMAN
	POTE ankyrin domain family member E	324	POTEE_HUMAN
	Serpin H1	203	SERPH_HUMAN
	40S ribosomal protein S25	198	RS25_HUMAN
	Peroxiredoxin-1	139	PRDX1_HUMAN
	Tubulin beta chain	128	TBB5_HUMAN
	Desmoplakin	123	DESP_HUMAN
	Alpha-enolase	121	ENOA_HUMAN
	40S ribosomal protein S17	118	RS17_HUMAN
	60S ribosomal protein L10	114	RL10_HUMAN
	Peptidyl-prolyl cis-trans isomerase A	112	PPIA_HUMAN
	60S ribosomal protein L13	111	RL13_HUMAN
	60S ribosomal protein L22	109	RL22_HUMAN
	40S ribosomal protein S24	104	RS24_HUMAN
	Tubulin beta-2C chain	103	TBB2C_HUMAN
	Calmodulin	103	CALM_HUMAN
	Myosin light chain 3	100	MYL3_HUMAN
	60S ribosomal protein L35	99	RL35_HUMAN
	40S ribosomal protein S6	93	RS6_HUMAN
	Filaggrin-2	93	FILA2_HUMAN
	Neurofilament heavy polypeptide	92	NFH_HUMAN
	40S ribosomal protein S29	91	RS29_HUMAN
	Beta-enolase	90	ENOB_HUMAN
	Cysteine and glycine-rich protein 1	89	CSRP1_HUMAN
	Dermcidin	86	DCD_HUMAN
	78 kDa glucose-regulated protein	86	GRP78_HUMAN
	Heat shock protein HSP 90-beta	84	HS90B_HUMAN
	Histone H4	82	H4_HUMAN
	40S ribosomal protein S3	82	RS3_HUMAN
	Protein S100-A7	82	S10A7_HUMAN
	Elongation factor 1-delta	80	EF1D_HUMAN
	Protein S100-A4	80	S10A4_HUMAN
	60S ribosomal protein L13a	79	RL13A_HUMAN
	40S ribosomal protein S28	79	RS28_HUMAN
	Myosin-10	78	MYH10_HUMAN
	60S ribosomal protein L26-like 1	78	RL26L_HUMAN
	40S ribosomal protein S8	78	RS8_HUMAN
	Glyceraldehyde-3-phosphate dehydrogenase	78	G3P_HUMAN
	Peptidyl-prolyl cis-trans isomerase B	77	PPIB_HUMAN

	Dynein light chain 1, cytoplasmic	76	DYL1_HUMAN
	Myosin-14	75	MYH14_HUMAN
	Heat shock protein HSP 90-alpha	73	HS90A_HUMAN
	Desmoglein-1	73	DSG1_HUMAN
	F-actin-capping protein subunit alpha-1	73	CAZA1_HUMAN
	Serine/threonine-protein kinase DCLK1	72	DCLK1_HUMAN
	60S ribosomal protein L27a	70	RL27A_HUMAN

Appendix 4: Southern Blot Analysis for Assessment of p16 Gene Deletion in Canine Mammary Tumor Cell Lines

We have previously shown that p16 mRNA is expressed in NCF and CMT28 cells but p16 mRNA expression was defective in CMT12 and CMT27 cells. In order to find out if the absence of p16 and p14ARF mRNA is the result of gene deletion in these two cell lines (CMT12 and CMT27), we used southern blotting. NCF, CMT12, CMT27 and CMT28 cells were cultured in L-15 medium (Gibco) with antibiotics (ampicillin) (Sigma), and 10% FBS (Hyclone), in tissue culture flasks (Corning) at 37^{o}C (air, 95%; CO_2, 5%) (DeInnocentes *et al.*, 2006). Genomic DNA was isolated from 4 X 10^7 cells of each investigated cell line using QIAamp DNA Blood Maxi Kit (Qiagen). Isolated genomic DNA of each cell line was digested using EcoR1 restriction enzyme (Promega) and separated by agarose gel electrophoresis (0.8%). DNA fragments were then transferred from agarose gel to nitrocellulose membrane as described previously (Maniatis *et al.*, 1982). Transferred DNA fragments were permanently cross linked to the membrane using a UV stratalinker (Stratagene). The membrane was pre-hybridized in pre-hybridization solution for 60 min at 65^{o}C (3 X standard saline citrate, 0.1% SDS, 10 X Denhardt's medium, and 0.02M phosphate buffer pH 6.8). Human p16 gene (pBK-RSV/P16 expression plasmid) (Modiano *et al.*, 2000) was used to prepare the probe, which was to detect the canine p16 specific DNA fragments. Probes were labeled with ^{32}P by random priming to a DNA having specific activity of > 10^9 cpm/µg, according to the manufacturer's instructions (Invitrogen) (Feinberg and Vogelstein, 1983). Membrane was then hybridized with ^{32}P labeled probe for 16 hrs at 65^{o}C. Hybridized membrane was

washed to a final stringency of 1 X SSC/ 1% SDS at 65^{o}C and exposed to X-ray film for one week.



Detection of p16 gene in NCF, CMT12, CMT27, and CMT28 using southern blotting

We have found one band (band 1) which is common in all cell lines NCF, CMT12, CMT27, and CMT28 cells. Additionally, in CMT28 cells we have found two more bands (band 2 and 3). CMT28 cells have sharper p16 bands in comparison to NCF, CMT12 and CMT27, which may suggest that there might be a gene duplication/amplification of the p16 gene present in CMT28 cells. Increased number of bands in CMT28 cells may also suggest the presence of additional EcoR1 restriction site in the p16 gene. We can conclude from this experiment that NCF and CMT28 cells have intact p16 gene locus (Appendix-4) and express p16 mRNA (fig.2). Further, although, p16 gene locus was found to be intact in CMT12 and CMT27 (Appendix-4) too, but the expression of 16 mRNA cannot be detected in these cells (fig.2).