The Role of mRNA Translation Inhibitor Programmed Cell Death 4 (PDCD4) during Differentiation in Skeletal Muscle Cells

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ABSTRACT

PDCD4 is a downstream substrate of mammalian/mechanistic target of rapamycin complex 1/ S6 ribosomal protein kinase 1 (mTORC1/ S6K1) pathway. It is known as an apoptotic protein, a tumour suppressor and an mRNA translation inhibitor.

PDCD4 expression at the onset of differentiation was significantly greater than on Day 0, the time of change from growth to differentiation medium ($p<0.05$). This observation is corresponding to the mRNA expression ($p<0.05$). Using the pulse-chase technique, PDCD4 degradation rate was significantly different between Day 0 and Day 1 ($p<0.05$). Taking together, these imply that PDCD4 abundance during the onset of differentiation is likely regulated at the level of both protein synthesis and degradation.

siRNA-mediated knockdown of PDCD4 led to a decrease in myogenic protein abundance during differentiation ($p<0.05$), and overexpression of PDCD4 stimulated differentiation ($p<0.05$).

My study showed that the regulation of PDCD4 level may help in the management of muscle atrophy.

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TABLE OF CONTENTS

LIST OF FIGURES

Supplementary Figure 2. EGFP image under florescent light.

LIST OF ABBREVIATIONS

4EBP1	eukaryotic initiation factor 4E binding protein 1
40S	40S eukaryotic small ribosomal subunit
AA	amino acid
AP-1	activated protein-1
ADP	adenosine diphosphate
AICAR	5-aminoimidazole-4-carbozamideribonucleoside
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
ATM	Ataxia telangiectasia mutated
BCAA	branched chain amino acid
bHLH	transcription factors are basic helix-loop-helix
βTrCP	beta transducing repeat containing protein
CaM	calmodulin
DEPTOR	DEP domain containing mTOR-interacting protein
EF3	elongation factor 3
eIF3	eukarytic translation initiation factor 3
eIF4A	eukarytic translation initiation factor 4A
eIF4B	eukarytic translation initiation factor 4B
eIF4E	eukarytic translation initiation factor 4E
eIF4F	eukarytic translation initiation factor 4F
eIF4G	eukarytic translation initiation factor 4G

FFM	fat-free mass
FKBP12	rapamycin binds 12 kDa FK506-binding protein
FRAP	FKBP12-rapamycin-binding domain of FKBP-rapamycin-associated protein
FRB	FKBP-rapamycin binding
GβL	G protein β-subunit-like protein
HEAT	20 Huntington, EF3, a subunit of protein phosphatase 2, TOR1
hVps34	human vacuolor protein sorting 34
IGF-1	insulin-like growth factor 1
JNK	Jun N-terminal Kinase
MAPK	mitogen-activated protein kinase
MAP4K1	MAPK kinase kinase kinase 1
miR-21	micro RNA21
mLST8	mammalian lethal with SEC13 protein 8
mSIN1	mammalian SAPK interacting protein 1
MP1	MAPK scaffold protein 1
mTORC1	mammalian/mechanistic target of rapamycin complex 1
MRFs	muscle regulatory factors
MRF4	muscle regulatory factor 4
MyoD	myogenic differentiation 1
Myf-5	myogenic factor 5
NF-kB	nuclear factor kappa-light chain-enhancer of activated B cell
NRD	a putative negative regulatory domain

PDCD4	programmed cell death 4
PDK1	phosphoinositide dependent kinase 1
PIP3	phosphoinositide 3-kinase
PI(4,5)P2	phosphatidylinositol 4,5-bisphosphate
PI(3,4,5)P3	phosphatidylinositol (3,4,5)-triphosphate
PP2A	a subunit of protein phosphatase 2
PRAS40	proline-rich AKT substrate 40 kDa
Raptor	regulatory-associated protein of mTOR
Rheb	Ras homolog enriched in brain
Rictor	rapamycin insensitive companion of mTOR
PRR5/protor	protor proline rich protein 5/protein observed with rictor
RTK	receptor tyrosine kinase
RMR	resting metabolic rate
S6K1	S6 ribosomal protein kinase 1
SAPK	mammalian stress-activated protein kinase
TOS	TOR signalling
TPA	12-O-tetradecanoylphorbol-13-acetate
TRRAP	Transformation/transcription domain-associated protein
TSC1	tuberous sclerosis protein 1
TSC2	tuberous sclerosis protein 2

INTRODUCTION

Maintaining skeletal muscle mass is a crucial factor in human health for people of all ages. The maintenance of muscle mass is essential for mobility, disease prevention and quality of life (1). By vaguely saying "making muscles", we can talk about two different processes. One is making existing muscles bigger, and the other is making new muscles. "Making existing muscles bigger" for adults is done through physical activity, particularly resistance training. "Making new muscles" occurs after injury for adults. The healing process in the elderly population is not optimal since satellite cells decrease with aging (2, 3).

In recent studies, apoptotic cells have provided new insight into the formation of new muscle. Apoptotic cells can induce myotube formation (4). PDCD4, a more recently discovered downstream substrate of mammalian/mechanistic target of rapamycin complex 1/ S6 ribosomal protein kinase 1 (mTORC1/ S6K1) pathway, is known as an apoptotic protein. It is also known as a tumour suppressor and an mRNA translation inhibitor (5). My thesis focuses on the role of PDCD4 in muscle cell (myotube) formation.

LITERATURE REVIEW

2.1 Factors affecting muscle mass

Muscle mass comprises approximately 40% of the average adult body weight (6, 7). The functions of skeletal muscle tissue include body movement and maintenance of posture (8). It is also a tissue that plays an important role in metabolizing glucose, fatty acids and amino acids. If we cannot maintain muscle mass, it is detrimental to our quality of life. In general, the mammalian body maintains a balance of protein synthesis and degradation. If there is a positive net anabolism, muscle hypertrophy (growth) can occur. On the other hand, if the net protein balance is negative, muscle wasting (atrophy) can occur (9). Two of the factors that influence net protein balance are physical exercise and nutritional conditions (10). Muscle mass and integrity are affected by age.

2.1.1 Exercise

Exercise/physical activities can maintain overall health and wellness. The reason for doing exercise is varied, and the type of exercise also varied. However the health benefits are the same: improve physical health such as a better immune system which leads to disease prevention, and mental health such as improving self-esteem and preventing depression. On the other hand, inactivity such as bed-rest and space flight-induced muscle atrophy is associated with decline in peak power and force (11-14). If humans experience 130 days of bed rest, we experience 90% of peak torque force loss (15).

The American College of Sports Medicine (ACSM) recommends that most adults carry out moderate-intensity cardiorespiratory exercise training for at least 30 minutes for

5 days a week, or vigorous intensity cardiorespiratory exercise training for at least 20 minutes 3 days a week. They also mention the importance of maintaining joint range of movement, flexibility, balance, coordination and agility as well (16).

Exercise is a stimulus that promotes protein synthesis and insulin sensitivity. Resistance training is beneficial for muscle mass and strength. Endurance training improves numerous metabolic functions, such as insulin-induced glucose metabolism and mitochondrial function (17). Compared to resistance training, aerobic exercise is less stressful on muscles. Therefore, endurance training leads to minor muscle adaptation compare to resistance training (7). However both low- and high-intensity exercise contribute to maintaining body weight and protein synthesis (18).

The resting metabolic rate (RMR) is the largest component and approximately 60-70% of daily energy expenditure (19). Fat-free mass (FFM) is an important component for RMR. FFM consists of high metabolic rate organs and tissues such as skeletal muscle, brain, heart, liver and kidney, and low metabolic rate tissues such as bone (20, 21). The lean tissue loss, which is usually associated with dieting, leads to lower RMR. Because of this, dieters may not achieve long-term weight maintenance. It is also widely accepted that RMR progressively decreases with age (22). Therefore, exercise, particularly strength training has the greatest effect for preventing aging and obesity. Macronutrient particularly protein has an important role to increase or maintain FFM and building muscle. However, all nutrient are likely important for optimal muscle mass.

2.1.2. Nutrition

Human beings, like all animals, are heterotrophs. Unlike autotrophs, such as plants, we cannot produce organic compounds from inorganic compounds. Therefore we have to rely on ingesting other organisms to obtain organic compounds to sustain our lives and maintain our health. Those organic compounds are mainly carbohydrate, fat and protein. The acceptable macronutrient distribution ranges (AMDR) are 45-65% for carbohydrate, 20-35% for fat and 10-35% for protein. However, people tend to ingest food more than they need, which accumulates in one's body and leads to obesity.

2.1.2.1 Nutritional effect on obesity

During evolution, our ancestors faced famine regularly. Therefore, individuals who could have an efficient fat deposition during food abundant time period had a higher chance to survive. This genetic evolution, thrifty genes, is not suitable in recent decades (23). Due to the technological improvement, we do not need to store food in our body (24, 25). Instead of having famine, we are facing to the prevalence of obesity. People move less and eat more, which is completely opposed to our ancestor's lifestyle.

Obesity is known as multi risk factor for many diseases including diabetes, cardiovascular disease and cancer. Obesity occurs through the imbalance between energy intake and energy expenditure. The excess energy accumulates as fat in our body. If the imbalance between energy intake and output causes problems, eat less and do exercise. In reality, it is not easy to achieve in a long-term maintenance of weight loss (25). Caloric restriction has many beneficial effects including insulin sensitivity and obesity in many species including human being (26, 27). Caloric restriction does not mean starvation. It is

the absence of malnutrition in nutrient intake reduction. However, the precise caloric intake is unknown for human.

2.1.2.2 Protein ingestion and protein synthesis

It is known that people tend to take in more protein than they need in a day (>0.8g/kg). On the other hand, older generations are not ingesting enough protein due to a reduced appetite (28). In addition to lower ingestion, studies show that elderly and obese populations have a lower protein synthesis rate even when ingesting the same amount of protein as others due to the insulin insensitivity (29-32). This leads to the idea that having excess amount of protein may help their condition. The problem is that excess amounts of protein do not build muscle. Research showed that protein supplementation solely does not help muscle wasting condition. Physical activity is the most important factor in improving muscle atrophy (33-35).

2.1.2.3 Carbohydrate/protein ingestion for protein synthesis

Low carbohydrate diet improves insulin resistance due to reduction of blood glucose (36-39). The criticism of this diet relates to the increase of saturated fat ingestion as a substitute for the carbohydrate (40). Nowadays, low fat diet is accepted as healthy diet, therefore low carbohydrate diet is more likely associated to high protein diet. The increase rate of some diseases such as kidney disease is related with a high protein diet (41, 42). This observation is controversial. For pre or existing kidney disease individuals, protein restriction may improve the condition. However if subjects are healthy people, studies did not observe the correlation between high protein diet and kidney disease (43,

44). Even so, obesity and aging are multi-risk factor, therefore being cautious is important. For example, elderly patients with type 2 diabetes who did physical activity with inadequate protein intake observed negative effects to their health (45). Since the debate is still inconclusive, following the RDA of 0.8g/kg/day and 10-25% of daily energy consumption is the safe choice (28, 45).

If people can keep the quantity of protein, the quality and timing of protein ingestion may change the absorbance.

2.1.2.4 Nutritional ingestion post exercise for protein synthesis

Protein ingestion, particularly the amino acid, leucine is known to induce protein synthesis (46, 47). People who want to build muscle tend to eat meat products in addition to taking protein supplements such as whey protein, branched chain amino acid (BCAA) powder and so on. However, ingestion of carbohydrate too after resistance training improves the net protein balance. It was primarily due to the decrease of protein degradation. Carbohydrate ingestion alone is not as effective as protein ingestion alone (48). However, not only amino acids, but also insulin plays a key role (46). Leucine stimulation of protein synthesis is most effective in the presence of insulin (49, 50).

During exercise, fuel selection is determined by exercise intensity and duration. High intensity exercise of short duration relies on muscle glycogen for adenosine triphosphate (ATP) provision, and carbohydrate is a major fuel (51, 52). In body builders, thirty minutes of resistance exercise caused muscle glycogen concentration to be reduced by 26% lower after the exercise (53). Ingestion of carbohydrate during resistance exercise also the increased the amount workload before exhaustion (54). Therefore, carbohydrate

ingestion during and post resistance training enhances muscle glycogen synthesis and a quick recovery (54, 55). Ultimately carbohydrate ingestion is important for building muscle.

Protein ingestion post exercise is important for athletes to maximize the muscle adaptive response, such as muscle hypertrophy (17). The resistance training effect on protein synthesis works for up to 24-48 hours after exercise (9, 56-58). However, not only protein synthesis, but also protein degradation too is increased by resistance training (9). Without nutrient intake, net protein balance is negative (59). Therefore the timing of nutrient ingestion is another important factors that can affect protein synthesis. The generally accepted idea is that amino acids/protein ingestion should be right after the exercise in order to activate mTORC1 and increase the rate of protein synthesis (56). Ingesting protein for elderly, the timing is crucial. The muscle mass gain is significantly lowered for people who ingested protein 2 hour post exercise compared to immediately after resistance exercise (60). The ingestion of whole milk post resistance training demonstrated more positive net protein balance compared to fat free milk (61). Even so, low fat or fat free milk ingestion after exercise is also beneficial for not only net protein balance, but also strength and muscle hypertrophy as well (62-64). Moreover, low fat/fat free milk ingestion gives higher net protein balance than ingesting soy milk (62, 64). This is due to the lower bioavailability of soy milk compared to animal milk (47). Instead of taking fancy protein supplementation, elderly can increase protein synthesis with one of their regular food items (i.e. milk). This should be consumed immediately after their exercise. It is important for elderly population to aware of the high probability of age-related muscle wasting condition, such as sarcopenia. The factors causing those

conditions are not only genetic, but also environmental, meaning they are preventable (65-68). Although aging cannot be prevented, delaying aging can be achieved through adequate intake of nutrition such as caloric restriction and protein intake and exercise (26, 27, 69).

2.1.3 Aging and sarcopenia

Eternal youth and immortality is a dream. Historical heroes and heroines tried many things for their longevity. In the beginning of the eighteen-century, half of the population died before 10 years of age. Improvements in hygiene and environment, reduction of the rate of infant mortality and improvements in medicine made the average life span longer. On the other hand, the worldwide prevalence of elderly population challenges health care system in this century. Aging is associated with changes in body composition, leading to a loss of lean tissue and a corresponding increase in body fat (18). Sarcopenia is characterized by a deterioration of muscle quality and quantity due to the age-related reduced amount of anabolic stimuli (7, 28, 70). Sarcopenia affects approximately 15% of those over 65 years old, and 50% of those over 85 years old (71). Among young adults, 40-50% of body weight is lean muscle mass. However in those 75-80 years old, only 25% of body mass is lean muscle mass. Sarcopenia is most notable in the lower limb muscle loss, and type II muscle atrophy is observed (72, 73). Because sarcopenia is a gradual muscle wasting condition, it is the least understood of muscle wasting conditions (71).

Aging is the process of degenerating or destroying of homeostatic functions of individuals. The DNA damage is cumulative, even though the DNA damage is supposed

to repair during meiosis. As a result, cell replication and transcription are interfered, it causes loss of cell function and apoptosis (74), malfunctioning can lead to cancer cell proliferation as well. The repairing function of DNA and longevity are correlated.

Both caloric restriction and nutrient sensing pathway are well known to decrease the incidence of diseases and age related lowering body function such as loss of insulin sensitivity, bone, immune and motor dysfunction (75).

2.1.3.1 Insulin/ IGF-1 and mTORC1 pathway and aging

Reduced insulin/ insulin-like growth factor I (IGF-I) signalling pathway increases lifespan and health span. Reduction of insulin/IGF-1 activities is through caloric restriction or growth hormone deficiency; those conditions showed lifespan increase in rodents (67, 75). Overexpression of IGF-I or its receptor has been found in malignancies in children and adults. On the other hand, congenital IGF-I deficient individuals did not have any cancers, even though family members experienced malignancy in human (76). Heterozygous IGF-I receptor (IGF-IR) knockout mice also showed longer life span compared to their littermates. Those mice did not have any difference in energy metabolism, nutrient uptake, fertility, reproduction or physical activity compared to their littermates. Moreover, IGF-IR mutant mice showed greater resistance to oxidative stress, which is aging determinant (77). Having IGF-I deficiency causes developmental defects. Their body size is smaller compared to their littermates. This is because insulin/IGF-I signaling is linked to the activities of mTORC1/S6K1signaling, a pathway important for regulate muscle mass (Fig 2).

mTORC1/S6K1 pathway is activated by amino acids independently. mTORC1/S6K1 pathway inhibition increases longevities of species. The difference between rodents and humans is that caloric restriction is effective on decrease of IGF-1 in rodents, however human IGF-1 reduction can be observed with reduced intake of protein. Even so, the change in study method was 1.67g/kg of body weight per day to 0.95 g/kg of body weight per day for 3 weeks (67). It shows that we should follow the RDA's recommendation (0.8g/kg) (28, 45).

2.2 mTORC1 and regulation of muscle mass

2.2.1 mTORC1

mTORC1 is known as a master regulator of cell growth, proliferation and metabolism. mTORC1 is regulated by growth factors, energy/oxygen status and nutrients. Therefore, having adequate amount of mTORC1 is important for cell growth and proliferation. This is because mTORC1 regulates the synthesis of nucleotides, lipids, proteins, mRNA and autophagy (78-80). On the other hand, improper regulation of the mTORC1 pathway leads to uncontrolled cell proliferation as can be seen in cancer (79, 81). There is a drug, rapamycin, which is an mTORC1 inhibitor that is also used for some cancer treatments. It was discovered in Easter Island (Rapa Nui) from soil samples. It is an antifungal metabolite produced by streptomyces hygroscopicus (mycin is a suffix for antibiotics produced by Streptomyces strains). It has been on the market in the U.S. since September 1999. It has immunosuppressant functions and is administered to prevent rejection in kidney transplants (82-86). It has also received attention in the public since

mice administered this drug showed prolonged life span (87). mTORC1 researchers often use rapamycin to inhibit the function of the complex (88). Rapamycin binds 12 kDa FK506-binding protein (FKBP12), and the rapamycin and FKBP12 complex directly binds to mTORC1 and inhibits mTORC1 pathway (79, 89).

2.2.2 The structure of mTORC1

As the name suggested, TOR was discovered as a target of rapamycin in yeast. mTOR (also known as FRAP, RAFT or RAPT) was identified as the ortholog of the yeast TOR1/2 in 1994 (84, 90). mTOR is identified as a member of two distinct complexes termed mTORC1 and mTORC2. mTORC1 consists of mTOR, proline-rich AKT substrate 40 kDa (PRAS40), DEP domain containing mTOR-interacting protein (DEPTOR), mammalian lethal with SEC13 protein 8 (mLST8) and regulatory-associated protein of mTOR (raptor). On the other hand, mTORC2 consists of mTOR, mLST8, DEPTOR, rapamycin insensitive companion of mTOR (rictor), mammalian stress-activated protein kinase (SAPK) interacting protein 1 (mSIN1) and proline rich protein 5/protein observed with rictor (PRR5/protor) (81, 91-94).

mTOR is a serine/threonine kinase with high molecular weight (289 kDa), and contains 2549 amino acids. From the N-terminal to C-terminal, mTOR contains *I*) 20 Huntington, elongation factor 3 (EF3), a subunit of protein phosphatase 2 (PP2A), TOR1 *(HEAT) repeats domain*, *II*) FKBP12-rapamycin-binding domain of FKBP-rapamycin-associated protein (FRAP), Ataxia telangiectasia mutated (ATM), Transformation/transcription domain-associated protein (TRRAP) *(FAT) domain*, *III*) FKBP-rapamycin binding *(FRB) domain*, *IV*) *the kinase domain, V)* FAT-C-terminal

(FATC) domain. FRB domain binds the FKBP12- rapamycin complex (79, 95, 96) (Fig 1).

Raptor is an mTOR binding protein with a mass of 150 kDa. It also binds p70 S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and act as an mTOR scaffold protein. The interaction between raptor and S6K1 or 4E-BP1 is through TOR signalling (TOS) motif, which is in the N terminus of S6K1 and is in the C terminus of 4E-BP1 (97-100). When raptor is knocked down, there is a decrease in S6K1 phosphorylation and cell size reduction (80). However overexpression of raptor increases the mTORC1 signalling (95). In nutrient abundant situation, raptor has a positive role particularly enhancing the mTORC1 activity toward S6K1 (80, 95). Under low energy state, the gamma subunit of 5' adenosine monophosphate-activated protein kinase (AMPK) phosphorylates raptor at Ser 722 and Ser 792 and represses mTORC1 activity (101-103).

mLST8 is a 36 kDa protein that binds to the kinase domain of mTOR. It is also identified as G protein β-subunit-like protein (GβL) (82). When mLST8 is knocked-down there is a decrease in mTORC1 signalling and cell size reduction, however overexpression of mLST8 increases mTORC1 signalling (81, 82). Both mTOR and raptor knockout mice die in early stage of embryo development, however mLST8 knockout live a little bit longer (104).

Opposing the effects of raptor and mLST8, DEPTOR and PRAS40 negatively regulate mTORC1. DEPTOR interacts with the FAT domain of mTOR via a postsynaptic density 95, discs large, zonula occludens-1 (PDZ) domain located at its C terminus (105). Upon serum stimulation, DEPTOR is degraded by proteasome through ubiquitylation by

E3 ligase β-transducin repeat-containing protein (β-TrCP). Disturbing β-TrCP impairs DEPTOR degradation and it also impairs S6K1 phosphorylation. It leads to activation of autophagy and cell growth reduction occurs (101, 106).

PRAS40 was first reported as a substrate of AKT. When insulin is depleted, PRAS40 disturbs mTORC1 activities. Therefore, PRA40 is regulated by insulin. PRAS40 is a raptor binding protein (93, 94, 107). For binding raptor, TOS motif in PRAS40 is required (108, 109). PRAS40 binds the mTOR kinase domain under the conditions such as serum or nutrient deprivation or mitochondrial metabolic inhibition (110). After AKT phosphorylates PRAS40 on Thr 246, mTORC1 phosphorylates PRAS40 on Ser 183 and 221, which then causes PRAS40 to dissociate from mTORC1 (111).

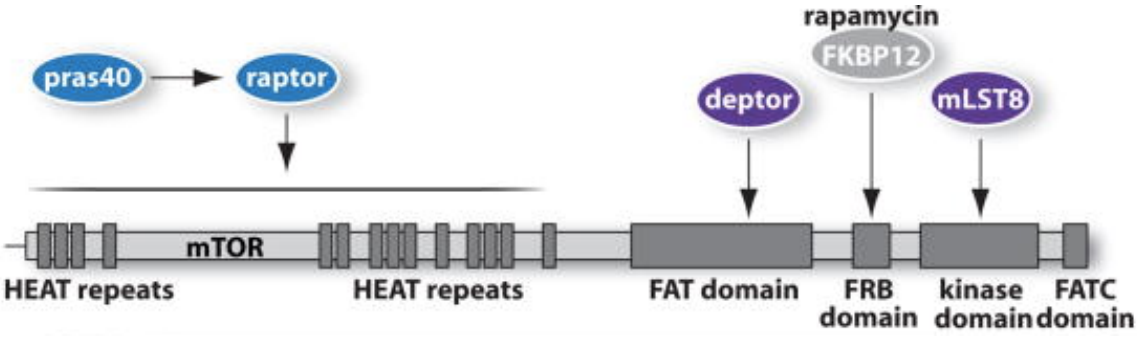

Fig 1. The domain structure of mTOR and mTORC1 components
mTORC1 components (mTOR, PRAS40, raptor, DEPTOR and mLST8) and mTORC1 inhibitor, the complex of rapamycin and FKBP12 and their interaction site on mTOR. Raptor and mLST8 are positive regulators of mTORC1, and DEPTOR, PRAS40 and the complex of rapamycin and FKBP12 negatively regulate mTORC1.
Retrieved from Laplante M and Sabatini DM. Cell. 2012;149(2):274-93.

2.2.3 Activation of mTORC1

mTORC1 activation is predominantly regulated by nutrient availability. In addition to it, mTORC1 is activated by growth factor and stress signaling pathways.

2.2.3.1 Activation of mTORC1 by growth factors

Growth factors have capabilities of stimulating mammalian cell growth and proliferation through regulating cellular signalling pathways such as mTORC1.

mTORC1 is activated by growth factors, such as insulin and insulin-like growth factor 1 (IGF-1), through the receptor tyrosine kinase (RKT)-AKT/PKB signalling pathway. Once the growth factor binds to receptor tyrosine kinases (RTK), the receptor is activated by autophosphorylation (112, 113). Activated RTK leads to recruitment and phosphorylation of insulin receptor substrate 1 (IRS-1), which also recruits phosphoinositide 3-kinase (PI3K). PI3K then converts phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) to phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P3). PIP3 then binds to the pleckstrin homology (PH) domain of AKT, which enables phosphoinositide dependent kinase 1 (PDK1) to phosphorylate and activate AKT (81, 91, 94, 114). Prior to AKT phosphorylation by PDK1, Akt needs to be phosphorylated at Ser 473 and Ser 450 by mTORC2. Following phosphorylation at the 2-serine residues, PDK phosphorylates Thr 308, which leads to Akt activation (115). Activated AKT indirectly stimulates mTORC1 by phosphorylating 2 proteins: PRAS40 and tuberous sclerosis protein 1 (TSC1)/ tuberous sclerosis protein 2 (TSC2) (10, 94, 116).

TSC1/TSC2 exist as a heterodimer, and mutations in either of two genes cause tuberous sclerosis, a genetic disease in wide range of tissues, including brain. The tumor

growth in the brain causes serious medical complications: mental retardation, epilepsy and autism (117). TSC2 contains a guanosine triphosphatase (GTPase)-activator domain at its C-terminal end (91, 117). It promotes hydrolysis of GTP bound to a G protein, Ras homolog enriched in brain (Rheb). If TSC2 is inactivated/phosphorylated by AKT on multiple sites, Rheb is in a GTP bound state and directly binds to mTOCR1 (91, 118, 119). This activates mTORC1.

2.2.3.2 Activation of mTORC1 by amino acids

Amino acids are essential nutrients for all cells. Among amino acids, particularly leucine is known as one of the most important activator of mTORC1 (118, 120). Insulin stimulation without amino acids fails to activate 4E-BP1 and S6K1 (121). On the other hand, amino acid activation suffers when insulin is not present. Activation of mTORC1 through amino acids has been speculated to be via inhibition of TSC1-TSC2, leading to the activation of Rheb (81). However, a study showed TSC2 is not necessary for amino acid activation of mTORC1 (122). For its activation, mTORC1 has to translocate to the surface of lysosome. mTORC1 activation by amino acids can occur through Rag guanosine triphosphatases (GTPases) and the Ragulator. Ragulator is a trimeric protein complex encoded by MAPKSP1, ROBLD3 and c11orf59 genes. Those proteins have been called MAPK scaffold protein 1 (MP1), p14 and p18 respectively (123, 124). There are 4 Rag proteins in mammals, RagA, RagB, RagC and RagD. Those form Rag heterodimers in which RagA or RagB binds with RagC or RagD. Rag heterodimers containing GTP-bound RagB interacts with mTORC1 (124, 125). Rag GTPases bind to the Ragulator on the surface of lysosome. Subsequently, the rag complex binds raptors,

but not mTOR (125) and mTORC1 is translocated to the lysosome, where Rheb-GTP is located. It leads to the activation of mTORC1 by Rheb (124, 125).

In addition to the effects of the Rag proteins, amino acid stimulation of mTORC1 signalling pathway is also sensitive to wortmannin, a PI3K inhibitor. A well known wortmannin target is class I PI3K, however it does not have any effect on amino acid induced mTORC1 activation. Instead, a class III PI3K protein, human vacuolor protein sorting 34 (hVps34) can mediate the activation of mTORC1 by amino acid (50). The hVps34/ human vacuolor protein sorting 15 (hVps15) heterodimer regulates endosomal sorting and trafficking and initiation of autophagy. Amino acids trigger elevation in intracellular Ca^{2+} concentration. This leads to elevation of binding of Ca^{2+}/calmodulin (CaM) to hVps34 (126). This leads to increased mTORC1 activity. The depletion of hVps34 or hVps15 decreased S6K1 phosphorylation, and overexpression of hVps34 and hVps15 increased S6K1 phosphorylation.

A Sterile 20 family protein kinase, mitogen-activated protein kinase 4-kinase 3 (MAP4K3) was identified as another possible activator that induces mTORC1 pathway in response to amino acid. Depletion of MAP4K3 reduced S6K1 and 4E-BP1 phosphorylation, on the other hand overexpression of MAP4K3 increased S6K1 phosphorylation in a rapamycin sensitive manner. MAP4K3 also regulates cell growth (127, 128).

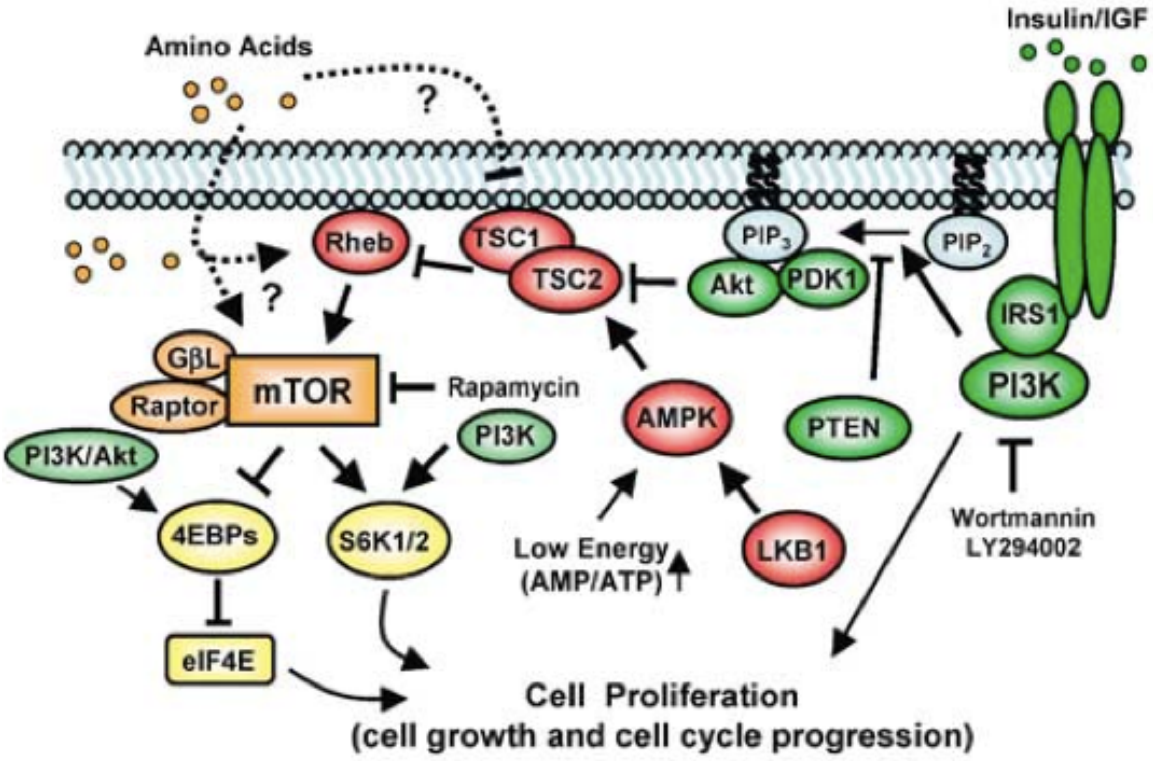

Fig 2. mTORC1 signalling pathway: mTORC1 signalling pathway is activated by insulin/IGF-1 or amino acids. Activated insulin receptor tyrosine kinase (RTK) by insulin/IGF-1 binding phosphorylates IRS1. Ph-IRS-1 recruits PI3K, which converts PIP_2 to PIP_3. Then, PIP_3 translocates AKT to the membrane. AKT is phosphorylated/activated by PDK1. AKT then phosphorylates TSC2. This phosphorylation of TSC2 inhibits the TSC1/2 complex function as an inhibitor of Rheb function towards activation of mTORC1. A GTP-bound state of Rheb activates mTORC1. mTORC1 phophorylates well known downstream substrates, S6K1/2 and 4E-BP1. Once 4E-BP1 is phosphorylated by mTORC1, it dissociates from eIF4E, which is a component of eIF4F complex. Amino acids activation is through the activation of Rheb.
Retrieved from Fingar DC, Blenis J. Oncogene. 2004;23(18):3151-71.

2.2.3.3 mTORC1 regulation by AMPK

Even if cells have an adequate amount of amino acid, protein synthesis, a high energy consuming process, cannot occur under cellular energy stress, such as hypoxia, low energy state and exercise (102, 129). AMPK detects and is activated by the increase in the AMP: ATP ratio. AMPK regulates fatty acid oxidation and glucose uptake in skeletal

muscle (129). To conserve existing ATP levels, AMPK phosphorylates many downstream substrates including raptor (131). An analog of AMP, 5-aminoimidazole-4-carbozamideribonucleoside (AICAR), is considered a useful tool to activate AMPK in research. Unlike other activators of AMPK, such as 2-deoxyglucose, or high fructose, or heat shock and inhibitors of oxidative phosphorylation, AICAR is able to activate AMPK without disturbing ATP, adenosine diphosphate (ADP) and AMP concentrations (130). AMPK directly phosphorylates TSC2 on Ser1345 and Thr1227, which increases TSC2 GAP activity toward Rheb. This suppresses Rheb/mTORC1 activation. AMPK also phosphorylates raptor and inhibits mTORC1 directly (131).

2.2.4 Downstream target of mTORC1 and mRNA translation

The substrates of mTORC1 activities are involved in mRNA translational control, ultimately protein synthesis. In this regard, the two well-studied downstream substrates of mTORC1 are S6K1 and 4E-BP1 (79, 81, 131). The phosphorylation of 4E-BP1 by mTORC1 frees eukarytic translation initiation factor 4E (eIF4E) which can then form the eukarytic translation initiation factor 4F (eIF4F) complex (the complex of eukarytic translation initiation factor 4A (eIF4A), eukarytic translation initiation factor 4G (eIF4G) and eIF4E) (131).

S6K1 and S6K2 are two S6K isoforms in mammalian cells and they are 80% identical (132). Both of them are direct targets of mTORC1 and are phosphorylated and activated by mTORC1. S6K1 is more studied, and characterized due to the fact that it was discovered earlier than S6K2. S6K1 is phosphorylated by mTORC1 on Thr 389. It is subsequently phosphorylated on Thr 229 in the T-loop region by PDK1 (133, 134).

Activated S6K1 phosphorylates numerous downstream targets, such as the ribosomal protein S6 (rpS6), eIF4B and PDCD4, all of which appear to play important roles in mRNA translation and ribosome biogenesis. rpS6 is the most characterized substrate of S6K1. This component of the 40S eukaryotic small ribosomal subunit protein (40S) has 5 phosphorylation sites: Ser 235, Ser 236, Ser 240, Ser 244 and Ser 247. Phosphorylation of S6 has a role in the translation of mRNA having a 5' terminal oligopyrimidine tract (5' TOP). However, the translational role of S6 is questioned (135, 136).

Phosphorylation of eIF4B on Ser 422 activates eIF3 directly. eIF3 binds to eIF4G which bridges the mRNA with 40S ribosome (137).

A more recently discovered substrate of S6K1 is PDCD4. It is phosphorylated on Ser 67 and then subsequently ubiquitylated by E3 ligase β-TrCP and degraded by the 26S proteasome. PDCD4 is also phosphorylated on Ser 71 and Ser 76 allowing for binding to β-TrCP. If PDCD4 is not phosphorylated, it binds to eIF4A which prevents formation of eIF4F (Fig3). To start translation, the 40S ribosome subunit and eIF2-GTP-Met-tRNA form 43S pre-initiation complex (PIC) (138). This complex binds to mRNA via the eIF4F complex (the complex of eIF4A, eIF4G and eIF4E). This complex is called the 48S PIC. Once 48S PIC finds a starting codon on mRNA, 60S ribosome bind to 40S ribosome and form 80S ribosome and leads to translation (79, 81, 139).

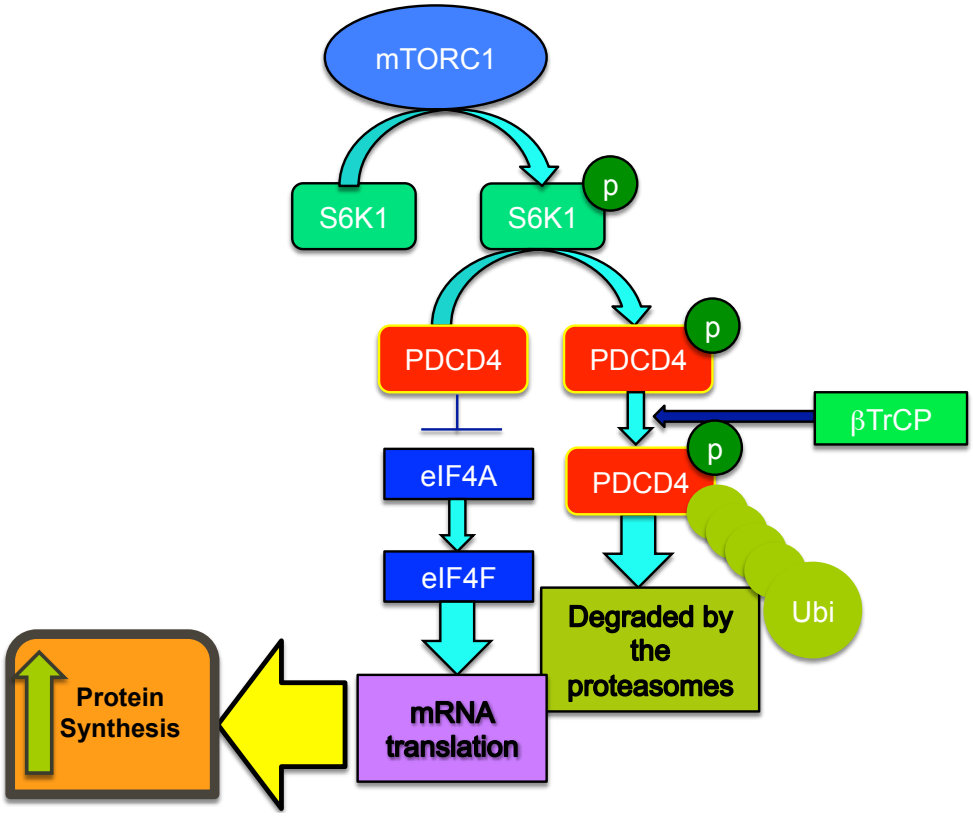

Fig 3. General overview of mTORC1 signaling and PDCD4 regulation
Once mTORC1 is activated, its downstream substrate S6K1 is phosphorylated. S6K1 can phosphorylate PDCD4. If PDCD4 is not phosphorylated, it attaches to eIF4A, a part of eIF4F complex. As a result, it inhibits mRNA translation. Phosphorylated PDCD4 (Ser67) is targeted for ubiquitin-mediated degradation by the ubiquitin protein ligase βTrCP (beta transducing repeat containing protein).

2.2.4.1 eIF4G

eIF4G is an adaptor protein and a mediator of mRNA binding to the 43S PIC. eIF4G coordinates to scaffold eIF4E, eIF4A, eIF3, 3'–end bound poly (A)-binding protein (PABP) and RNA. eIF4G contains 3 domains; N-terminal one-third of eIF4G (4G-N), middle one-third of eIF4G (4G-m) and C-terminal one third of eIF4G (4G-C). 4G-N is amino acids (AA) 1 to 634, 4G-m is AA 635 to 1039, and 4G-C is AA 1040 to

1560. PABP binds to the 4G-N AA 132 to 160, eIF4E binds to the 4G-N AA 570 to 582, eIF3 binds to the 4G-m AA 672 to 1065 and eIF4A has two binding sites within 4G-m AA 672 to 970 and 4G-C AA 1201 to 1411 (140-142) (Fig 4).

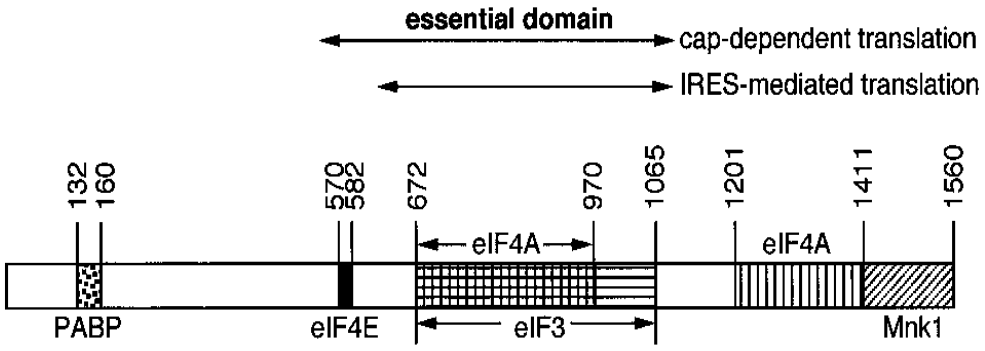

Fig 4. Structure of eIF4G domain
eIF4G contains 3 domains in N-terminal (AA 1 to 634), middle-terminal (AA 635 to 1039) and C-terminal (1040 to 1560). Binding sites of proteins (PABP, eIF4E, eIF3 and eIF4A) are depicted.
Retrieved from Morino S, Imataka H, Svitkin YV, Pestova TV, Sonenberg N. Molecular and cellular biology. 2000;20(2):468-77.

2.2.4.2 eIF4E

eIF4E is the 5'-methy-7-guanosine (m7GppN) cap-binding protein responsible for binding of the eIF4F complex to the mRNA cap structure (139, 143). The abundance of eIF4E is due to mTORC1 activation via the phosphorylation of a downstream target, 4E-BP1. 4E-BP1, which acts as small molecular mimics to eIF4G, binds to eIF4E (139). When phosphorylated by mTORC1, it dissociates from eIF4E. This allows eIF4E to bind

to eIF4G. However, if mTORC1 is reduced or inactivated through nutrient depletion or growth factor withdrawal, dephosphorylation of 4E-BP1 can occur. Unphosphorylated 4E-BP1 then binds to eIF4E, ultimately it inhibits protein synthesis (144, 145). eIF4E binds to the mRNA cap and it binds to eIF4G on 43S for the cap-dependent translation.

2.2.4.3 eIF4A

eIF4A is a DEAD-box containing protein. A DEAD-box contains nine motifs, and one of them, motif II has amino acid sequence of asp (D)-glu (E) –ala (A)-asp(D), from where the name "DEAD" arises (146). Proteins containing DEAD-box participate in RNA metabolism (147). eIF4A was the first DEAD box protein discovered. It has an RNA dependent ATPase activity and ATP dependent RNA helicase activity: unwinding RNA secondary structure in 5' untranslated region (UTR) and this facilitates ribosome access to the mRNA template (139, 148). If this helicase activity is inhibited, the small ribosome cannot scan on the mRNA. eIF4A has two binding sites in the middle one- third of eIF4G (4G-m, AA 672 to 970) and C-terminal one third of eIF4G (4G-C, AA 1201 to 1411) (Fig 4). A mutated eIF4G which possessed eIF4E, eIF3 and eIF4A in middle one-third of eIF4G binding site, but not the C-terminal had 70% of 40S ribosome binding compared to control. However, mRNA translation occurred only 20-30% without C-terminal compared to full length of eIF4G. This demonstrated that eIF4A binding site in the middle one- third of eIF4G is crucial whereas the site in the C-terminal is important to having a robust translation (140, 149).

2.2.4.4 PDCD4

Abundance of PDCD4 is controlled by S6K1 and β-TrCP. Phosphorylation of PDCD4 by S6K1 at Ser 67 and ubiquitylation by the ubiquitin ligase β-TrCP lead to degradation of PDCD4 by proteasome (150). If phosphorylation does not occur, PDCD4 binds to eIF4A and it prevents mRNA translation (5, 151, 152). As discussed earlier, eIF4A has 2 binding sites in eIF4G. PDCD4 binds eIF4G in the middle region independent of eIF4A. It was discovered by PDCD4 mutant inactivated for binding to eIF4A bound to the middle region of eIF4G. Therefore PDCD4 prevents eIF4A binding in C-terminal in eIF4G, which is not as crucial as the one in the middle, but important to have a robust translation (141). Not only eIF4G, but also PDCD4 contains alpha-helical MA-3 domains. eIF4G at C-terminal has MA-3 domain in the eIF4A binding domain, and PDCD4 contains two MA-3 domains at N and C terminals (149, 153, 154). The two MA-3 domains of PDCD4 are very similar in structure and function. They bind to eIF4A in N-terminal. If one of them is mutated, PDCD4 binding to eIF4A is decreased approximately 90%. The mechanism of inhibition of mRNA translation by PDCD4 is through both binding to eIF4A and binding to MA-3 domain in C-terminal in eIF4G. Therefore, the two domains working together make the binding to eIF4A to be tighter and stable, and PDCD4 also inhibits eIF4G binding to eIF4A as well (141, 149, 153, 155).

2.3 Muscle differentiation: the role of mTORC1

2.3.1 Muscle differentiation

Adult skeletal muscle is a mature and stable tissue. It has the capacity to regenerate after injury or physical activity, particularly resistance training. This relies on

satellite cell activation, which posses the potential to differentiate into new fibers. Myogenesis, the process of generating muscle, begins at an early stage of embryonic development (131, 156). After birth, satellite cells act as muscle stem cells (157, 158). Satellite cells are so termed due to their location; quiescent satellite cells reside between the sarcolemma and the basal lamina. Up until the mature state, myogenic progenitor cells proliferate greatly until they reach the number of myonuclei and myofibrillar protein synthesis peak. Adult skeletal muscle fibers are terminally differentiated; therefore muscle repair and hypertrophy are accomplished by satellite cells. Satellite cells are normally in a non proliferative quiescence state. Once activated by extrinsic signals such as myotrauma, they proliferate. After proliferation, they either differentiate or withdraw from the cell cycle and return to a state of quiescence. If they differentiate, they fuse together to form new myofibers or fuse into existing myofibers and provide additional myonuclei to muscle fiber (159, 160). Satellite cell capacities are strongly related to protein expression.

The molecular mechanisms regulating myogenesis are relatively well known. Myogenic differentiation 1 (MyoD), myogenic factor 5 (Myf-5), myogenin, and muscle regulatory factor 4 (MRF4) transcription factors are basic helix-loop-helix (bHLH) proteins. They are recognized as muscle regulatory factors (MRFs) and are also considered the driving force behind the specification and differentiation of all myogenic compartments (161). MyoD and Myf-5 are essential to establishing the myogenic cell lineage and producing committed undifferentiated myogenic stem cells. On the other hand, MRF4 and myogenin are critical to terminal differentiation events (162, 163).

2.3.2 mTORC1 and muscle differentiation

Cell growth and proliferation are important factors in maintaining the proportions of organs. Even though most mitogens promote myoblast proliferation and inhibit differentiation, IGF-I and IGF-II stimulate both proliferation and differentiation in muscle cell culture (159). An effector of IGF-I, mTORC1 activity level was 10 times more during the differentiation of C2C12 and L6 muscle cells compared to proliferating cells (164, 165). In particular, N terminus of mTOR (residues 11-91, part of the first HEAT domain) is required for differentiation (165). mTORC2 component rictor downregulation suppressed myoblast fusion through downregulation of AKT Ser 473 phosphorylation. As mentioned earlier, AKT Ser 473 phosphorylation is important for the activation of mTORC1 (166).

Earlier studies in Drosophila showed that inactivation of dTOR or its substrate dS6K1 cause a high rate of embryonic lethality, reduction of cell size and body size (85, 167). Later, studies in mammalian system showed that mTORC1-knockout embryonic mouse had a proliferation of cell lesions and the inability to have embryonic stem cell. In stem cells, an mTORC1 inhibitor slowed the rate of proliferation and showed undifferentiated condition, and rapamycin resistant mutant mTOR gene conserved the cell size (104, 167-169). Overexpression of either S6K1 or eIF4E increased cell size, and co-overexpression of those genes further increased cell size (169). On the other hand, mutations of phosphorylation site in 4E-BP1, which did not lose binding capacity to eIF4E, prevented mRNA translation initiation activities and reduced the cell size (169). Those studies showed that mTORC1 and its downstream targets regulate the cell size. In

vivo, skeletal muscle activation relies on satellite cell activation, proliferation and differentiation.

On the other hand, in cell culture, differentiation occurs after growth factors are removed. Not only C2C12, but also various myoblast cells treated with rapamycin had differentiation inhibited (164). C2C12 transfected with rapamycin-resistant mTOR retained the ability to differentiate even in the presence of rapamycin. Nutrient dependent IGF-II is an important factor in myogenesis at the onset of myoblast differentiation. However activities of mTORC1 or S6K1 activities are necessary at the myotube maturation stage (170).

2.3.3 The role of PDCD4 in differentiation and apoptosis

PDCD4 gene (also known as Dug, H731, TIS and MA-3) was initially found as an apoptotic gene. It was upregulated during apoptosis and identified in mice in 1995 (171-173). The gene expression of PDCD4 is found not only in vertebrates but also in Drosophila melanogaster (151, 152). It implies that PDCD4 has been highly conserved during evolution and it also implies an important function for this protein (151, 174). It is expressed in a wide variety of tissues and at the highest level in the liver and to a lesser extent in the heart and skeletal muscle in mouse (171, 172, 175).

PDCD4 expression is normally at low level, however apoptosis upregulates its expression (171, 175-177). PDCD4 has a nuclear localization signal (NLS) at its N and C termini. It shuttles between the nucleus and cytosol (5, 178-180). PDCD4 localizes primarily in the nucleus. It can be exported from the nucleus to the cytosol under certain condition such as serum withdrawal (151, 172, 180). However, this observation is

dependent on cell type and cell conditions. In general, PDCD4 localization is in the nucleus in normal tissue and shuttle to cytoplasm in carcinoma tissue (172, 181, 182). However in breast tissue, PDCD4 is predominantly expressed in the cytoplasm in normal condition, but in nucleus in breast cancer cell (183).

The roles of PDCD4 in the cytosol are well documented: contribution for transcription and translation (152, 172). PDCD4 may have a role in the nucleus, however this is not clear. It might be a contributor for nuclear RNA processing events such as splicing and nucleo-cytoplasmic transport (151, 172, 180, 183). A loss of PDCD4 expression or accumulation of PDCD4 in the nucleus may positively regulate cell proliferation (183)

Programmed cell death, referred to as apoptosis, is a cellular homeostatic response. Apoptosis serves to eliminate unwanted or damaged cell for normal development of multicellular organisms. Proliferating myoblasts in culture cells withdraw from the cell cycle once the medium is changed to one with low concentration of mitogens. Those cells undergo either differentiation or apoptosis.

There is some evidence that PDCD4 may regulate other cell cycle regulator (184). p21 inhibits cyclin-dependent kinase4/6 and the transcription of the mitosis promoting factor CDK1/cdc2 (184). The expression of p21 was correlated with apoptosis-resistance condition. Moreover, overexpression of p21 can protect from apoptosis during myocyte differentiation (185). PDCD4 is known as an upregulator of p21. p21 is an important factor for differentiation (186). Since PDCD4 upregulates it, this implies that PDCD4 may have a role during muscle differentiation. However, this observation is also cell specific and cannot be generalized (172).

The serine/threonine kinase AKT controls cellular processes such as cell proliferation, growth and survival through phosphorylation of numerous downstream substrates (180). AKT is also considered as an anti-apoptotic regulator, and PDCD4 phosphorylation by phosphorylated AKT may have a role in differentiation (182). Phosphorylation of PDCD4 at Ser 457 by activated AKT causes nuclear localization (180, 182). In colorectal cancer, phosphorylated AKT is correlated with the loss of total PDCD4 expression and with the less localization from nucleus to cytoplasm (182).

In caspase-dependent apoptotic pathway, caspase-3 is the executioner caspase. Activated caspase-3 translocates to nucleus and induces DNA fragmentation (187). Inhibition of caspase 3 activity reduces myoblast fusion and myotube formation (188). The PDCD4 accumulation in nucleus correlated to apoptosis in human hepatocellular carcinoma. This is because its nuclear accumulation was observed in nuclear fragmented cells (189). PDCD4 activates Bax and release cytochrome C from the mitochondria. This leads to caspase-dependent apoptosis (187). However the mechanism by which PDCD4 activates, Bax has not yet been elucidated (178, 189).

Recently, apoptotic cells have been shown to be promoters of myotube formation (4). As the name suggests, programmed cell death 4 (PDCD4) is pro-apoptotic and it may regulate myotube formation. Therefore it may have the potential for a therapeutic approach to muscle degenerating conditions.

2.4 PDCD4 research

2.4.1 PDCD4 in cancer research

Events leading to apoptosis are important to understand cancer development (174). As an apoptotic gene, PDCD4 is studied as a tumor suppressor in cancer research (5, 172, 190, 191).

PDCD4 inhibits tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced neoplastic transformation (189, 192). Reduced PDCD4 expression was observed in neoplastic transformation sensitive cells; on the other hand, its expression was high in neoplastic transformation resistant cells (193). PDCD4 expression is reduced in human tumors (178, 189, 194). PDCD4-deficient mice developed spontaneous lymphomas and had a significant shorter life span (195) and the overexpressing PDCD4 rescued cell transformation (196).

In cancer progression, upregulation of transcription factor is an important factor. Nuclear factor kappa-light chain-enhancer of activated B cell (NF-kB) and activated protein-1 (AP-1) are known as those transcription factors. PDCD4 inhibits the AP-1 dependent transcription, but not NF-kB (197, 198). AP-1 promotes cell proliferation (154, 178), and it is recognized as a target of mitogen activated protein kinases (MAPK) (199, 200). PDCD4 does not inhibit c-Jun, which is a component of AP-1 or c-Jun activator Jun N-terminal Kinase (JNK) directly (201, 202). Instead, PDCD4 inhibit JNK upstream kinase mitogen activated protein kinase kinase kinase kinase 1 (MAP4K1). This ultimately inhibits AP-1 dependent transcriptions (152, 155, 197, 202).

PDCD4 is a translation initiation inhibitor. It binds to eIF4A and inhibits the formation of eIF4F complex, a complex that is required for mRNA translation initiation. As explained earlier, phosphorylation (inactivation) of PDCD4 promotes cell growth. In cancer cells, inactivated PDCD4 promotes cancer development. Therefore, an

overexpression PDCD4 induced apoptosis of cancer cells. To induce apoptosis and arrest cell cycle in cancer cells may be the most efficient way to deal with tumor progression (203).

Also, related to cancer, PDCD4 is a target of micro RNA21 (miR-21) (152, 202). A microRNA is a short noncoding RNA, which regulates gene expression post transcriptionally (204). MiR-21 is upregulated in most malignant cancers and cardiac disease (205, 206). The targets of miR-21 are tumor suppressors, including PDCD4. MiR-21 inhibition may be a potential therapeutic target to prevent PDCD4 reduction in cancer cells (205).

2.4.2PDCD4 in muscle research

Muscle formation and protein synthesis have a correlative relationship. In skeletal muscle, mTORC1 and its substrate S6K1 are known regulators of mRNA translation and protein synthesis (207). However the mechanisms by which S6K1 regulates protein synthesis are not clearly documented in muscle studies. Since PDCD4 inhibits mRNA translation, it is an important factor to regulate protein synthesis. The first PDCD4 research in muscle was done by Zargar et. al. in 2011. In a study with rat, starvation decreased phosphorylation of PDCD4 along with a reduction in protein synthesis in skeletal muscle. Conversely, total PDCD4 was increased. Those effects were reversed once the rats were refed. In vitro, when myoblasts depleted of PDCD4 were grown in amino-acids and serum free medium, those cells incorporated twice as much phenylalanine into proteins than control cells. This showed that PDCD4 is sensitive to nutrients, and PDCD4 inhibits protein synthesis (208). However this did not examine the

relationship between mTORC1/S6K1 and PDCD4. In a follow-up study, it was shown that total PDCD4 expression, but not phosphorylated PDCD4, is sensitive to the medium nutrient composition. PDCD4 expression was abundant if cells were in the starvation medium or incubated with rapamycin (mTORC1 inhibitor) or MG132 (proteasome inhibitor). In the refeeding stage, they also compared the stimulus. Further experiments showed that in muscle cells, serum, but not amino acids, promote PDCD4 proteolysis. Therefore, growth factor is the definite regulator of PDCD4 expression (209). Data implicating apoptosis in the regulation of muscle cell differentiation prompted me to examine whether PDCD4, a proapoptotic protein may regulate skeletal muscle cell differentiation.

RATIONALE AND OBJECTIVES

3.1 Rationale

PDCD4 is known to reside predominantly in the nucleus in proliferating cell, and it shuttles to cytosol under serum withdrawal (151, 172). However, this effect depends on the cell type and their growth state (151). Because transition from proliferating to differentiating myoblasts is initiated by a reduction of serum quality and concentration, this transition may be regulated by PDCD4.

PDCD4 is known as an mRNA translation inhibitor. When cells are proliferating, PDCD4-depleted-cells had more protein synthesis compared to the control. However, the opposite phenomenon was observed in myotubes: PDCD4-depleted myotubes had less protein synthesis (208, 209). Thus, this implies PDCD4's involvement in myotubes metabolism may be different from that of myoblasts.

Apoptotic cells are known to be a regulator of satellite cell fusion to form new muscle (4), and PDCD4 is a pro-apoptotic protein. PDCD4 is an upregulator of p21, which is an important factor for differentiation (184).

3.2 Objectives

1. Examined expressions of protein and mRNA level of PDCD4 during skeletal muscle cell differentiation.
2. Examined the effect of PDCD4 depletion on myoblast fusion.
3. Examined the effect of overexpression of PDCD4 during muscle cell differentiation.

3.3 Hypothesis

1. I hypothesized that PDCD4 expression would be high on Day 1 compared to Day 0, since withdrawal from the cell cycle is a stressor for cells.
2. I hypothesized that PDCD4 expression in the nucleus would be lower and it would shuttle to the cytosol after medium is changed from growth medium to differentiation medium.
3. Because apoptosis is required for myotube formation and PDCD4 is a pro-apoptotic protein, depletion of PDCD4 would lead to impaired myotube formation.
4. I hypothesized that overexpression of PDCD4 would induce myotube formation.

MANUSCRIPT

The Role of mRNA Translation Inhibitor Programmed Cell Death 4 (PDCD4) during Differentiation in Skeletal Muscle Cells

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Figures: 8

Introduction

Maintaining skeletal muscle mass is a crucial factor in human health for people of all ages. The maintenance of muscle mass is essential for mobility, disease prevention and quality of life, since muscle mass comprises approximately 40% of the average adult body weight (1). In general, the mammalian body maintains a balance of protein synthesis and degradation. If protein synthesis exceeds protein degradation, it leads to muscle hypertrophy. On the other hand, protein degradation exceeds protein synthesis, muscle wasting (atrophy) can occur (9).

The mammalian/mechanistic target of rapamycin complex 1 (mTORC1) is well known to be a regulator of muscle mass autophagy (78-80). The importance of its downstream substrate S6 ribosomal protein kinase 1 as a regulator of mRNA translation and as an inhibitor of insulin receptor substrate 1 (IRS-1) is also well documented. However little is known about their roles and significance in muscle differentiation and myogenesis. Programmed cell death 4 (PDCD4), a downstream substrate of S6K1 was discovered to be upregulated in apoptotic cells (149). There is evidence that apoptosis is required for muscle cell differentiation (7). PDCD4 has been extensively studied as a tumor suppressor and an mRNA translation inhibitor in many different cells in cancer research, however its role in skeletal muscle cells has not been elucidated. Therefore, the objectives of this study were to examined expressions of protein and mRNA level of PDCD4 during skeletal muscle cell differentiation. Also, examined the effect of PDCD4 depletion on myoblast fusion and the effect of overexpression of PDCD4 during muscle cell differentiation.

Materials and methods

Reagents: Fetal Bovine Serum (FBS), Horse Serum (HS), Lipofectamine RNAimax, Lipofectamine 2000 DNA Transfection Reagent, Optimem medium, and antibiotic/antimycotic (Ab-Am) reagents were purchased from Life Technologies (Burlington, Ontario, Canada). PDCD4 siRNA oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphatase and protease inhibitor cocktails were purchased from Sigma-Aldrich (St. Louis, MO, USA). α- Modification of Eagle's Medium (AMEM) and Phosphate Buffered Saline (PBS) were obtained from Wisent (St-Bruno, Quebec, Canada).

Antibodies: Antibodies to PDCD4 (cat #9535) and GAPDH (cat #2118) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to troponin, myogenin and MHC-1 were purchased from Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA). Antibodies to γ-tubulin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and Treatments: Differentiation experiments were performed as follows: L6 rat skeletal muscle myoblasts were obtained from the American Type Culture Collection. Stocks of the earliest passage cells have been stored and used. The cells were cultured in 6 well-plates (150×10^3 cells/well for differentiation experiments or PDCD4 overexpression experiments and 250×10^3 cells/well for PDCD4 siRNA experiments) or in 10cm plates (600×10^3 cells/plate). The cells were propagated at 37°C and 5% CO_2 in humidified atmosphere in complete proliferation medium composed of AMEM supplemented with 10% FBS, 1% Ab-Am until they reached 80-90% confluency. Cells

were then shifted into differentiation medium (DM) which is AMEM supplemented with 2% HS and 1% Ab-Am.

Cell Fractionation: Twenty-one 10 cm plates (3 plates per day) were used for this experiment. After they reached up to 80-90% confluency, the plates for day 0 were harvested following the procedure below. The medium for the remaining plates was changed to DM. The DM was changed every forty-eight hours. Remaining plates were harvested Day 1 to Day 6. To harvest the cells, they were trypsinized with 1 ml/10cm-plate. Five mls of ice cold PBS were added to stop the trypsin and the cells put in 15 ml tubes. They were centrifuged at 621.6g (2000 rpm) for 5 minutes in a microcentrifuge. After the centrifugation, the PBS was sucked out, and 1 ml PBS was added and cells were resuspended. Everything was put in 1.5 ml eppendorf tubes and centrifuged 431.74g (2300 rpm) for 3 minutes at 4°C in a microcentrifuge. PBS was then removed. Five hundred μl of Buffer 1 (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 10 μl/ml protease inhibitor and 10 μl/ml phosphatase inhibitor) were added to the pellet and resuspended. The samples were put on ice and vortexed for 15 sec every minute for 5 mins at low speed. From the resulting lysate, 100 μl aliquot was put in eppendorf tubes for further examination. The remaining volume was centrifuged 431.74g (2300 rpm) for 3 minutes at 4 °C in a microcentrifuge. After centrifugation, the supernatants were collected as cytosol. To make sure there was no contamination, samples were washed 2 times with 200 μl Buffer 1. After the wash, the pullet was resuspended in 150 μl of Buffer 2 (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 40% (wt/vol) glycerol, 10 μl/ml protease inhibitor and 10 μl/ml phosphatase inhibitor). This was the nuclear fraction.

^{35}S-Methionine Pulse-Chase and Immunoprecipitation experiments: Twenty-one 10 cm-plates (3 plates harvested at each time point) were used for this experiment. Twenty-four hours after cells were plated, the medium for twenty-one plates was changed to 4 mL of GM and 30 μCi of [^{35}S] methionine/cystine. Twenty-four hours after adding radioactive methionine (48 hours after seeding cells), GM and 30 μCi of [^{35}S] methionine/cystine were removed. Plates were rinsed in PBS and harvested or incubated in DM supplemented with methionine/cysteine (2 mM). Medium was changed every 4 hour till harvested. For harvesting, all plates were washed twice with ice cold PBS and harvested with 450 μl/plate of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) Buffer (40 mM HEPES pH 7.5, 120 mM NaCl, 1mM EDTA, 10 mM Pyrophosphate, 10 mM Glycerophosphate, 50 mM sodium fluoride (NaF), 0.5 mM Orthovanadate) with supplementation (0.3% CHAPS, 1 mM DTT, 1 mM Benzamidine (Benz), 0.5 mM sodium vanadate (Nav), 10 μl/mL of protease inhibitor, 10 μl/mL of phosphatase inhibitor, 6.25 mM NEM). Lysates were put in eppendorf tubes (this is the LOAD). New tubes were prepared for immunoprecipitation (IP). To do this, 400 μl of CHAP buffer, 3 μl of anti-PDCD4 antibody (Cell Signalling cat #9535) and 300 μl lysate were added. Those tubes were incubated overnight in the cold room (4°C) with rotation. The following day, magnetic beads solution was prepared. BioMag Protein G (QIAGEN, cat #311812) was used (100 μl/tube). The re-suspended beads (100 μl) were washed 3 times in Low Salt Buffer (LSB: 20 mM TrisHCl, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% β-mercaptoethanol). After washing, the beads were resuspended in 100 μl/tube of LSB+ (LSB supplemented with: 1 mM DTT, 0.5 mM Nav, 1mM Benz, protease inhibitor 10 μl/ml, phosphatase inhibitor 10 μl/ml, 6.25 mM NEM

and 0.1% milk powder). After adding LSB+, the tubes were incubated for 2 hours in the cold room (4°C) with rotation. After the incubation, samples were put on the magnetic rack. Supernatant was collected. After taking out the supernatant, each sample was washed twice with 500 µl of LSB+ and washed once with High Salt Buffer (HSB: 50 mM TrisHCL, 500 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.04% β-mercaptoethanol) with supplementation (1 mM DTT, 0.5 mM Nav, 1mM Benz, protease inhibitor 10 µl/ml, phosphatase inhibitor 10 µl/ml, 6.25 mM NEM). After removing the washes, 100 µl of 1x Sample Loading buffer supplemented with β-mercaptoethanol were added to each tube. They were vortexed and boiled for 5 mins at 95°C. They were vortexed again, and centrifuged for 2 mins at 13000g. Samples were placed on the magnetic rack and supernatant was transferred into 1.5 ml eppendorf tube (eluate).

RNA Interference: To knockdown PDCD4, siRNA oligonucleotides were used. L6 myoblasts (250 x 10^3 cells) were seeded in 6-well plates. The cells in the negative control condition were transfected with a scrambled RNAi oligonucleotide that will not induce the knockdown of any rat proteins. The PDCD4 #1 siRNA oligonucleotide (Sigma-Aldrich): PDCD4 #1 sense (GUCUUCUACUAUUACCAUA [dT] [dT]), PDCD4 #1 antisense (5′UAUGGUAAUAGUAGAAGAC [dT] [dT]), PDCD4 #2 sense (CUACUAUUACCAUAGACCA [dT] [dT]), and PDCD4 #2 antisense (UGGUCUAUGGUAAUAGUAG [dT] [dT]) were used at a final concentration of 30 nM, following the manufacturer's protocol. After forty-eight hours of incubation with the transfection mix, cells were harvested or switched to DM. They were harvested in lysis buffer (25 mM Tris, pH 7.5, 1 mM EDTA, 2% sodium dodecyl sulfate (SDS)

supplemented with protease inhibitor cocktail (10 µl/ml), phosphatase inhibitor cocktail (10 µl /ml) and DTT 1M (1 µl /ml) from day 0 through day 5. Cell lysates were passed through a 25-gauge needle to break apart the globular proteins and collected in 1.5 ml eppendorf tube.

mRNA expression experiment: The cell preparation is the same as above as for cells transfected with siRNA oligonucleotide. On the day of harvesting, cells were harvested and RNA was extracted by TRIzol Plus RNA Purification Kit (Life Technologies, Burlington, Ontario, Canada) following the manufacturer's protocol. After the extraction, RNA concentration was measured by Smart Spec^{TM} Plus (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group).

Reverse transcription (RT) was done with iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories ((Canada) Ltd Life Science Group) following the manufacturer's protocol. RT reaction was carried out for 60 minutes at 42 °C followed by 5 minutes at 85 °C in 5345 PCR Mastercycker, Gradient Thermal Cycler (Eppendorf) Resulting cDNA was diluted 1.5 times with autoclaved deionized water. In carrying out quantitative PCR (qPCR), the following primers were used:

	primer	
PDCD4	forward	ATGAGACTGTGGTTCTGCCC
	reverse	TCCCTTAACATCTCCGCGAC
Myogenin	forward	CCCAGTGAATGCAACTCCCA
	reverse	CGAGCAAATGATCTCCTGGGT
Myosin Heavy Chain	forward	GAGTCCCAGGTCAACAAGCTG
	reverse	GTGCCTCTCTTCGGTCATTC
HPRT	forward	CTTCCTCCTCAGACCGCTTTT
	reverse	ATCACTAATCACGACGCTGG

All primers were purchased from Life Technologies (Burlington, Ontario, Canada). qPCR was conducted with 10 μl of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group), 1.25 μl of 10 μM of each forward and each reverse primer, 6 μl autoclaved deionized water and 1.5 μl of diluted cDNA. qPCR products and data were obtained by CFX9^{TM} Real-Time System C1000 Thermal Cycler and CFX Manager 3.1 (Bio-Rad Laboratories ((Canada) Ltd Life Science Group).

Overexpression experiment: To overexpress PDCD4, EGFP-empty and EGFP- PDCD4 plasmid were used. Plasmid pcDNA3-EGFP was a gift from Dr Doug Golenbock (Addgene plasmid # 13031), 1436 pcDNA3 Flag HA from Dr William Sellers (Addgene plasmid # 10792), and pGEX6p1-hPdcd4 from Dr Haiwei Song (Addgene Plasmid #20693). Human PDCD4 was digested with Bamh1 and Xho1 from pGEX6p1-hPDCD4 and cloned into the BamH1 and Xho1 sites in pcDNA3 Flag HA or pcDNA3-EGFP to generate, respectively PDCD4 Flag pcDNA or PDCD4 EGFP pcDNA plasmids. The integrity of these resulting plasmids was confirmed by PCR, restriction digestion and sequencing different regions of the plasmids. L6 myoblast (160 x 10^3 cells) were seeded and transfected in 6-well plates when the wells were 70-75% confluency. Plasmid was used at 2.5 μg per well. After forty-eight hours of incubation with the transfection mix, cells were harvested or switched to DM. They were harvested in lysis buffer from day 1 through day 5 of differentiation in DM. Cell lysates were passed through a 25-gauge needle to break apart the globular proteins and collected in 1.5 ml eppendorf tube.

Western blot determination for all experiments: For experiments involving nuclear-cytosolic fractionation, 30 μl (nuclear fraction) or 25 μl (each of cytosol and whole cell lysate) samples were loaded per well of 15% SDS-polyacrylamide gel electrophoresis

(PAGE). For immunoprecipitation experiments, 20 μl (load) or 30 μl (eluate) samples were loaded per well of 10% gel. For RNAi experiments, protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA). Equal amounts of protein (25 μg) were loaded per well of 12.5% gel. Proteins were then transferred onto polyvinylidene difluoride (PVDF) 0.2 μm membrane. Membranes were subsequently blocked in 5% non-fat milk in Tris-Buffered Saline + Tween20 (TBST) for 1 hour at room temperature. After 1 hour, membranes were washed quickly twice and then washed 3 times for 5 min with TBST, and then incubated with the diluted desired primary antibody (PDCD4: 1:2000 for all experiments; histone H2A: 1:1000 for nuclear loading control; GAPDH: 1:1000 for cytosolic loading control; MHC-1, troponin and myogenin: 1:500 for RNAi experiments, γ–tubulin: 1:10,000 for loading control except for nuclear and cytosol). All the primary antibodies were diluted with 2.5% Bovine Serum Albumin (BSA) in TBST. Following overnight incubation in the cold room (4°C), membranes were washed quickly twice and then washed 3 times for 5 minutes with TBST, and incubated with HRP-conjugated goat anti rabbit (GAR) (for PDCD4, GAPDH, histone H2A) or goat anti mouse (GAM) (for MHC-1, myogenin, troponin and γ–tubulin) secondary antibodies for 1 to 3 hours at room temperature. Immunoblots were then incubated with the Immobilon Western Chemiluminescence HRP Substrate (Millipore).

For visualizing, the Kodak imaging station (Molecular Imaging System Carestream Health Inc.) was used. For the immunoprecipitation experiment only, membranes were exposed to High performance chemiluminescence film (GE Healthcare

Limited) for 48 hours. Signals were quantified using the Carestream Molecular Imaging software (Version 3, Software, La Jolla CA).

Statistical Analyses: Data are presented as means ± SD of all experiments. One-way analysis of variance (ANOVA) was used, followed by Tukey's post-hoc tests to assess statistically significant differences amongst means. The level of significance was set at $p<0.05$ for all analyses. Statistical analyses were done using GraphPAD (Version 3, GraphPAD Software, La Jolla, CA, U.S.A.).

Results

PDCD4 expression increases during C2C12 and L6 cell differentiation.

PDCD4 expression in L6 (Fig 1) and C2C12 (Fig 2) was significantly increased at the onset of differentiation ($p<0.05$) after medium was changed to differentiation medium (Fig 1A, B, Fig 2A, B). MHC-1, as a myotube formation marker, expression was observed starting Day 3 in L6 cells (Fig 1C), Day 2 in C2C12 cells (Fig 2C). Significant difference started D4 in L6 cells, Day 3 in C2C12 cells ($p<0.05$).

Nuclear-Cytosolic expression of PDCD4 during the skeletal muscle cell differentiation.

PDCD4 is known to shuttle between the nucleus and cytosol. To examine nuclear-cytoplasmic distribution of PDCD4 during differentiation, I fractionated L6 (Fig 3) and C2C12 (Fig 4) cell lysate. Western blots and graphical representation of nuclear and cytoplasmic expression of PDCD4 during differentiation are shown in Fig 3 and Fig 4. In general, PDCD4 expression is high in nucleus in proliferating cells. In muscle cells, PDCD4 expression was low in lysate from myoblast, however, expression was observed

in nucleus and cytosol on day 0 in both L6 (Fig 3) and C2C12 (Fig 4). The cytosolic expression was higher than nuclear one (Fig 3, 4). At the onset of differentiation, cytosolic expressions were high, which was expected. On the other hand, unexpectedly, nuclear expressions remained higher on D1 to D6 compared to day 0. Cytosolic expression of PDCD4 was significantly higher on day 1 compared to day 0 ($p<0.05$) in both C2C12 and L6 muscle cells (Fig 3C, 4C).

PDCD4 abundance during differentiation is regulated by proteolysis

To figure out why PDCD4 expression is higher on day 1 and day 2 of differentiation (Fig 1, Fig 2), I used pulse chase technique to examine whether the changes were due to changes in PDCD4 degradation. In particular, in immunoprecipitation for ^{35}S-labeled cells, there was a significant decrease in PDCD4-associated radioactivity between 0H and 8H vs. 32H after the medium change to DM ($p<0.05$) (Fig 5B, C). On the other hand, mRNA expression of PDCD4 showed that there was a significant difference between D0 and D2 (Fig 7A of CTL).

Knockdown of PDCD4 in L6 myoblasts leads to significant changes in myotube formation.

To examine the role of PDCD4 during myotube formation, I employed RNA interference (RNAi) mediated knockdown of the protein. Western blot analysis showed that PDCD4 depletion was conducted successfully (Fig 6A, B). From day 3, cells transfected with the control siRNA started to differentiate as can be seen in MHC-1 expression (Fig 6A, C). This was observed much less in PDCD4-depleted-cells (Fig 6C).

The myofibullar proteins (MHC-1 and myogenin expressions) were higher in the control compared to PDCD4-depleted-cells at all time points (Fig 6C, D). However MHC-1 mRNA expression was not different between treatments (Fig 7B). The morphological image showed that PDCD4-depleted-cells had very few and smaller myotubes (Fig 6E, on the bottom right).

Overexpression of PDCD4 in L6 myoblast leads to increased myotube formation

The knockdown experiment showed the importance of PDCD4 in myotube formation (Fig 6). Therefore, I overexpressed PDCD4 in L6 cells (Fig 8B, C) and observed more myotubes formation (Fig 8A, B and D) ($p<0.05$). Morphologically, cells overexpressing PDCD4 appeared to have more myotubes compared to control (Fig 8A). The transfection of efficacy was low (approximately 20%). Nethertheless, I observed more myotube formations in transfected cells (Fig 8A).

Discussion

Regulation of mRNA binding to 40S ribosomal subunit is the initial step of protein synthesis (138). The mRNA binding is regulated by mTORC1 signalling pathway. It does this via at least two of its substrates, 4E-BP1 and S6K1. mTORC1 phosphorylates 4E-BP1 and S6K1 directly. The phosphorylated 4E-BP1 dissociates from eIF4E. The activated S6K1 phosphorylates PDCD4. The phosphorylated PDCD4 is subsequently degraded by proteasome via ubiquitylation by E3 ligase β-TrCP (150). PDCD4 binds to and inhibit eIF4A. Phosphorylation of PDCD4 frees eIF4A. eIF4E and

eIF4A are components of eIF4F, a complex needed to initiate mRNA translation (141, 198, 201).

PDCD4, a pro-apoptotic protein, is also known as a tumor suppressor protein (5, 172, 190, 191). Tumor promoters such as TPA or Epidermal growth factor (EGF) induces neoplastic transformation through increasing the expression of transcription factors such as NF-kB and AP-1 (189, 192). PDCD4 inhibits AP-1 dependent transcription by inhibiting MAP4K1, which is upstream of AP-1 (197, 198). Therefore, PDCD4 inhibits TPA-induced neoplastic transformation by inhibiting AP-1 activation (189, 192). This is the main idea of PDCD4 related cancer research, however the role in muscle research is not well established yet. The ability of PDCD4 to inhibit the cell cycle and to promote apoptosis led me to examine its role in regulating muscle cell differentiation. PDCD4 is expressed in a wide variety of tissues and at the highest level in the liver and to a lesser extent in the heart and skeletal muscle in mouse (171, 172, 175). In both L6 and C2C12 cells, the expression of PDCD4 was low during proliferation (D0) (Fig 1A, B and 2A, B). Beginning of differentiation (D1-2), PDCD4 expression increased significantly. Previous studies showed that PDCD4 expression is normally at low level, however apoptosis or transformation upregulates its expression (171, 175-177). PDCD4 expression is high in non-proliferating cells such as differentiating cells (151). Those observations are consistent with our observation in muscle cells.

PDCD4 has a nuclear localization signal (NLS) at its N and C termini. It shuttles between the nucleus and cytosol (5, 178-180). There are many studies showing the movement of this protein, however they are controversial. The reason is that each tumor cell is unique, and depending on the stage of the progression of the cancer the

characteristics can vary. Studies demonstrated that PDCD4 expression is predominantly in the nucleus in proliferating cells. It can be exported from the nucleus to the cytosol under certain condition such as serum withdrawal (151, 172, 180). However, this observation is dependent on cell type and cell conditions. For example, PDCD4 is predominantly expressed in the cytoplasm in breast cancer cell (183). In muscle cells, PDCD4 shuttling is not the same as in majority of cancer cells: cytosolic expression was higher in proliferating cells (Fig 3C, 4C), and nuclear expression was observed in differentiating cells (Fig 3B, 4B). Also both cytosolic and nuclear expressions in differentiating cells were higher than in proliferating cells (Fig 3B,C and Fig 4B,C). It may imply nuclear PDCD4 involvement in nuclear RNA processing events such as splicing and nucleo-cytoplasmic transport (151, 172, 180, 183). A loss of PDCD4 expression or accumulation of nuclear PDCD4 may positively regulate cell proliferation (183). PDCD4 is upregulated in apoptotic cells (171-173). The apoptotic protein may have a role in forming myotubes (4). Increased expression of PDCD4 in the nucleus may inhibit myotubes formation. However those observations were not clear in this study.

Protein abundance depends on the balance between protein synthesis and degradation. To understand the mechanism of PDCD4 expression at the onset of differentiation, I used the pulse chase assay (Fig 5A). There are different ways to examine PDCD4 degradation. PDCD4 phosphorylation is regulated by its upstream kinases S6K1 and AKT. Phosphorylation of PDCD4 on Ser 457 by AKT may have a role in nucleus, but this is not conclusive in literature (182). PDCD4 phosphorylation on Ser 67 by S6K1 is subsequently degraded by proteasome through ubiquitylation by E3 ligase β-TrCP. The phosphorylation of Ser 67 promotes phosphorylation on Ser 71 and Ser 76

allowing for binding to β-TrCP (178). Therefore, we could use the anti-phPDCD4 antibodies to measure the amount of PDCD4. This is an indirect way of looking at PDCD4 degradation. Even though phospho-PDCD4 antibodies are available on market, they are unreliable. Instead, I used anti-β-TrCP (cell signalling cat #4394) to examine regulation of PDCD4 degradation. The data showed β-TrCP expression is low during cell proliferation (supplemental figure 1A, B). It makes sense since PDCD4 expression is low during cell proliferation. During differentiation, β-TrCP expression is significantly higher at any day point compared to cells in proliferation. This implies that PDCD4 abundance during differentiation is regulated by proteolysis (supplemental figure 1A, B). However, if degradation of PDCD4 is rapid, one might not be able to see ubiquitylated PDCD4. If degradation occurs very quickly, one may not be able to see ubiquitylated proteins (210). An increase in β-TrCP should correspond to increased ubiquitylation of PDCD4 and therefore increased degradation of PDCD4. However this is not a direct measure of protein degradation. β-TrCP has many substrates. For example, one of the important components of mTORC1, DEPTOR, is also a substrate of β-TrCP (101, 106, 211). To strengthen my analysis of degradation, I used pulse chase assay combined with immunoprecipitation. Because PDCD4 expression is low during proliferation (when I labeled cells with the radioactive isotope), I immunoprecipitated samples. Results from the pulse chase assay and immunoprecipitation experiments indicated that the decrease in PDCD4 abundance as differentiation progressed might be due to increased proteolysis (Fig 5B, C). However, mRNA levels of PDCD4 also significantly increased between D0 and D2 (Fig 7A of CTL). Taking together, these imply that PDCD4 abundance during the onset of differentiation is due to the increases of both protein synthesis and degradation.

To examine the significance of PDCD4 expression during differentiation, I used RNAi to deplete the protein in differentiating cells. Results with siRNA experiment showed that the expression of myotube formation indicators (MHC-1, myogenin) was less in PDCD4-depleted-cells. It may imply that the expression of PDCD4 is an important factor for muscle cell to form myotubes. Further assessment, mRNA expression was measured. The myogenin mRNA expression showed that mRNA expression in control condition was significantly higher than in cells depleted of PDCD4 at any day points (Fig 7C). The same trend was observed in protein expression (Fig 6E). In control (scrambled) cells, myogenin mRNA expression peaked on Day 2 and decreased by Day3 and Day4. In PDCD4 knocked-down cells, myogenin expression increased during differentiation. This observation is consistent with the literature: myogenin mRNA expression increases during differentiation, and declines as cells become well differentiated (212). MHC-1 mRNA level was very low (Fig 7B). Even though I checked 2 sets of primers, both gave me the same results. It is important to obtain another set of primers and check the result for the future studies.

If the depletion of PDCD4 suppressed muscle cell differentiation, overexpression of the protein might rescue myotube formation. I conducted a transient overexpression experiment of PDCD4. Considering the quantification of western blot, the transfection efficiency was around 20% (Fig 8C, also see Supplemental figure 2A, B). The PDCD4-overexpressed-cells formed more myotube than control condition. This was seen both from morphological and western blot analyses ($p<0.05$) (Fig 8A, D). A criticism of this study is the low transfection efficiency. However, increasing the amount of a pro-apoptotic protein as the name suggests, lead to cell death. However I did not measure

apoptosis. PDCD4 expression in Day 4 and 5 decreased (Fig 8B, C). This is because I used transient overexpression protocol in this study. If one uses stable overexpression protocol, one may obtain better results.

PDCD4 is known as a tumor suppressor and an mRNA translation inhibitor. Overexpression of PDCD4 inhibits cancer cell progression. However this intervention has not reached human clinical trial yet. If we can overexpress PDCD4 in whole body, it acts as tumor suppressor to inhibit transcription factor regulatory proliferation of cancer cells. My data also show PDCD4 has an effect of maintaining skeletal muscle mass by inducing myotube formation. Therefore increased expression of PDCD4 may not only limit cancer cell growth, it also appears to hold potentials in promoting muscle cell recovery (Fig 9).

Future Direction

For overexpression experiment, I used a transient overexpression technique. A criticism of this study is the low transfection efficiency, which was approximately 30-45% (Supplemental Figure 2C). Thus may be corrected (increased) by using stable overexpression protocol such as viral vectors. It is possible that increasing the amount of a pro-apoptotic protein may lead to cell death. Therefore, not only the efficiency of the transfection, but also the effect of increased expression on apoptosis needs to be examined. To examine the cell viability, I should use Trypan Blue ((Bio-Rad Laboratories ((Canada)) Ltd Life Science Group. Cat # 145-0021) to see the effect of transfection in apoptosis. Other way of overexpressing PDCD4, I can use mutant PDCD4. If I mutate Serine 67 of PDCD4, we can overexpress PDCD4.

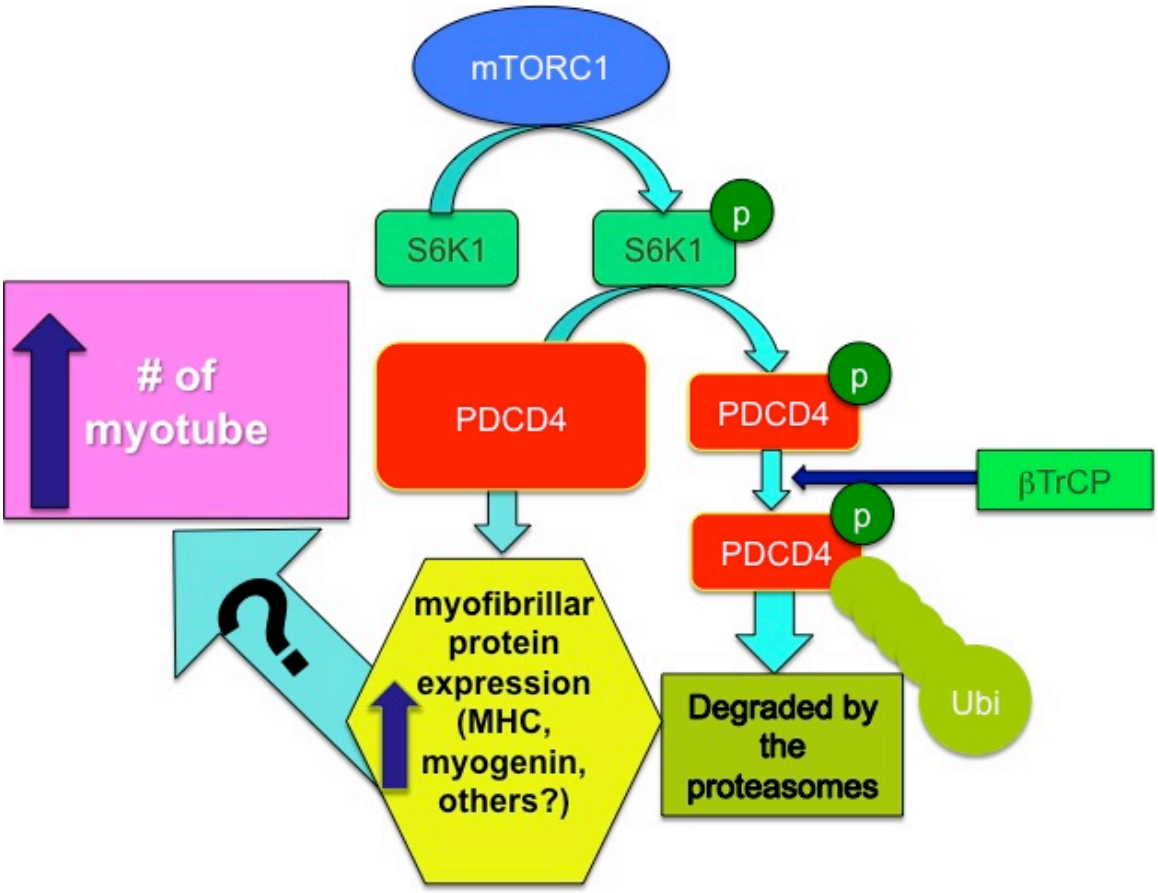


Fig 9. Schematic of possible role of PDCD4 during myotube
Once mTORC1 is activated, its downstream substrate S6K1 is phosphorylated. S6K1 can phosphorylate PDCD4. Phosphorylated PDCD4 (Ser 67) is targeted for ubiquitin-mediated degradation by the ubiquitin protein ligase βTrCP (beta transducing repeat containing protein). If PDCD4 is in the unphosphorylated state, or overexpressed as done in this study, it may have an effect on myofibrillar protein expressions in myotube. It may affect the number of myotubes formed. It effects on myotube size needs to be studied.

There was no significant difference in mRNA level of MHC-1 between treatments (control and PDCD4 knock-down cells). This may imply that protein level of MHC-1 was disturbed by PDCD4 post-transcriptionally. It may be not only MHC-1, but the levels of other myotube formation markers may have been decreased their protein expressions by knock-down. Therefore, I should examine the mRNA and protein levels of myofibrillar proteins such as actin and MHC isoforms in control and PDCD4-depleted-

cells. I suspect that even though PDCD4 may have an effect on increasing the number of myotubes, but it may not necessarily increase muscle size, except myotube size is also affected.

After examining in vitro, it is crucial to examine in vivo and confirm the results obtained in cell culture. For example, after causing muscle atrophy or injury with immobilization (tie up one leg) or muscle ablation with control and PDCD4-knockout-mice, one can study the effect of PDCD4 inactivation on muscle regeneration.

FIGURES

Fig 1.

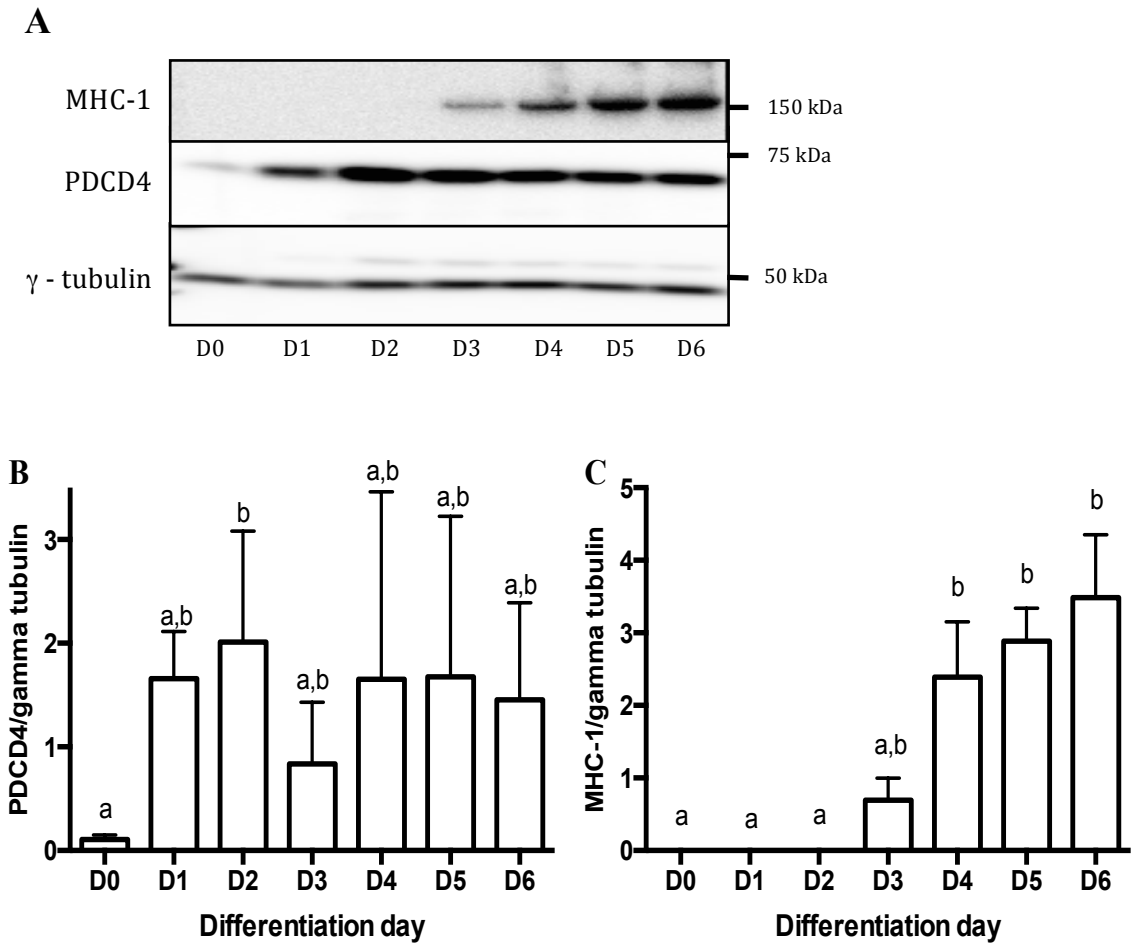


Figure 1. PDCD4 expression in proliferating L6 cells (Day 0) and during differentiation (Day 1 to Day 6). PDCD4 expression was significantly increased from Day 0 to beginning of differentiation (Day 2) ($p<0.05$) after growth medium was changed to differentiation medium (A, B). MHC-1 was used as myotube formation indicator. Abundance of MHC-1 was observed starting Day 3, and significantly different starting Day 4 ($p<0.05$) (A, C). D0=day 0, 48 hours after cells were seeded in 6-well plate. D1=1 day after medium was changed to differentiation medium. Means ± SEM; n =3 independent experiments; bars with different letters differ.

Fig 2.

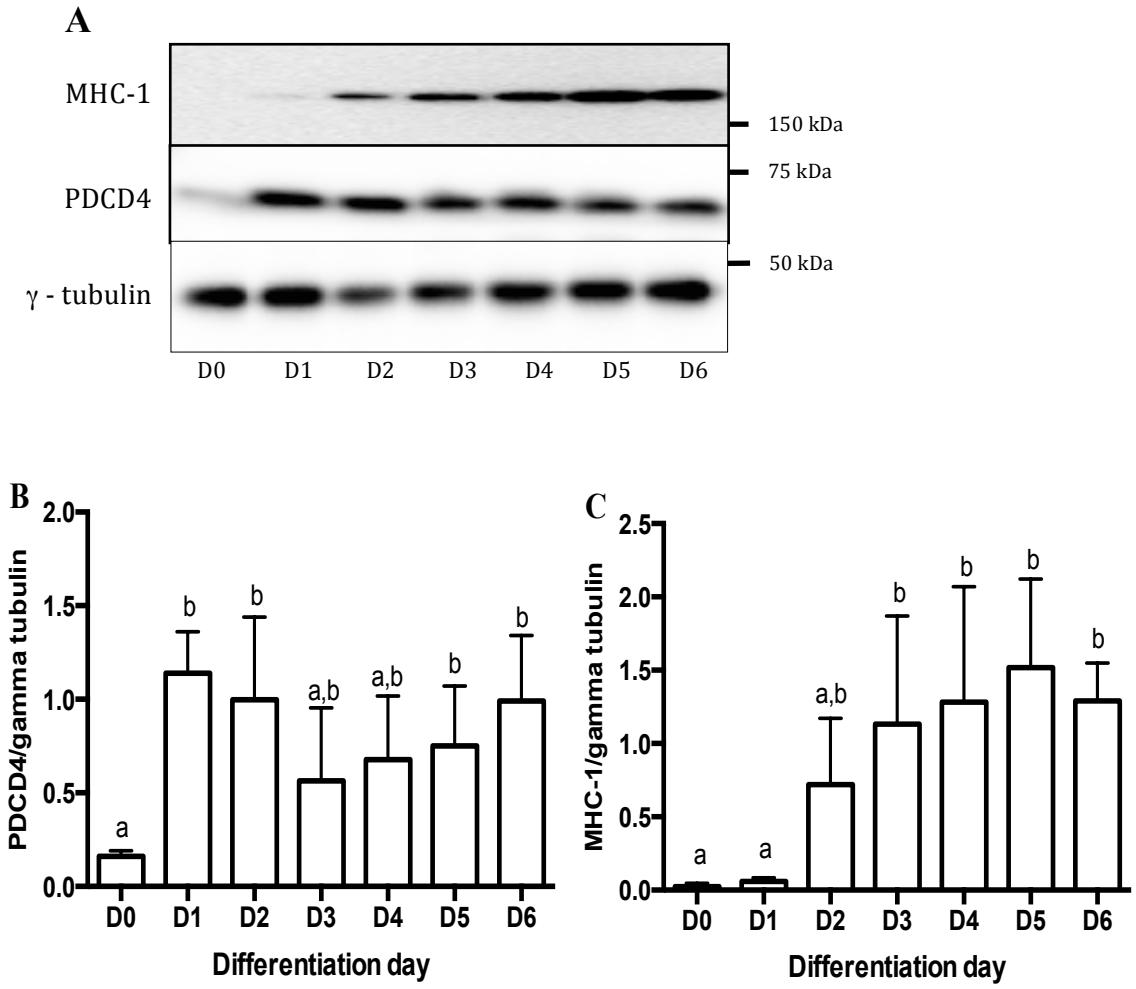


Figure 2. PDCD4 expression in proliferating C2C12 cells (Day 0) and during differentiation (Day 1 to Day 6). PDCD4 expression was significantly increased from Day 0 to beginning of differentiation (Day 1 and Day2) ($p<0.05$) after growth medium was changed to differentiation medium (A, B). MHC-1 was used as myotube formation indicator. Abundance of MHC-1 was observed starting Day 2 ad significantly different starting Day 3 ($p<0.05$) (A, C). D0=day 0, 48 hours after cells were seeded in 6-well plate. D1=1 day after medium was changed to differentiation medium. Means ± SEM; n=3 independent experiments; bars with different letters differ.

Fig 3.

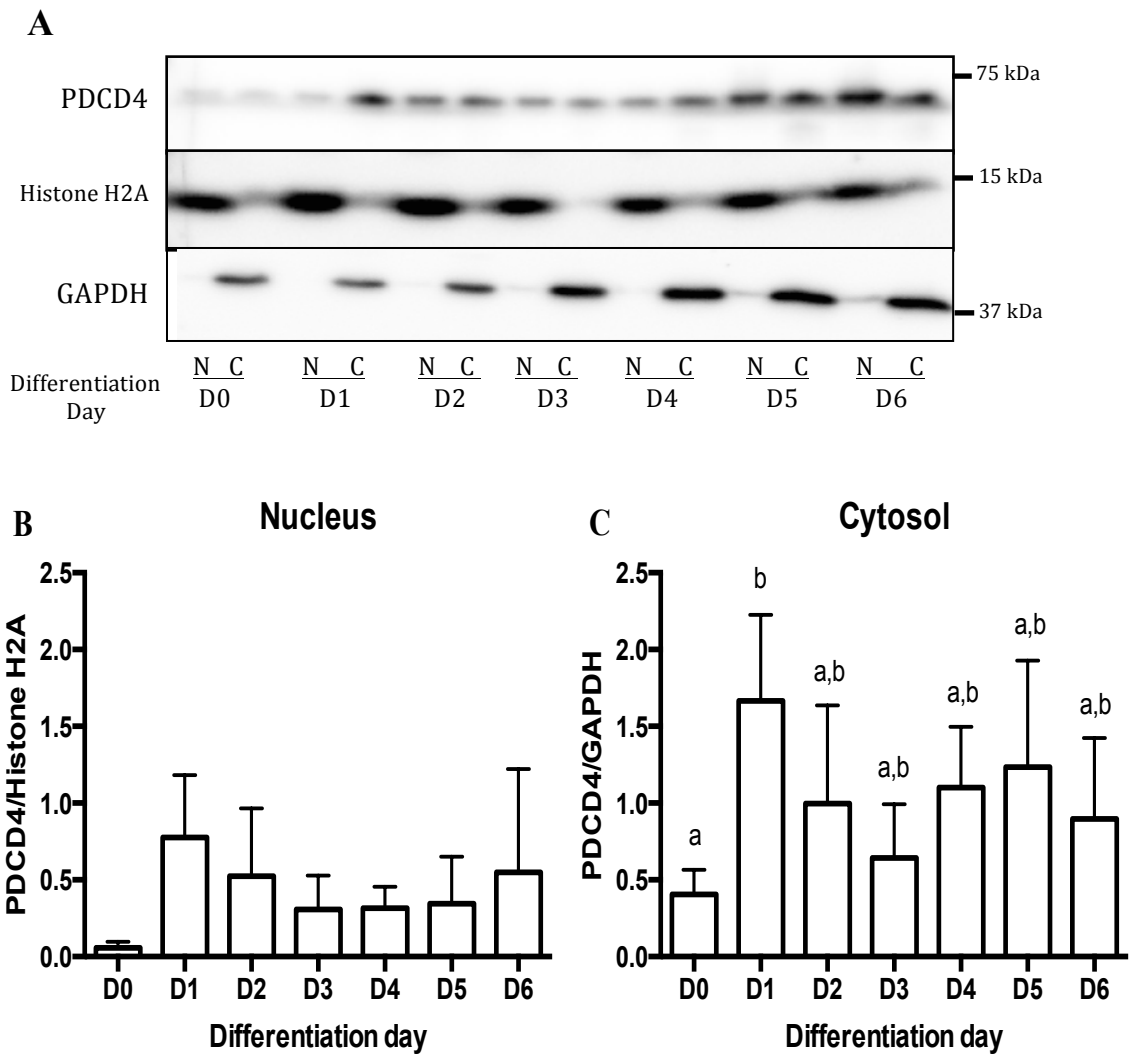


Figure 3. Nuclear-Cytosolic expression of PDCD4 during L6 cell differentiation. Forty eight hours after cells were seeded, cells were harvested (D0) or shifted to differentiation medium (DM). After cells were scraped off the plates, the samples (whole lysate) were fractionated into cytosolic and nuclear fractions. Histone H2A was used as loading control for nuclear expression of PDCD4 and GAPDH for cytosolic one. Cytosolic expression at onset of differentiation was significantly different from myoblasts ($p<0.05$). N=nucleus C=cytosol, Means ± SEM; n=3 independent experiments; bars with different letters differ.

Fig 4.

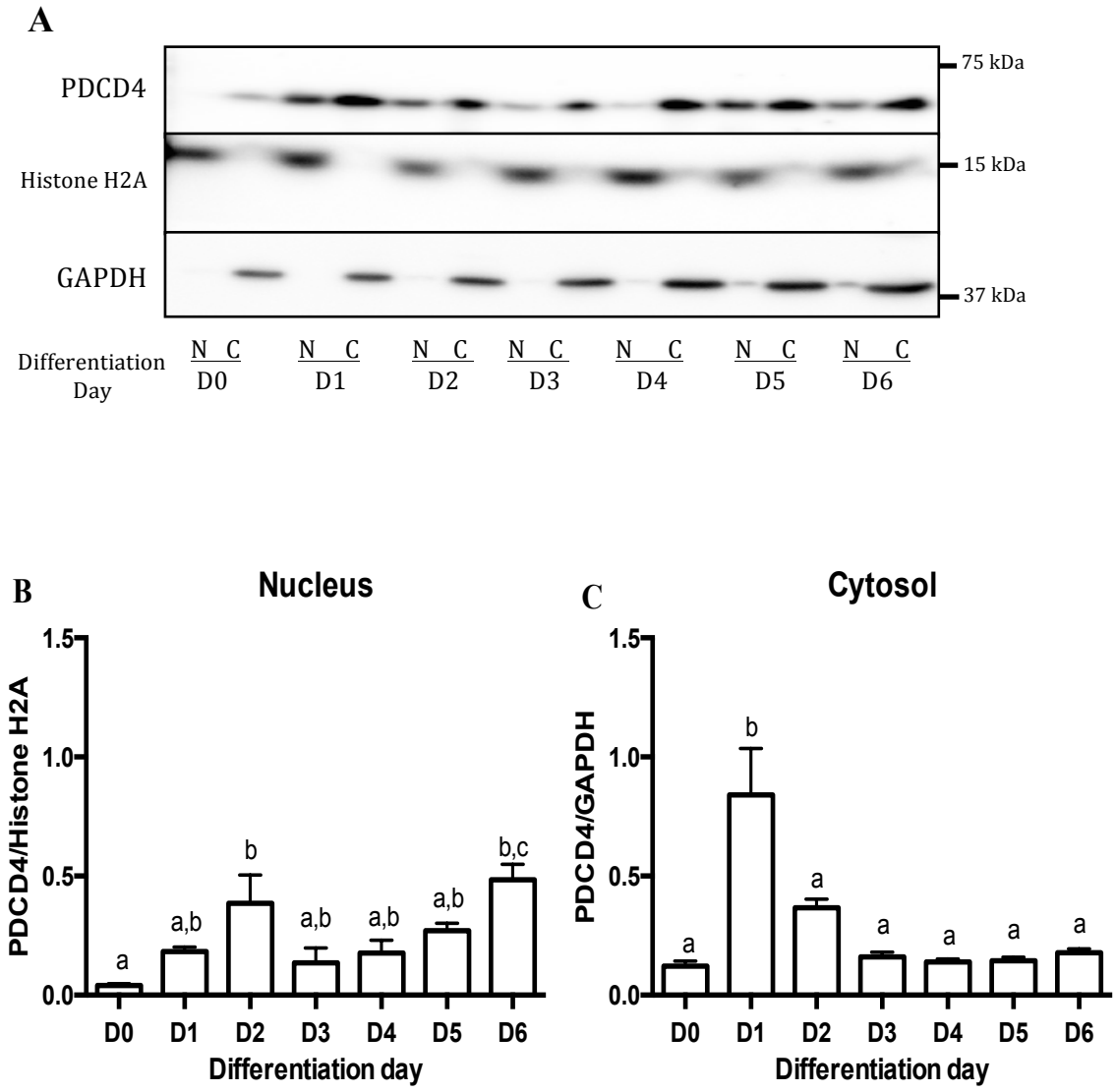
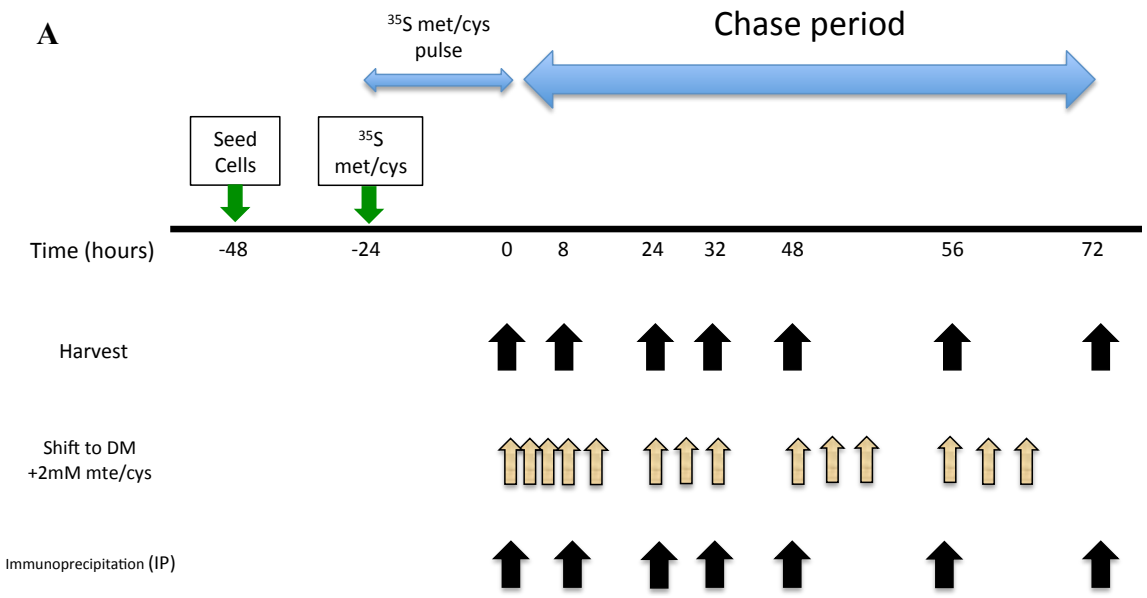


Figure 4. Nuclear-Cytosolic expression of PDCD4 in C2C12 during cell differentiation. Forty eight hours after cells were seeded, cells were harvested (D0) or shift to differentiation medium (DM). After cells were scraped off the plates, the samples (whole lysate) were fractionated to cytosolic and nuclear fractions. Histone H2A was used as loading control for nuclear expression of PDCD4 and GAPDH for cytosolic one. Both cytosolic and nucleus expression at onset of differentiation were significantly different from myoblasts ($p<0.05$). N=nucleus C=cytosol, Means ± SEM; n=3 independent experiments; bars with different letters differ.

Fig 5.


A
35S met/cys pulse
Chase period
Seed Cells
35S met/cys
Time (hours)
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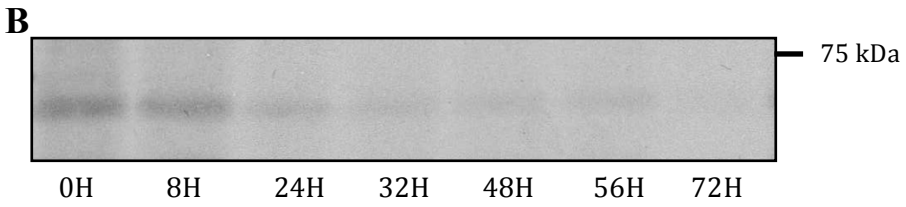
-24
0
8
24
32
48
56
72
Harvest
Shift to DM +2mM mte/cys
Immunoprecipitation (IP)


B
75 kDa
0H
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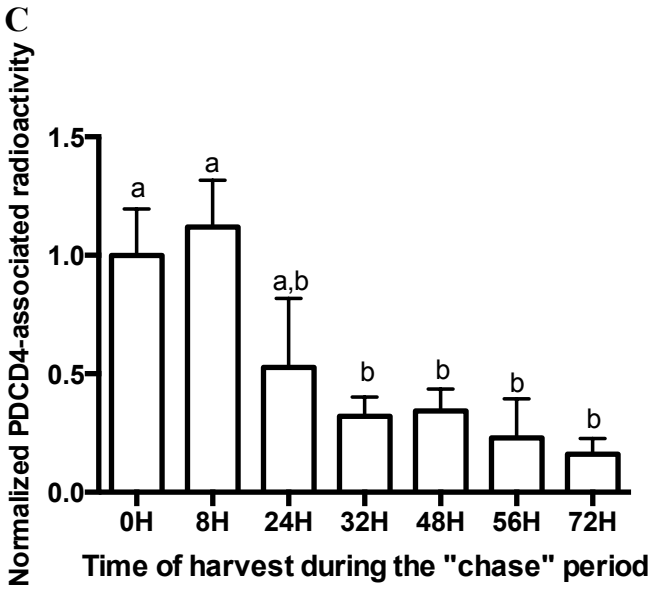
C
Normalized PDCD4-associated radioactivity
1.5
1.0
0.5
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a
a
a,b
b
b
b
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0H
8H
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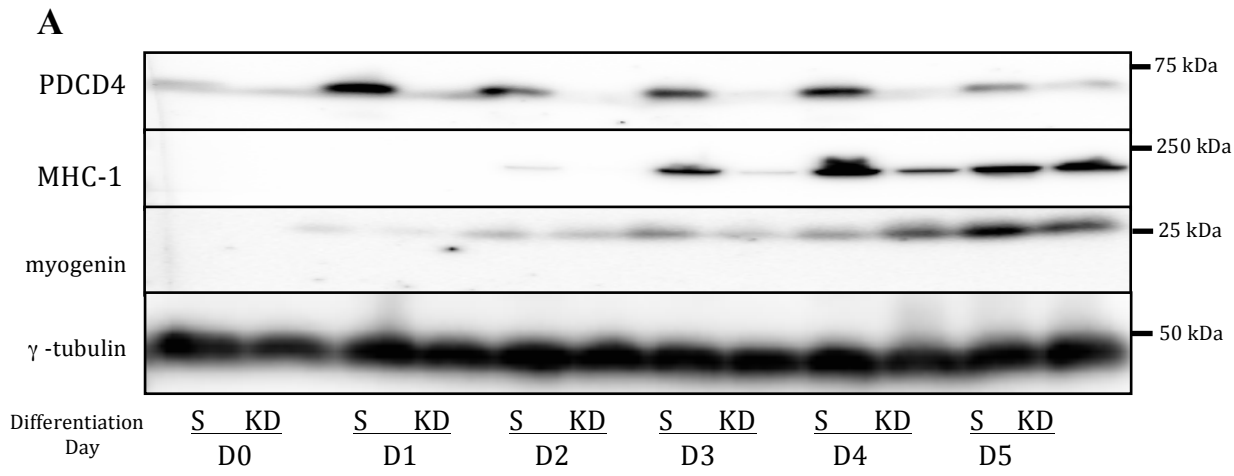
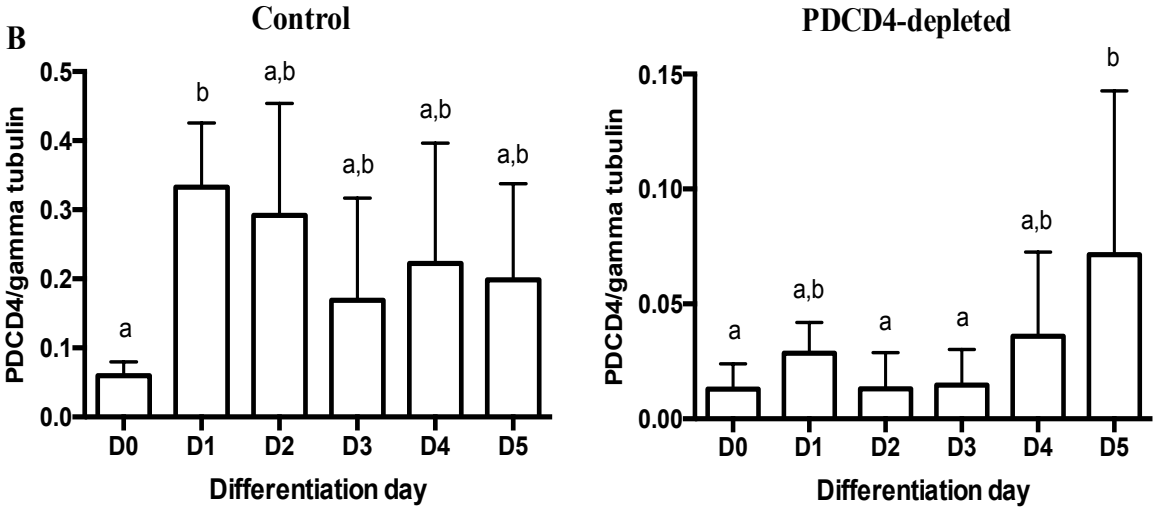
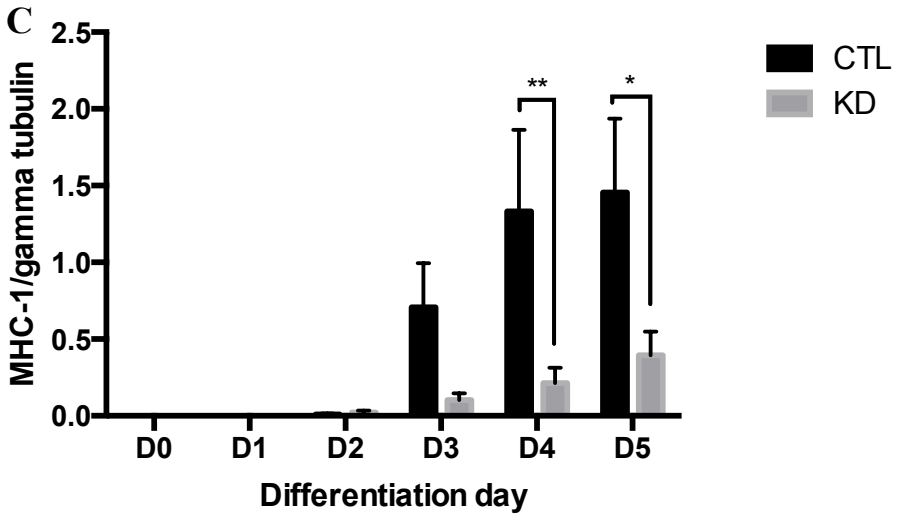
32H
48H
56H
72H
Time of harvest during the "chase" period

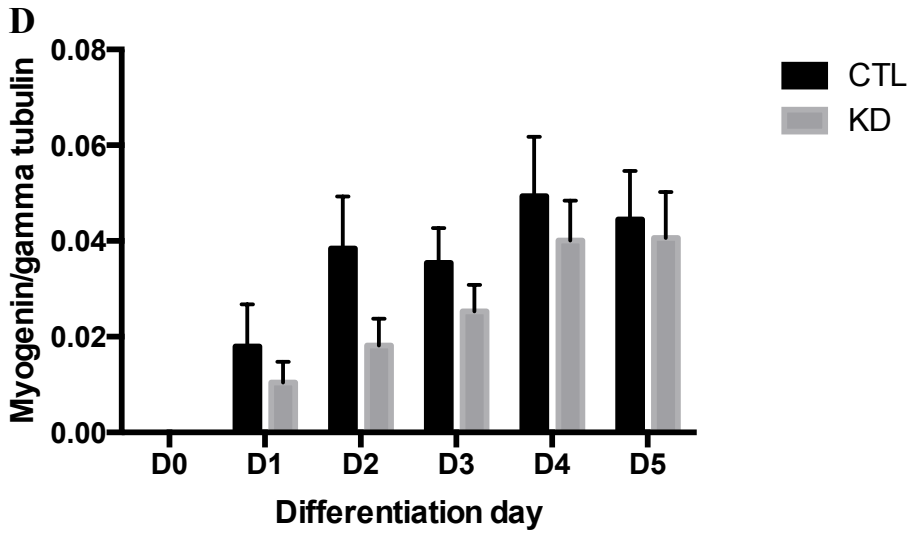
Figure 5. Rate of degradation of PDCD4 measurement by pulse chase assay. Experimental timeline of the pulse chase experiment (A). Cells were seeded at -48 hour point and ^{35}S-methionine/cysteine was given to cells -24 hour point which is 24 hours after seeding cells. Zero hour is the point when cells were harvested following the "pulse" with ^{35}S-methionine/cysteine. Image of the PDCD4-associated radioactivity of samples obtained by immunoprecipitation (IP) (B). Radioactive signals in IP samples were quantified at the end of "pulse" (0H) and at different times during the "chase" period (8H, 24H, 32H, 48H, 56H and 72H). Signals were expressed as a fraction of 0H values (C). Means ± SEM; n=3 independent experiments, bars with different letters differ ($p < 0.05$) IP 0H= sample of immunoprecipitation at 0 hour point.

Fig 6.

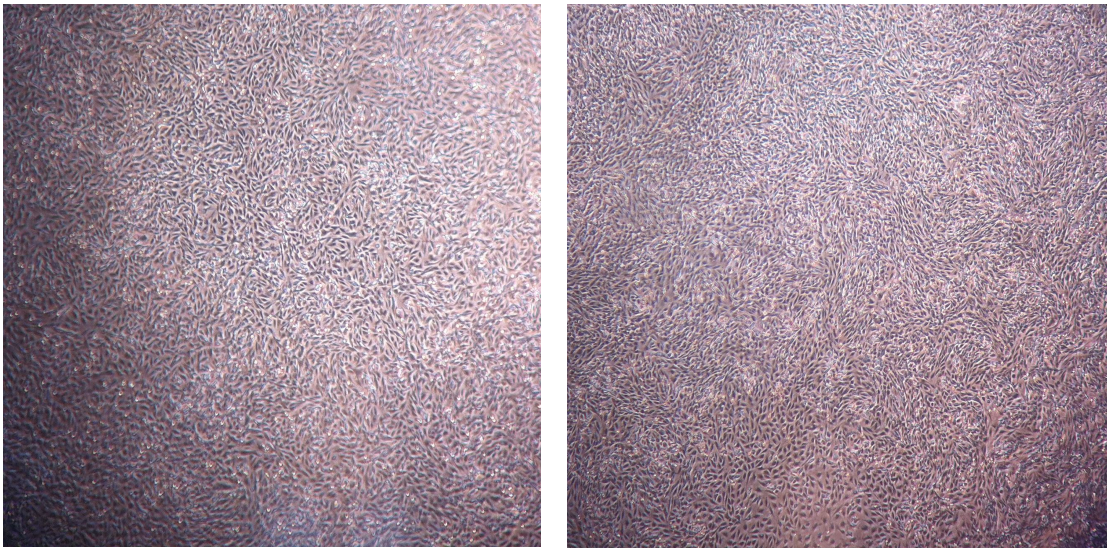

A
PDCD4
MHC-1
myogenin
γ -tubulin
75 kDa
250 kDa
25 kDa
50 kDa
Differentiation Day
S KD
D0
D1
D2
D3
D4
D5


B
Control
PDCD4-depleted
PDCD4/gamma tubulin
Differentiation day
a
b
a,b
a,b
a,b
a,b
a
a,b
a
a
a,b
b
D0
D1
D2
D3
D4
D5

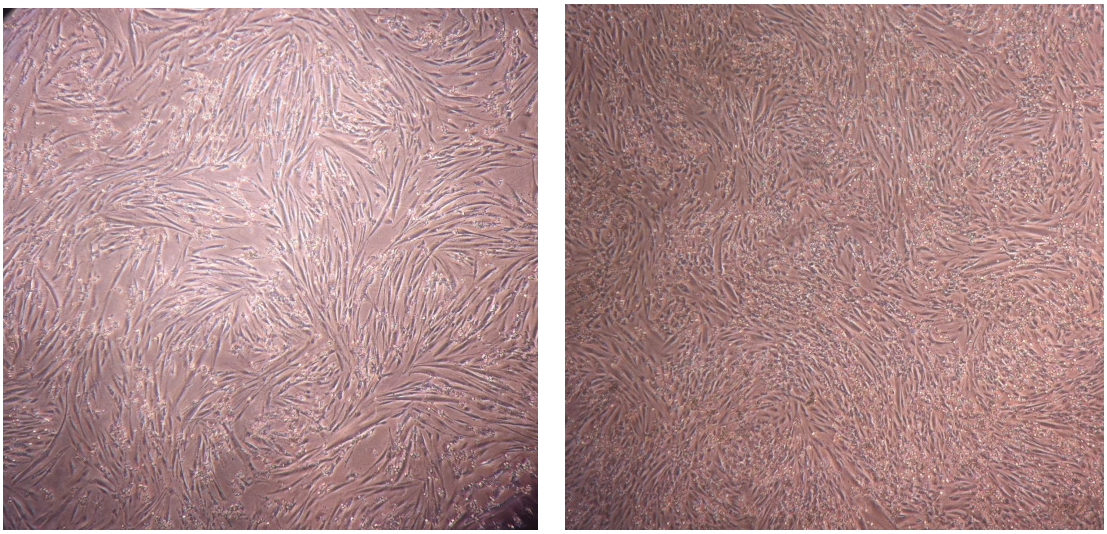

C
MHC-1/gamma tubulin
2.5
2.0
1.5
1.0
0.5
0.0
**
*
D0
D1
D2
D3
D4
D5
Differentiation day
CTL
KD


D
Myogenin/gamma tubulin
0.08
0.06
0.04
0.02
0.00
D0
D1
D2
D3
D4
D5
Differentiation day
CTL
KD

E



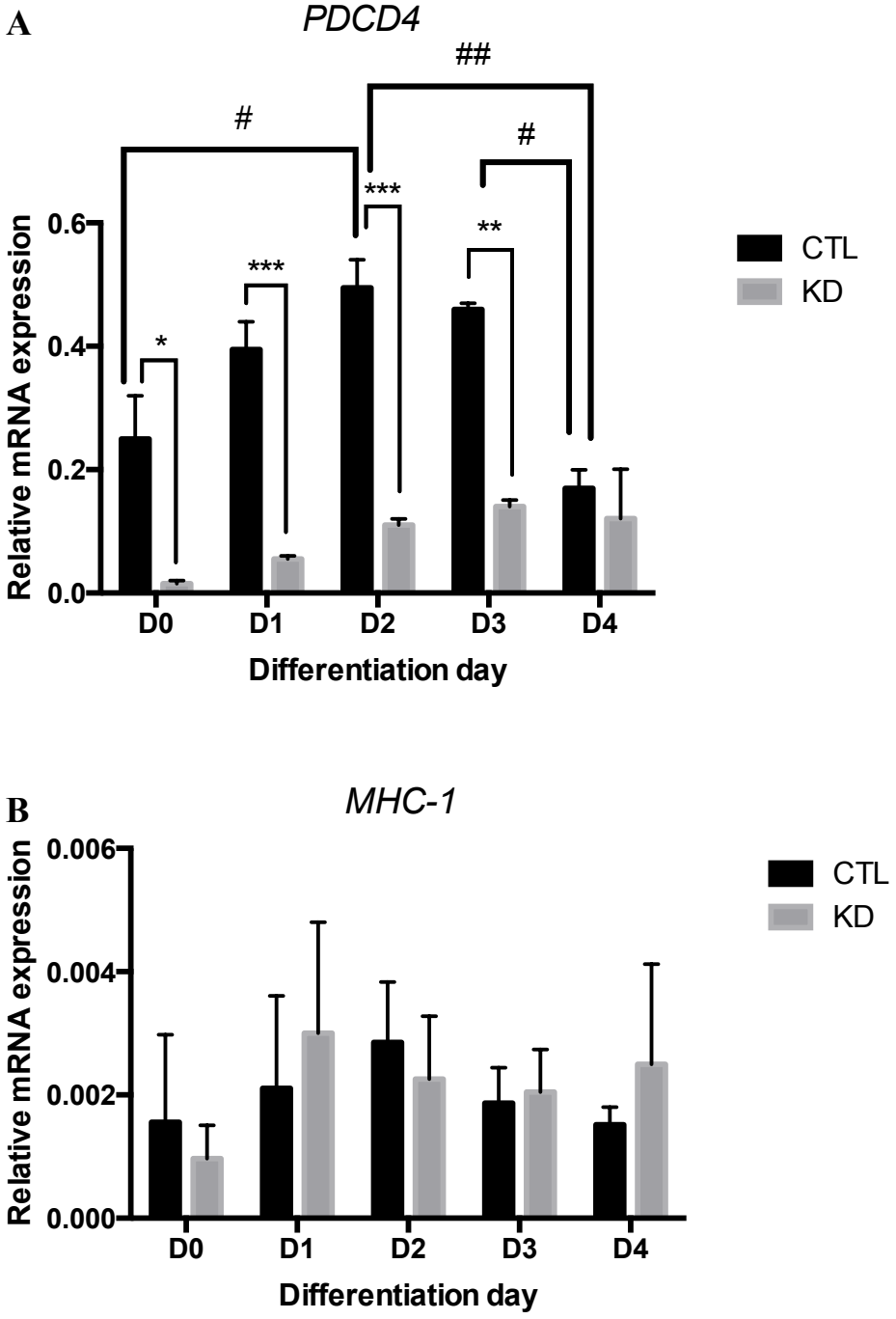
CTL D0 PDCD4-siRNA D0



CTL D4 PDCD4-siRNA D4

Figure 6. Effects of PDCD4 knockdown on L6 myoblast differentiation. PDCD4 was effectively knocked down (A, B). Cells treated with CTL or PDCD4 siRNA oligonucleotide were harvested and blotted for MHC-1 (A, C) and myogenin (A, D). Images of cells treated with CTL or PDCD4 siRNA oligonucleotide Day 0 and Day 4. Cell confluency was similar after 48 hours of siRNA treatment (D0 image, E). The western blot showed the myotube formation was impaired in PDCD4-depleted-cells ($p<0.05$). Means ± SEM; n=3 independent experiments, bars with different letters differ.

Fig 7.


A
PDCD4
##
#
#
***
**
***
*
0.6
0.4
0.2
0.0
Relative mRNA expression
D0
D1
D2
D3
D4
Differentiation day
CTL
KD
B
MHC-1
0.006
0.004
0.002
0.000
Relative mRNA expression
D0
D1
D2
D3
D4
Differentiation day
CTL
KD

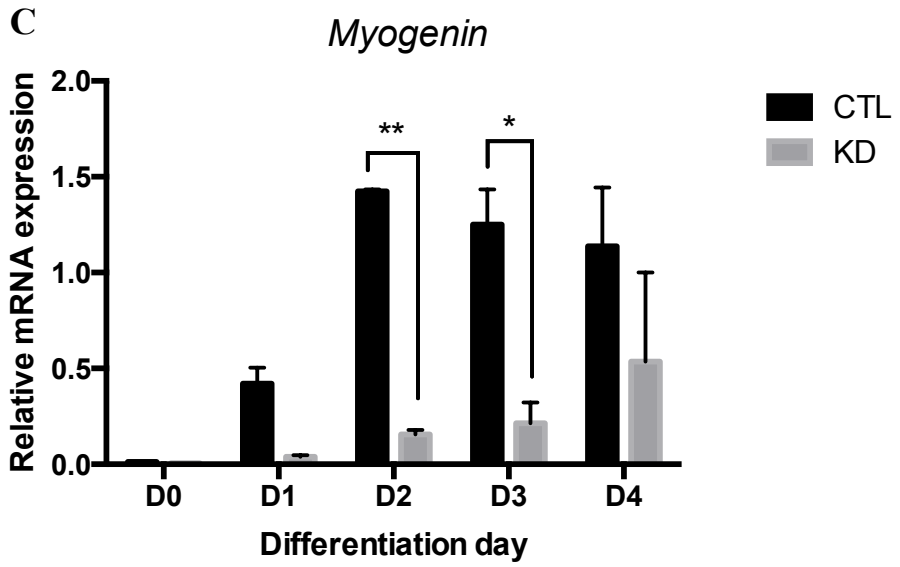
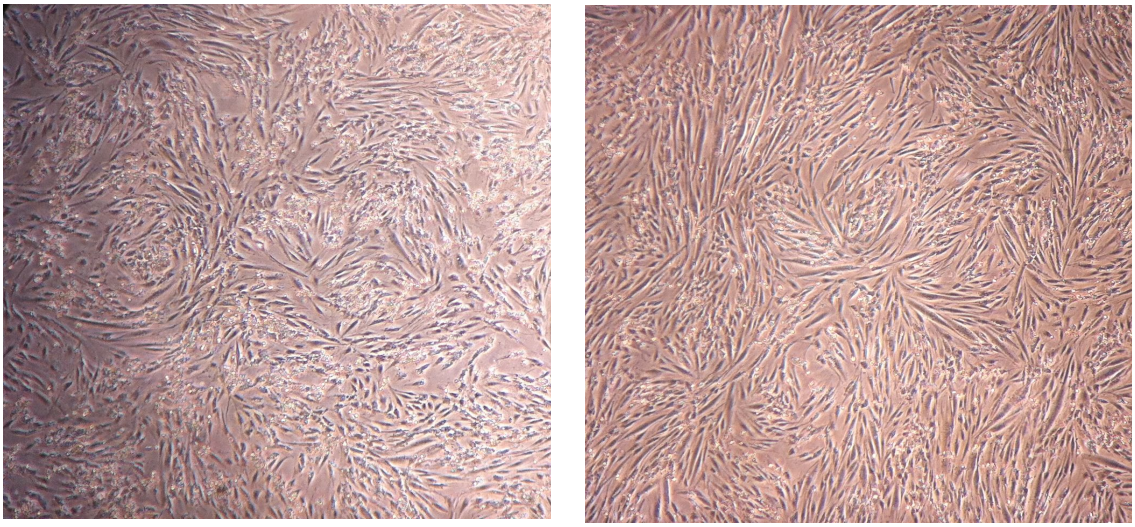


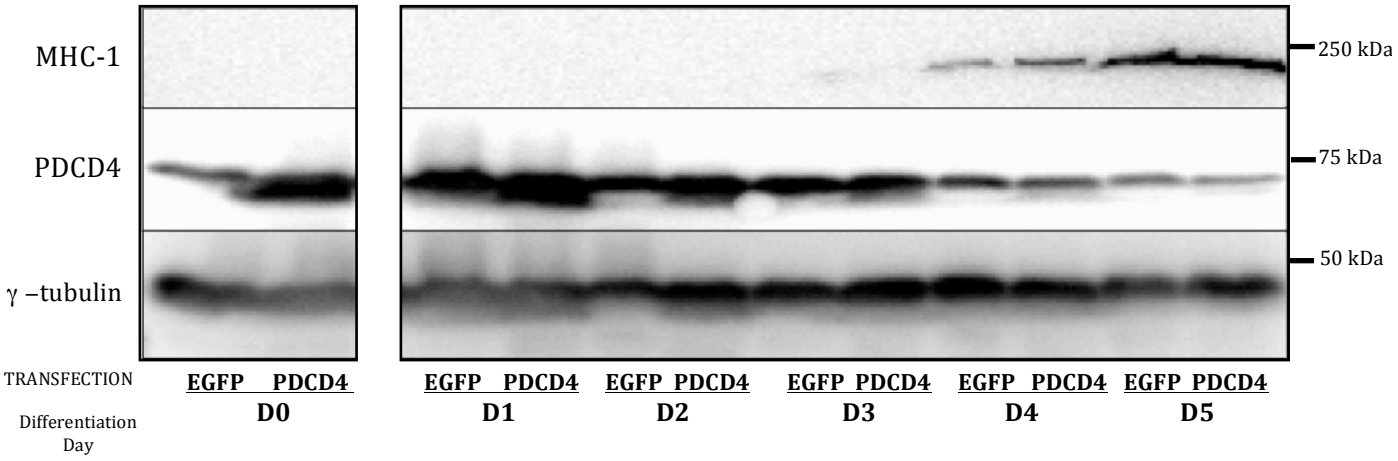
Figure 7. mRNA expression of PDCD4 and myofibrillar proteins in control and PDCD4-depleted-cells. Cells were treated with control (CTL) or PDCD4 siRNA oligonucleotide (KD) in L6 cells and harvested D0 (48 hours after siRNA treatment) to D4. RNA was isolated from harvested cells and subjected to relative quantitative PCR (qPCR). mRNA data are expressed relative to mRNA level of house-keep gene (HPRT). PDCD4 mRNA levels on any day points except day 4 were significantly higher than in control cells ($p<0.05$) (A). MHC-1 mRNA levels were shown in (B). Myogenin mRNA levels on all day points were higher in control cells. It was significantly higher in D2 and D3 ($p<0.05$) (C). Means ± SEM; n=2 independent experiments, * $p<0.05$, **<0.01, ***<0.001, # $p<0.05$ and ##<0.01.

Fig 8.

A. EGFP on Day 4 EGFP- PDCD4 on Day 4



B



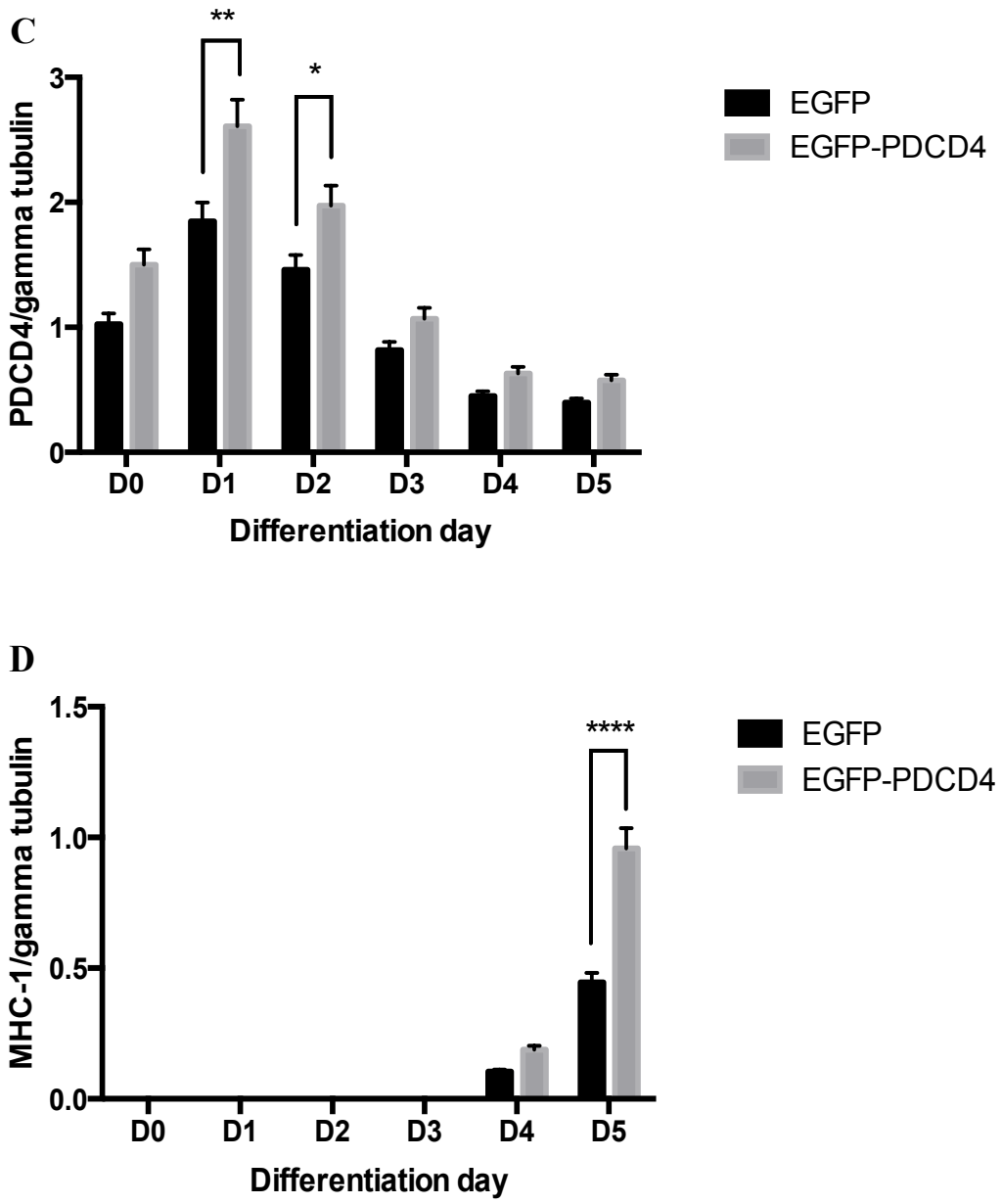


Figure 8. Effect of overexpression of PDCD4 on myotube formation. L6 cells were transfected with EGFP or EGFP- PDCD4 plasmid. Images were captured on Day 4, and the myotube formation was observed in both cells (A). Western blot images of control (EGFP) and overexpressed PDCD4 (PDCD4) are shown (B). PDCD4 expression (A, C) and MHC-1 (A, D) were quantified by using gamma tubulin as a loading control ($P< 0.05$). Means ± SEM; * is $p<0.05$, **<0.01 and ****<0.0001.

REFERENCES

1. **Jacobs BL, Goodman CA, Hornberger TA.** The mechanical activation of mTOR signaling: an emerging role for late endosome/lysosomal targeting. Journal of muscle research and cell motility. 2014;35(1):11-21.
2. **Chakkalakal JV, Jones KM, Basson MA, Brack AS.** The aged niche disrupts muscle stem cell quiescence. Nature. 2012;490(7420):355-60.
3. **Kadi F, Charifi N, Denis C, Lexell J, Andersen JL, Schjerling P, et al.** The behaviour of satellite cells in response to exercise: what have we learned from human studies? Pflugers Archiv : European journal of physiology. 2005;451(2):319-27.
4. **Hochreiter-Hufford AE, Lee CS, Kinchen JM, Sokolowski JD, Arandjelovic S, Call JA, et al.** Phosphatidylserine receptor BAI1 and apoptotic cells as new promoters of myoblast fusion. Nature. 2013;497(7448):263-7.
5. **Nakashima M, Hamajima H, Xia J, Iwane S, Kwaguchi Y, Eguchi Y, et al.** Regulation of tumor suppressor PDCD4 by novel protein kinase C isoforms. Biochimica et biophysica acta. 2010;1803(9):1020-7.
6. **Brooks NE, Myburgh KH.** Skeletal muscle wasting with disuse atrophy is multi-dimensional: the response and interaction of myonuclei, satellite cells and signaling pathways. Frontiers in physiology. 2014;5:99.
7. **Sakuma K, Aoi W, Yamaguchi A.** Current understanding of sarcopenia: possible candidates modulating muscle mass. Pflugers Archiv : European journal of physiology. 2015;467(2):213-29.

8. **Bentzinger CF, Wang YX, Rudnicki MA.** Building muscle: molecular regulation of myogenesis. Cold Spring Harbor perspectives in biology. 2012;4(2).

9. **Phillips SM, Tipton KD, Aarsland A, Wolf SE, Wolfe RR.** Mixed muscle protein synthesis and breakdown after resistance exercise in humans. The American journal of physiology. 1997;273(1 Pt 1):E99-107.

10. **Adegoke OA, Abdullahi A, Tavajohi-Fini P.** mTORC1 and the regulation of skeletal muscle anabolism and mass. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2012;37(3):395-406.

11. **Fuster G, Busquets S, Almendro V, Lopez-Soriano FJ, Argiles JM.** Antiproteolytic effects of plasma from hibernating bears: a new approach for muscle wasting therapy? Clinical nutrition. 2007;26(5):658-61.

12. **Roy RR, Baldwin KM, Edgerton VR.** Response of the neuromuscular unit to spaceflight: what has been learned from the rat model. Exercise and sport sciences reviews. 1996;24:399-425.

13. **Rittweger J, Frost HM, Schiessl H, Ohshima H, Alkner B, Tesch P, et al.** Muscle atrophy and bone loss after 90 days' bed rest and the effects of flywheel resistive exercise and pamidronate: results from the LTBR study. Bone. 2005;36(6):1019-29.

14. **Fitts RH, Riley DR, Widrick JJ.** Functional and structural adaptations of skeletal muscle to microgravity. The Journal of experimental biology. 2001;204(Pt 18):3201-8.

15. **Harlow HJ, Lohuis T, Beck TD, Iaizzo PA.** Muscle strength in overwintering bears. Nature. 2001;409(6823):997.

16. **Garber CE, Blissmer B, Deschenes MR, Franklin BA, Lamonte MJ, Lee IM, et al.** American College of Sports Medicine position stand. Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: guidance for prescribing exercise. Medicine and science in sports and exercise. 2011;43(7):1334-59.

17. **Koopman R, Saris WH, Wagenmakers AJ, van Loon LJ.** Nutritional interventions to promote post-exercise muscle protein synthesis. Sports medicine. 2007;37(10):895-906.

18. **Short KR, Vittone JL, Bigelow ML, Proctor DN, Nair KS.** Age and aerobic exercise training effects on whole body and muscle protein metabolism. American journal of physiology Endocrinology and metabolism. 2004;286(1):E92-101.

19. **Stiegler P, Cunliffe A.** The role of diet and exercise for the maintenance of fat-free mass and resting metabolic rate during weight loss. Sports medicine. 2006;36(3):239-62.

20. **Muller MJ, Bosy-Westphal A, Kutzner D, Heller M.** Metabolically active components of fat-free mass and resting energy expenditure in humans: recent lessons from imaging technologies. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2002;3(2):113-22.

21. **Muller MJ, Bosy-Westphal A, Kutzner D, Heller M.** Metabolically active components of fat free mass (FFM) and resting energy expenditure (REE) in humans. Forum of nutrition. 2003;56:301-3.

22. **Keys A, Taylor HL, Grande F.** Basal metabolism and age of adult man. Metabolism: clinical and experimental. 1973;22(4):579-87.

23. **Summermatter S, Handschin C.** PGC-1alpha and exercise in the control of body weight. International journal of obesity. 2012;36(11):1428-35.

24. **Speakman JR.** Thrifty genes for obesity and the metabolic syndrome--time to call off the search? Diabetes & vascular disease research : official journal of the International Society of Diabetes and Vascular Disease. 2006;3(1):7-11.

25. **van Dam RM, Seidell JC.** Carbohydrate intake and obesity. European journal of clinical nutrition. 2007;61 Suppl 1:S75-99.

26. **Guarente L, Picard F.** Calorie restriction--the SIR2 connection. Cell. 2005;120(4):473-82.

27. **Fontana L, Partridge L, Longo VD.** Extending healthy life span--from yeast to humans. Science. 2010;328(5976):321-6.

28. **Evans WJ**. Protein nutrition, exercise and aging. Journal of the American College of Nutrition. 2004;23(6 Suppl):601S-9S.

29. **Anderson SR, Gilge DA, Steiber AL, Previs SF.** Diet-induced obesity alters protein synthesis: tissue-specific effects in fasted versus fed mice. Metabolism: clinical and experimental. 2008;57(3):347-54.

30. **Tardif N, Salles J, Guillet C, Tordjman J, Reggio S, Landrier JF, et al.** Muscle ectopic fat deposition contributes to anabolic resistance in obese

sarcopenic old rats through eIF2alpha activation. Aging cell. 2014;13(6):1001-11.

31. **Welle S, Thornton C, Statt M.** Myofibrillar protein synthesis in young and old human subjects after three months of resistance training. The American journal of physiology. 1995;268(3 Pt 1):E422-7.

32. **Volpi E, Mittendorfer B, Rasmussen BB, Wolfe RR.** The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly. The Journal of clinical endocrinology and metabolism. 2000;85(12):4481-90.

33. **Blottner D, Bosutti A, Degens H, Schiffl G, Gutsmann M, Buehlmeier J, et al.** Whey protein plus bicarbonate supplement has little effects on structural atrophy and proteolysis marker immunopatterns in skeletal muscle disuse during 21 days of bed rest. Journal of musculoskeletal & neuronal interactions. 2014;14(4):432-44.

34. **Ham DJ, Caldow MK, Lynch GS, Koopman R.** Leucine as a treatment for muscle wasting: a critical review. Clinical nutrition. 2014;33(6):937-45.

35. **Stein TP, Blanc S.** Does protein supplementation prevent muscle disuse atrophy and loss of strength? Critical reviews in food science and nutrition. 2011;51(9):828-34.

36. **Accurso A, Bernstein RK, Dahlqvist A, Draznin B, Feinman RD, Fine EJ, et al.** Dietary carbohydrate restriction in type 2 diabetes mellitus and metabolic syndrome: time for a critical appraisal. Nutrition & metabolism. 2008;5:9.

37. **Feinman RD, Pogozelski WK, Astrup A, Bernstein RK, Fine EJ, Westman EC, et al.** Dietary carbohydrate restriction as the first approach in diabetes management: critical review and evidence base. Nutrition. 2015;31(1):1-13.

38. **Volek JS, Fernandez ML, Feinman RD, Phinney SD.** Dietary carbohydrate restriction induces a unique metabolic state positively affecting atherogenic dyslipidemia, fatty acid partitioning, and metabolic syndrome. Progress in lipid research. 2008;47(5):307-18.

39. **Westman EC, Vernon MC.** Has carbohydrate-restriction been forgotten as a treatment for diabetes mellitus? A perspective on the ACCORD study design. Nutrition & metabolism. 2008;5:10.

40. **Feinman RD.** Fad diets in the treatment of diabetes. Current diabetes reports. 2011;11(2):128-35.

41. **Bernstein AM, Treyzon L, Li Z.** Are high-protein, vegetable-based diets safe for kidney function? A review of the literature. Journal of the American Dietetic Association. 2007;107(4):644-50.

42. **Metges CC, Barth CA.** Metabolic consequences of a high dietary-protein intake in adulthood: assessment of the available evidence. The Journal of nutrition. 2000;130(4):886-9.

43. **Brinkworth GD, Noakes M, Keogh JB, Luscombe ND, Wittert GA, Clifton PM.** Long-term effects of a high-protein, low-carbohydrate diet on weight control and cardiovascular risk markers in obese hyperinsulinemic subjects. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2004;28(5):661-70.

44. **Luscombe ND, Clifton PM, Noakes M, Farnsworth E, Wittert G.** Effect of a high-protein, energy-restricted diet on weight loss and energy expenditure after weight stabilization in hyperinsulinemic subjects. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2003;27(5):582-90.

45. **Huang JH, Cheng FC, Tsai LC, Lee NY, Lu YF.** Appropriate physical activity and dietary intake achieve optimal metabolic control in older type 2 diabetes patients. Journal of diabetes investigation. 2014;5(4):418-27.

46. **Figueiredo VC, Cameron-Smith D.** Is carbohydrate needed to further stimulate muscle protein synthesis/hypertrophy following resistance exercise? Journal of the International Society of Sports Nutrition. 2013;10(1):42.

47. **Stark M, Lukaszuk J, Prawitz A, Salacinski A.** Protein timing and its effects on muscular hypertrophy and strength in individuals engaged in weight-training. Journal of the International Society of Sports Nutrition. 2012;9(1):54.

48. **Borsheim E, Cree MG, Tipton KD, Elliott TA, Aarsland A, Wolfe RR.** Effect of carbohydrate intake on net muscle protein synthesis during recovery from resistance exercise. Journal of applied physiology. 2004;96(2):674-8.

49. **Byfield MP, Murray JT, Backer JM.** hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. The Journal of biological chemistry. 2005;280(38):33076-82.

50. **Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, et al.** Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(40):14238-43.

51. **Brooks GA, Mercier J.** Balance of carbohydrate and lipid utilization during exercise: the "crossover" concept. Journal of applied physiology. 1994;76(6):2253-61.

52. **Coyle EF.** Substrate utilization during exercise in active people. The American journal of clinical nutrition. 1995;61(4 Suppl):968S-79S.

53. **Tesch PA, Colliander EB, Kaiser P**. Muscle metabolism during intense, heavy-resistance exercise. European journal of applied physiology and occupational physiology. 1986;55(4):362-6.

54. **Lambert CP, Flynn MG.** Fatigue during high-intensity intermittent exercise: application to bodybuilding. Sports medicine. 2002;32(8):511-22.

55. **Haff GG, Lehmkuhl MJ, McCoy LB, Stone MH.** Carbohydrate supplementation and resistance training. Journal of strength and conditioning research / National Strength & Conditioning Association. 2003;17(1):187-96.

56. **Aguirre N, van Loon LJ, Baar K.** The role of amino acids in skeletal muscle adaptation to exercise. Nestle Nutrition Institute workshop series. 2013;76:85-102.

57. **Chesley A, MacDougall JD, Tarnopolsky MA, Atkinson SA, Smith K.** Changes in human muscle protein synthesis after resistance exercise. Journal of applied physiology. 1992;73(4):1383-8.

58. **MacDougall JD, Gibala MJ, Tarnopolsky MA, MacDonald JR, Interisano SA, Yarasheski KE.** The time course for elevated muscle protein synthesis following heavy resistance exercise. Canadian journal of applied physiology = Revue canadienne de physiologie appliquee. 1995;20(4):480-6.

59. **Tipton KD, Wolfe RR.** Exercise, protein metabolism, and muscle growth. International journal of sport nutrition and exercise metabolism. 2001;11(1):109-32.

60. **Esmarck B, Andersen JL, Olsen S, Richter EA, Mizuno M, Kjaer M.** Timing of postexercise protein intake is important for muscle hypertrophy with resistance training in elderly humans. The Journal of physiology. 2001;535(Pt 1):301-11.

61. **Elliot TA, Cree MG, Sanford AP, Wolfe RR, Tipton KD.** Milk ingestion stimulates net muscle protein synthesis following resistance exercise. Medicine and science in sports and exercise. 2006;38(4):667-74.

62. **Hartman JW, Tang JE, Wilkinson SB, Tarnopolsky MA, Lawrence RL, Fullerton AV, et al.** Consumption of fat-free fluid milk after resistance exercise promotes greater lean mass accretion than does consumption of soy or carbohydrate in young, novice, male weightlifters. The American journal of clinical nutrition. 2007;86(2):373-81.

63. **Rankin JW, Goldman LP, Puglisi MJ, Nickols-Richardson SM, Earthman CP, Gwazdauskas FC.** Effect of post-exercise supplement consumption on adaptations to resistance training. Journal of the American College of Nutrition. 2004;23(4):322-30.

64. **Wilkinson SB, Tarnopolsky MA, Macdonald MJ, Macdonald JR, Armstrong D, Phillips SM.** Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. The American journal of clinical nutrition. 2007;85(4):1031-40.

65. **Dillin A, Gottschling DE, Nystrom T.** The good and the bad of being connected: the integrons of aging. Current opinion in cell biology. 2014;26:107-12.

66. **Fontana L, Klein S.** Aging, adiposity, and calorie restriction. Jama. 2007;297(9):986-94.

67. **Fontana L, Weiss EP, Villareal DT, Klein S, Holloszy JO.** Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. Aging cell. 2008;7(5):681-7.

68. **Johnson SC, Rabinovitch PS, Kaeberlein M.** mTOR is a key modulator of ageing and age-related disease. Nature. 2013;493(7432):338-45.

69. **Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M, Ristow M.** Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. Cell metabolism. 2007;6(4):280-93.

70. **Roche AF.** The significance of sarcopenia in relation to health. Asia Pacific j ournal of clinical nutrition. 1995;4(1):129-32.

71. **Cohen S, Nathan JA, Goldberg AL.** Muscle wasting in disease: molecular mechanisms and promising therapies. Nature reviews Drug discovery. 2015;14(1):58-74.

72. **Lexell J.** Human aging, muscle mass, and fiber type composition. The journals of gerontology Series A, Biological sciences and medical sciences. 1995;50 Spec No:11-6.

73. **Roubenoff R.** Sarcopenia: effects on body composition and function. The journals of gerontology Series A, Biological sciences and medical sciences. 2003;58(11):1012-7.

74. **Gensler HL, Bernstein H.** DNA damage as the primary cause of aging. The Quarterly review of biology. 1981;56(3):279-303.

75. **Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, et al.** Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. Science. 2009;326(5949):140-4.

76. **Shevah O, Laron Z.** Patients with congenital deficiency of IGF-I seem protected from the development of malignancies: a preliminary report. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society. 2007;17(1):54-7.

77. **Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloen A, Even PC, et al.** IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature. 2003;421(6919):182-7.

78. **Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J.** Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science. 2001;294(5548):1942-5.

79. **Hay N, Sonenberg N.** Upstream and downstream of mTOR. Genes & development. 2004;18(16):1926-45.

80. **Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, et al.** mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell. 2002;110(2):163-75.

81. **Wullschleger S, Loewith R, Hall MN.** TOR signaling in growth and metabolism. Cell. 2006;124(3):471-84.

82. **Kim DH, Sarbassov DD, Ali SM, Latek RR, Guntur KV, Erdjument-Bromage H, et al.** GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Molecular cell. 2003;11(4):895-904.

83. **Oldham S, Montagne J, Radimerski T, Thomas G, Hafen E.** Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. Genes & development. 2000;14(21):2689-94.

84. Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell. 1994;78(1):35-43.

85. **Zhang H, Stallock JP, Ng JC, Reinhard C, Neufeld TP.** Regulation of cellular growth by the Drosophila target of rapamycin dTOR. Genes & development. 2000;14(21):2712-24.

86. **Li J, Kim SG, Blenis J.** Rapamycin: one drug, many effects. Cell metabolism. 2014;19(3):373-9.

87. **Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al.** Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009;460(7253):392-5.

88. **Shiota C, Woo JT, Lindner J, Shelton KD, Magnuson MA.** Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. Developmental cell. 2006;11(4):583-9.

89. **Bai X, Ma D, Liu A, Shen X, Wang QJ, Liu Y, et al.** Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38. Science. 2007;318(5852):977-80.

90. **Fingar DC, Blenis J.** Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene. 2004;23(18):3151-71.

91. **Huang J, Manning BD.** A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochemical Society transactions. 2009;37(Pt 1):217-22.

92. **Oshiro N, Takahashi R, Yoshino K, Tanimura K, Nakashima A, Eguchi S, et al.** The proline-rich Akt substrate of 40 kDa (PRAS40) is a physiological

substrate of mammalian target of rapamycin complex 1. The Journal of biological chemistry. 2007;282(28):20329-39.

93. **Wiza C, Herzfeld de Wiza D, Nascimento EB, Lehr S, Al-Hasani H, Ouwens DM.** Knockdown of PRAS40 inhibits insulin action via proteasome-mediated degradation of IRS1 in primary human skeletal muscle cells. Diabetologia. 2013;56(5):1118-28.

94. **Wiza C, Nascimento EB, Ouwens DM.** Role of PRAS40 in Akt and mTOR signaling in health and disease. American journal of physiology Endocrinology and metabolism. 2012;302(12):E1453-60.

95. **Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, et al.** Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell. 2002;110(2):177-89.

96. **Yang H, Rudge DG, Koos JD, Vaidialingam B, Yang HJ, Pavletich NP.** mTOR kinase structure, mechanism and regulation. Nature. 2013;497(7448):217-23.

97. **Schalm SS, Fingar DC, Sabatini DM, Blenis J.** TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. Current biology : CB. 2003;13(10):797-806.

98. **Nojima H, Tokunaga C, Eguchi S, Oshiro N, Hidayat S, Yoshino K, et al.** The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. The Journal of biological chemistry. 2003;278(18):15461-4.

99. **Schalm SS, Blenis J.** Identification of a conserved motif required for mTOR signaling. Current biology : CB. 2002;12(8):632-9.

100. **Beugnet A, Wang X, Proud CG.** Target of rapamycin (TOR)-signaling and RAIP motifs play distinct roles in the mammalian TOR-dependent phosphorylation of initiation factor 4E-binding protein 1. The Journal of biological chemistry. 2003;278(42):40717-22.

101. **Gao D, Inuzuka H, Tan MK, Fukushima H, Locasale JW, Liu P, et al.** mTOR drives its own activation via SCF(betaTrCP)-dependent degradation of the mTOR inhibitor DEPTOR. Molecular cell. 2011;44(2):290-303.

102. **Inoki K, Zhu T, Guan KL.** TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003;115(5):577-90.

103. **Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al.** AMPK phosphorylation of raptor mediates a metabolic checkpoint. Molecular cell. 2008;30(2):214-26.

104. **Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, et al.** Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Developmental cell. 2006;11(6):859-71.

105. **Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, et al.** DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell. 2009;137(5):873-86.

106. **Duan S, Skaar JR, Kuchay S, Toschi A, Kanarek N, Ben-Neriah Y, et al.** mTOR generates an auto-amplification loop by triggering the betaTrCP- and

CK1alpha-dependent degradation of DEPTOR. Molecular cell. 2011;44(2):317-24.

107. **Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, et al.** PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Molecular cell. 2007;25(6):903-15.

108. **Fonseca BD, Smith EM, Lee VH, MacKintosh C, Proud CG.** PRAS40 is a target for mammalian target of rapamycin complex 1 and is required for signaling downstream of this complex. The Journal of biological chemistry. 2007;282(34):24514-24.

109. **Wang L, Harris TE, Roth RA, Lawrence JC, Jr.** PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. The Journal of biological chemistry. 2007;282(27):20036-44.

110. **Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH.** Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nature cell biology. 2007;9(3):316-23.

111. **Nascimento EB, Snel M, Guigas B, van der Zon GC, Kriek J, Maassen JA, et al.** Phosphorylation of PRAS40 on Thr246 by PKB/AKT facilitates efficient phosphorylation of Ser183 by mTORC1. Cellular signalling. 2010;22(6):961-7.

112. **Schlessinger J.** Cell signaling by receptor tyrosine kinases. Cell. 2000;103(2):211-25.

113. **Zwick E, Bange J, Ullrich A.** Receptor tyrosine kinase signalling as a target for cancer intervention strategies. Endocrine-related cancer. 2001;8(3):161-73.

114. **Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, et al.** Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science. 1998;279(5351):710-4.

115. **Sarbassov DD, Guertin DA, Ali SM, Sabatini DM.** Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005;307(5712):1098-101.

116. **Sengupta S, Peterson TR, Sabatini DM.** Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Molecular cell. 2010;40(2):310-22.

117. **Inoki K, Li Y, Xu T, Guan KL.** Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes & development. 2003;17(15):1829-34.

118. **Bonfils G, Jaquenoud M, Bontron S, Ostrowicz C, Ungermann C, De Virgilio C.** Leucyl-tRNA synthetase controls TORC1 via the EGO complex. Molecular cell. 2012;46(1):105-10.

119. **Han JM, Jeong SJ, Park MC, Kim G, Kwon NH, Kim HK, et al.** Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. Cell. 2012;149(2):410-24.

120. **Avruch J, Long X, Ortiz-Vega S, Rapley J, Papageorgiou A, Dai N.** Amino acid regulation of TOR complex 1. American journal of physiology Endocrinology and metabolism. 2009;296(4):E592-602.

121. **Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J.** Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. The Journal of biological chemistry. 1998;273(23):14484-94.

122. **Smith EM, Finn SG, Tee AR, Browne GJ, Proud CG.** The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. The Journal of biological chemistry. 2005;280(19):18717-27.

123. **Heinrichs A.** Cell signalling: Of Rags and Ragulator. Nature reviews Molecular cell biology. 2010;11(6):388.

124. **Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM.** Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell. 2010;141(2):290-303.

125. **Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, et al.** The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science. 2008;320(5882):1496-501.

126. **Gulati P, Gaspers LD, Dann SG, Joaquin M, Nobukuni T, Natt F, et al.** Amino acids activate mTOR complex 1 via Ca2+/CaM signaling to hVps34. Cell metabolism. 2008;7(5):456-65.

127. **Findlay GM, Yan L, Procter J, Mieulet V, Lamb RF.** A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. The Biochemical journal. 2007;403(1):13-20.

128. **Suzuki T, Inoki K.** Spatial regulation of the mTORC1 system in amino acids sensing pathway. Acta biochimica et biophysica Sinica. 2011;43(9):671-9.

129. **Bolster DR, Crozier SJ, Kimball SR, Jefferson LS**. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. The Journal of biological chemistry. 2002;277(27):23977-80.

130. **Corton JM, Gillespie JG, Hawley SA, Hardie DG.** 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? European journal of biochemistry / FEBS. 1995;229(2):558-65.

131. **Laplante M, Sabatini DM.** mTOR signaling in growth control and disease. Cell. 2012;149(2):274-93.

132. **Pende M, Um SH, Mieulet V, Sticker M, Goss VL, Mestan J, et al.** S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Molecular and cellular biology. 2004;24(8):3112-24.

133. **Pullen N, Dennis PB, Andjelkovic M, Dufner A, Kozma SC, Hemmings BA, et al.** Phosphorylation and activation of p70s6k by PDK1. Science. 1998;279(5351):707-10.

134. **Alessi DR, Kozlowski MT, Weng QP, Morrice N, Avruch J.** 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. Current biology : CB. 1998;8(2):69-81.

135. **Stolovich M, Tang H, Hornstein E, Levy G, Cohen R, Bae SS, et al.** Transduction of growth or mitogenic signals into translational activation of TOP mRNAs is fully reliant on the phosphatidylinositol 3-kinase-mediated pathway but requires neither S6K1 nor rpS6 phosphorylation. Molecular and cellular biology. 2002;22(23):8101-13.

136. **Tang H, Hornstein E, Stolovich M, Levy G, Livingstone M, Templeton D, et al.** Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. Molecular and cellular biology. 2001;21(24):8671-83.

137. **Shahbazian D, Roux PP, Mieulet V, Cohen MS, Raught B, Taunton J, et al.** The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. The EMBO journal. 2006;25(12):2781-91.

138. **Dennis MD, Jefferson LS, Kimball SR.** Role of p70S6K1-mediated phosphorylation of eIF4B and PDCD4 proteins in the regulation of protein synthesis. The Journal of biological chemistry. 2012;287(51):42890-9.

139. **Marcotrigiano J, Gingras AC, Sonenberg N, Burley SK.** Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. Molecular cell. 1999;3(6):707-16.

140. **Morino S, Imataka H, Svitkin YV, Pestova TV, Sonenberg N.** Eukaryotic translation initiation factor 4E (eIF4E) binding site and the middle one-third of eIF4GI constitute the core domain for cap-dependent translation, and the C-terminal one-third functions as a modulatory region. Molecular and cellular biology. 2000;20(2):468-77.

141. **Yang HS, Jansen AP, Komar AA, Zheng X, Merrick WC, Costes S, et al.** The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. Molecular and cellular biology. 2003;23(1):26-37.

142. **Lamphear BJ, Kirchweger R, Skern T, Rhoads RE.** Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. The Journal of biological chemistry. 1995;270(37):21975-83.

143. **Lejbkowicz F, Goyer C, Darveau A, Neron S, Lemieux R, Sonenberg N.** A fraction of the mRNA 5' cap-binding protein, eukaryotic initiation factor 4E, localizes to the nucleus. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(20):9612-6.

144. **Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, et al.** Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes & development. 1999;13(11):1422-37.

145. **Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andrabi K, Weng QP, et al.** Regulation of eIF-4E BP1 phosphorylation by mTOR. The Journal of biological chemistry. 1997;272(42):26457-63.

146. **Linder P, Lasko PF, Ashburner M, Leroy P, Nielsen PJ, Nishi K, et al.** Birth of the D-E-A-D box. Nature. 1989;337(6203):121-2.

147. **Heung LJ, Del Poeta M.** Unlocking the DEAD-box: a key to cryptococcal virulence? The Journal of clinical investigation. 2005;115(3):593-5.

148. **Rogers GW, Jr., Richter NJ, Lima WF, Merrick WC.** Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F. The Journal of biological chemistry. 2001;276(33):30914-22.

149. **Suzuki C, Garces RG, Edmonds KA, Hiller S, Hyberts SG, Marintchev A, et al.** PDCD4 inhibits translation initiation by binding to eIF4A using both its MA3 domains. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(9):3274-9.

150. **Dorrello NV, Peschiaroli A, Guardavaccaro D, Colburn NH, Sherman NE, Pagano M.** S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science. 2006;314(5798):467-71.

151. **Bohm M, Sawicka K, Siebrasse JP, Brehmer-Fastnacht A, Peters R, Klempnauer KH.** The transformation suppressor protein Pdcd4 shuttles between nucleus and cytoplasm and binds RNA. Oncogene. 2003;22(31):4905-10.

152. **Singh P, Wedeken L, Waters LC, Carr MD, Klempnauer KH.** Pdcd4 directly binds the coding region of c-myb mRNA and suppresses its translation. Oncogene. 2011;30(49):4864-73.

153. **Ge C, Song J, Chen L, Wang L, Chen Y, Liu X, et al.** Atheroprotective pulsatile flow induces ubiquitin-proteasome-mediated degradation of programmed cell death 4 in endothelial cells. PloS one. 2014;9(3):e91564.

154. **Yang HS, Cho MH, Zakowicz H, Hegamyer G, Sonenberg N, Colburn NH.** A novel function of the MA-3 domains in transformation and translation suppressor Pdcd4 is essential for its binding to eukaryotic translation initiation factor 4A. Molecular and cellular biology. 2004;24(9):3894-906.

155. **Wedeken L, Singh P, Klempnauer KH.** Tumor suppressor protein Pdcd4 inhibits translation of p53 mRNA. The Journal of biological chemistry. 2011;286(50):42855-62.

156. **Barness LA, Opitz JM, Gilbert-Barness E.** Obesity: genetic, molecular, and environmental aspects. American journal of medical genetics Part A. 2007;143A(24):3016-34.

157. **Parker MH, Seale P, Rudnicki MA.** Looking back to the embryo: defining transcriptional networks in adult myogenesis. Nature reviews Genetics. 2003;4(7):497-507.

158. **Zammit PS, Partridge TA, Yablonka-Reuveni Z.** The skeletal muscle satellite cell: the stem cell that came in from the cold. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2006;54(11):1177-91.

159. **Hawke TJ, Garry DJ.** Myogenic satellite cells: physiology to molecular biology. Journal of applied physiology. 2001;91(2):534-51.

160. **Zammit PS.** All muscle satellite cells are equal, but are some more equal than others? Journal of cell science. 2008;121(Pt 18):2975-82.

161. **Knight JD, Kothary R.** The myogenic kinome: protein kinases critical to mammalian skeletal myogenesis. Skeletal muscle. 2011;1:29.

162. **Averous J, Gabillard JC, Seiliez I, Dardevet D.** Leucine limitation regulates myf5 and myoD expression and inhibits myoblast differentiation. Experimental cell research. 2012;318(3):217-27.

163. **Wyzykowski JC, Winata TI, Mitin N, Taparowsky EJ, Konieczny SF.** Identification of novel MyoD gene targets in proliferating myogenic stem cells. Molecular and cellular biology. 2002;22(17):6199-208.

164. **Erbay E, Chen J.** The mammalian target of rapamycin regulates C2C12 myogenesis via a kinase-independent mechanism. The Journal of biological chemistry. 2001;276(39):36079-82.

165. **Shu L, Zhang X, Houghton PJ.** Myogenic differentiation is dependent on both the kinase function and the N-terminal sequence of mammalian target of rapamycin. The Journal of biological chemistry. 2002;277(19):16726-32.

166. **Shu L, Houghton PJ.** The mTORC2 complex regulates terminal differentiation of C2C12 myoblasts. Molecular and cellular biology. 2009;29(17):4691-700.

167. **Murakami M, Ichisaka T, Maeda M, Oshiro N, Hara K, Edenhofer F, et al.** mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Molecular and cellular biology. 2004;24(15):6710-8.

168. **Gangloff YG, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz JF, et al.** Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. Molecular and cellular biology. 2004;24(21):9508-16.

169. **Fingar DC, Salama S, Tsou C, Harlow E, Blenis J.** Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes & development. 2002;16(12):1472-87.

170. **Park IH, Chen J.** Mammalian target of rapamycin (mTOR) signaling is required for a late-stage fusion process during skeletal myotube maturation. The Journal of biological chemistry. 2005;280(36):32009-17.

171. **Shibahara K, Asano M, Ishida Y, Aoki T, Koike T, Honjo T.** Isolation of a novel mouse gene MA-3 that is induced upon programmed cell death. Gene. 1995;166(2):297-301.

172. **Lankat-Buttgereit B, Goke R.** The tumour suppressor Pdcd4: recent advances in the elucidation of function and regulation. Biology of the cell / under the auspices of the European Cell Biology Organization. 2009;101(6):309-17.

173. **Soejima H, Miyoshi O, Yoshinaga H, Masaki Z, Ozaki I, Kajiwara S, et al.** Assignment of the programmed cell death 4 gene (PDCD4) to human

chromosome band 10q24 by in situ hybridization. Cytogenetics and cell genetics. 1999;87(1-2):113-4.

174. **Lankat-Buttgereit B, Lenschen B, Schmidt H, Goke R.** The action of Pdcd4 may be cell type specific: evidence that reduction of dUTPase levels might contribute to its tumor suppressor activity in Bon-1 cells. Apoptosis : an international journal on programmed cell death. 2008;13(1):157-64.

175. **Onishi Y, Hashimoto S, Kizaki H.** Cloning of the TIS gene suppressed by topoisomerase inhibitors. Gene. 1998;215(2):453-9.

176. **Goke A, Goke R, Knolle A, Trusheim H, Schmidt H, Wilmen A, et al.** DUG is a novel homologue of translation initiation factor 4G that binds eIF4A. Biochemical and biophysical research communications. 2002;297(1):78-82.

177. **Lankat-Buttgereit B, Muller S, Schmidt H, Parhofer KG, Gress TM, Goke R.** Knockdown of Pdcd4 results in induction of proprotein convertase 1/3 and potent secretion of chromogranin A and secretogranin II in a neuroendocrine cell line. Biology of the cell / under the auspices of the European Cell Biology Organization. 2008;100(12):703-15.

178. **Matsuhashi S, Hamajima H, Xia J, Zhang H, Mizuta T, Anzai K, et al.** Control of a tumor suppressor PDCD4: Degradation mechanisms of the protein in hepatocellular carcinoma cells. Cellular signalling. 2014;26(3):603-10.

179. **Yoshinaga H, Matsuhashi S, Fujiyama C, Masaki Z.** Novel human PDCD4 (H731) gene expressed in proliferative cells is expressed in the small duct

epithelial cells of the breast as revealed by an anti-H731 antibody. Pathology international. 1999;49(12):1067-77.

180. **Palamarchuk A, Efanov A, Maximov V, Aqeilan RI, Croce CM, Pekarsky Y.** Akt phosphorylates and regulates Pdcd4 tumor suppressor protein. Cancer research. 2005;65(24):11282-6.

181. **Matsuhashi S, Narisawa Y, Ozaki I, Mizuta T.** Expression patterns of programmed cell death 4 protein in normal human skin and some representative skin lesions. Experimental dermatology. 2007;16(3):179-84.

182. **Mudduluru G, Medved F, Grobholz R, Jost C, Gruber A, Leupold JH, et al.** Loss of programmed cell death 4 expression marks adenoma-carcinoma transition, correlates inversely with phosphorylated protein kinase B, and is an independent prognostic factor in resected colorectal cancer. Cancer. 2007;110(8):1697-707.

183. **Wen YH, Shi X, Chiriboga L, Matsahashi S, Yee H, Afonja O.** Alterations in the expression of PDCD4 in ductal carcinoma of the breast. Oncology reports. 2007;18(6):1387-93.

184. **Goke R, Barth P, Schmidt A, Samans B, Lankat-Buttgereit B.** Programmed cell death protein 4 suppresses CDK1/cdc2 via induction of p21(Waf1/Cip1). American journal of physiology Cell physiology. 2004;287(6):C1541-6.

185. **Walsh K, Perlman H.** Cell cycle exit upon myogenic differentiation. Current opinion in genetics & development. 1997;7(5):597-602.

186. **Steinman RA, Hoffman B, Iro A, Guillouf C, Liebermann DA, el-Houseini ME.** Induction of p21 (WAF-1/CIP1) during differentiation. Oncogene. 1994;9(11):3389-96.

187. **Adhihetty PJ, O'Leary MF, Hood DA.** Mitochondria in skeletal muscle: adaptable rheostats of apoptotic susceptibility. Exercise and sport sciences reviews. 2008;36(3):116-21.

188. **Fernando P, Kelly JF, Balazsi K, Slack RS, Megeney LA.** Caspase 3 activity is required for skeletal muscle differentiation. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(17):11025-30.

189. **Zhang H, Ozaki I, Mizuta T, Hamajima H, Yasutake T, Eguchi Y, et al.** Involvement of programmed cell death 4 in transforming growth factor-beta1-induced apoptosis in human hepatocellular carcinoma. Oncogene. 2006;25(45):6101-12.

190. **Afonja O, Juste D, Das S, Matsuhashi S, Samuels HH.** Induction of PDCD4 tumor suppressor gene expression by RAR agonists, antiestrogen and HER-2/neu antagonist in breast cancer cells. Evidence for a role in apoptosis. Oncogene. 2004;23(49):8135-45.

191. **Schmid T, Jansen AP, Baker AR, Hegamyer G, Hagan JP, Colburn NH.** Translation inhibitor Pdcd4 is targeted for degradation during tumor promotion. Cancer research. 2008;68(5):1254-60.

192. **Bernstein LR, Colburn NH.** AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. Science. 1989;244(4904):566-9.

193. **Cmarik JL, Min H, Hegamyer G, Zhan S, Kulesz-Martin M, Yoshinaga H, et al.** Differentially expressed protein Pdcd4 inhibits tumor promoter-induced neoplastic transformation. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(24):14037-42.

194. **Jansen AP, Camalier CE, Stark C, Colburn NH.** Characterization of programmed cell death 4 in multiple human cancers reveals a novel enhancer of drug sensitivity. Molecular cancer therapeutics. 2004;3(2):103-10.

195. **Hilliard A, Hilliard B, Zheng SJ, Sun H, Miwa T, Song W, et al.** Translational regulation of autoimmune inflammation and lymphoma genesis by programmed cell death 4. Journal of immunology. 2006;177(11):8095-102.

196. **Jansen AP, Camalier CE, Colburn NH.** Epidermal expression of the translation inhibitor programmed cell death 4 suppresses tumorigenesis. Cancer research. 2005;65(14):6034-41.

197. **Yang HS, Matthews CP, Clair T, Wang Q, Baker AR, Li CC, et al.** Tumorigenesis suppressor Pdcd4 down-regulates mitogen-activated protein kinase kinase kinase kinase 1 expression to suppress colon carcinoma cell invasion. Molecular and cellular biology. 2006;26(4):1297-306.

198. **Yang HS, Jansen AP, Nair R, Shibahara K, Verma AK, Cmarik JL, et al.** A novel transformation suppressor, Pdcd4, inhibits AP-1 transactivation but not NF-kappaB or ODC transactivation. Oncogene. 2001;20(6):669-76.

199. **Liou J, Kiefer F, Dang A, Hashimoto A, Cobb MH, Kurosaki T, et al.** HPK1 is activated by lymphocyte antigen receptors and negatively regulates AP-1. Immunity. 2000;12(4):399-408.

200. **Hess J, Angel P, Schorpp-Kistner M.** AP-1 subunits: quarrel and harmony among siblings. Journal of cell science. 2004;117(Pt 25):5965-73.

201. **Yang HS, Knies JL, Stark C, Colburn NH.** Pdcd4 suppresses tumor phenotype in JB6 cells by inhibiting AP-1 transactivation. Oncogene. 2003;22(24):3712-20.

202. **Gao F, Wang X, Zhu F, Wang Q, Zhang X, Guo C, et al.** PDCD4 gene silencing in gliomas is associated with 5'CpG island methylation and unfavourable prognosis. Journal of cellular and molecular medicine. 2009;13(10):4257-67.

203. **Jin H, Kim TH, Hwang SK, Chang SH, Kim HW, Anderson HK, et al.** Aerosol delivery of urocanic acid-modified chitosan/programmed cell death 4 complex regulated apoptosis, cell cycle, and angiogenesis in lungs of K-ras null mice. Molecular cancer therapeutics. 2006;5(4):1041-9.

204. **Ambros V.** The functions of animal microRNAs. Nature. 2004;431(7006):350-5.

205. **Liu T, Liu Q, Zheng S, Gao X, Lu M, Yang C, et al.** MicroRNA-21 promotes cell growth and migration by targeting programmed cell death 4 gene in Kazakh's esophageal squamous cell carcinoma. Disease markers. 2014;2014:232837.

206. **Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, et al.** MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature. 2008;456(7224):980-4.

207. **Bolster DR, Jefferson LS, Kimball SR.** Regulation of protein synthesis associated with skeletal muscle hypertrophy by insulin-, amino acid- and exercise-induced signalling. The Proceedings of the Nutrition Society. 2004;63(2):351-6.

208. **Zargar S, Moreira TS, Samimi-Seisan H, Jeganathan S, Kakade D, Islam N, et al.** Skeletal muscle protein synthesis and the abundance of the mRNA translation initiation repressor PDCD4 are inversely regulated by fasting and refeeding in rats. American journal of physiology Endocrinology and metabolism. 2011;300(6):E986-92.

209. **Kakade D, Islam N, Maeda N, Adegoke OA.** Differential effects of PDCD4 depletion on protein synthesis in myoblast and myotubes. BMC cell biology. 2014;15:2.

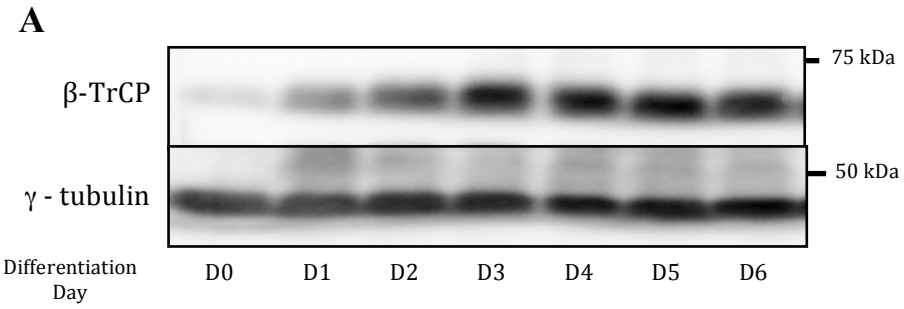
210. **Yi JS, Park JS, Ham YM, Nguyen N, Lee NR, Hong J, et al.** MG53-induced IRS-1 ubiquitination negatively regulates skeletal myogenesis and insulin signalling. Nature communications. 2013;4:2354.

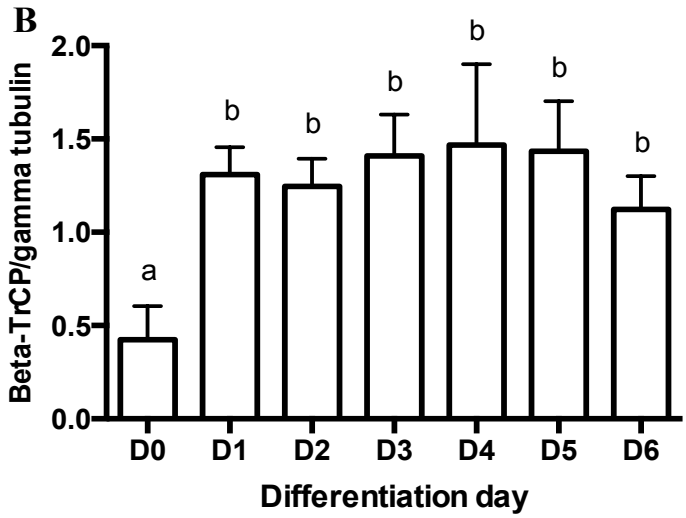
211. **Zhao Y, Xiong X, Sun Y.** DEPTOR, an mTOR inhibitor, is a physiological substrate of SCF(betaTrCP) E3 ubiquitin ligase and regulates survival and autophagy. Molecular cell. 2011;44(2):304-16.

212. **Brown DM, Parr T, Brameld JM.** Myosin heavy chain mRNA isoforms are expressed in two distinct cohorts during C2C12 myogenesis. Journal of muscle research and cell motility. 2012;32(6):383-90.

APPENDIX

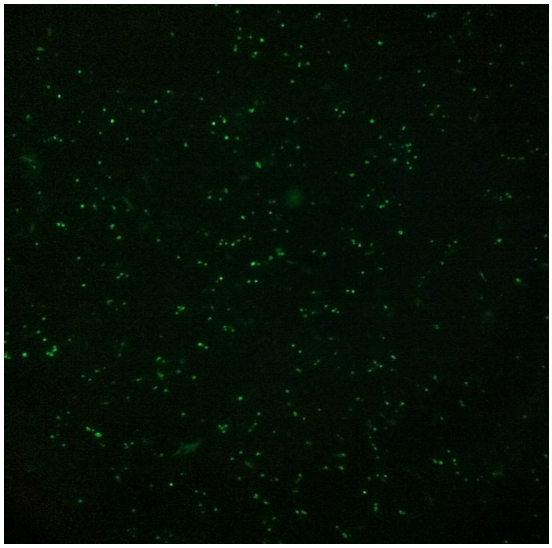
Appendix A: Supplementary Figures



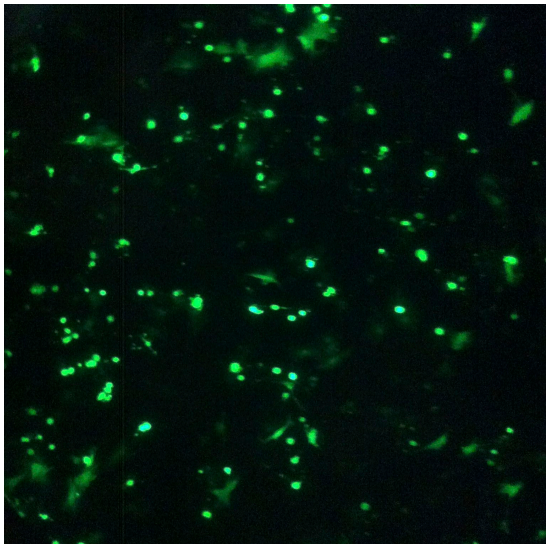


Supplemental Figure 1: βTrCP expression in proliferating L6 cells (Day 0) and during differentiation (Day 1 to Day 6). βTrCP expression was significantly increased from Day 0 to differentiation time (Day 1 to Day6) ($p<0.05$) after growth medium was changed to differentiation medium (A, B). D0=day 0, 48 hours after cells were seeded in 6-well plate. D1=1 day after medium was changed to differentiation medium. Means ± SEM; n = 2 independent experiments; bars with different letters differ.

A

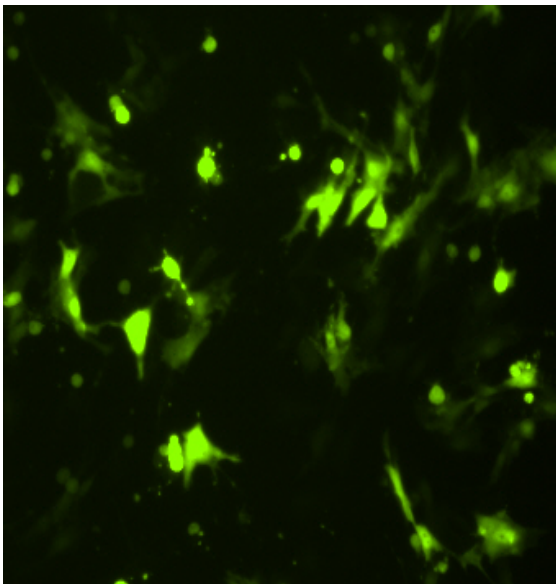


48H after transfection of EGFP
4X magnification

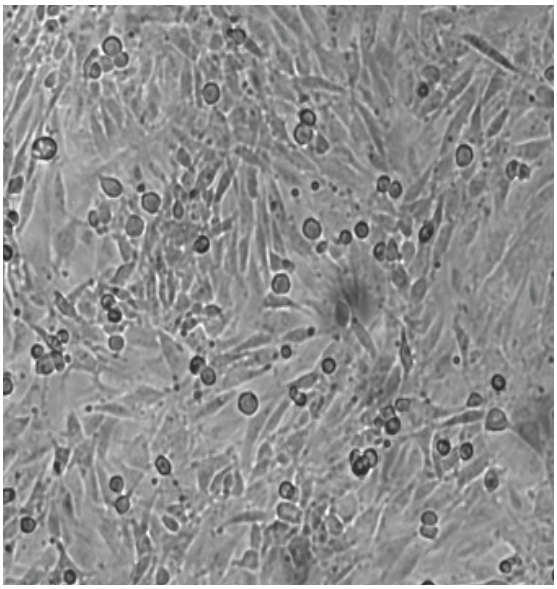


48H after transfection of EGFP
10X magnification

B



48H after tranfection of EGFP
10x magnification with florescent



48H after tranfection of EGFP
10x magnification with white light

C

	EGFP average (PDCD4/gamma tubulin)	EGFP-PDCD4 average (PDCD4/gamma tubulin)	EGFP-PDCD4/EGFP	EGFP-PDCD4/EGFP	*rate of overexpression*
D0	1.0271	1.5008	1.4613	***1.46***	46%
D1	1.8495	2.6093	1.4108	***1.41***	41%
D2	1.4601	1.9742	1.3521	***1.35***	35%
D3	0.8185	1.0686	1.3055	***1.31***	31%
D4	0.4518	0.6321	1.3990	***1.40***	40%
D5	0.3981	0.5752	1.4447	***1.44***	44%

Supplemental Figure 2: EGFP image under florescent light. Images for cells transfected with EGFP were taken under florescent light with different magnification 48 hours after transfection (A). Comparison with different sources of illumination (left panel with florescent light, right panel with white light) (B). The rate of overexpression was calculated (C).

Appendix B: Other Contributions

Journal articles published during the completion of this Master's thesis:

1. Jeganathan S, Abdullahi A, Zargar S, **Maeda N**, Riddell MC, Adegoke OA. Amino acid-induced impairment of insulin sensitivity in healthy and obese rats is reversible. Physiological reports. 2014;2(7).

2. Kakade D, Islam N, **Maeda N**, Adegoke OA. Differential effects of PDCD4 depletion on protein synthesis in myoblast and myotubes. BMC cell biology. 2014;15:2.

Appendix C: Laboratory methods and protocols

Splitting cells

Cells

L6 or C2C12 muscle cells

Materials

- α- Modification of Eagle's Medium (AMEM: Wisent Inc. Cat # 310-010-CL) for L6 cells, Dulbecco's Modified Eagle's Medium (DMEM: Wisent Inc. Cat # 319-015-CL) for C2C12 cells
- antibiotic/antimycotic (Ab-Am: Wisent Inc. Cat # 450-115-EL)
- Fetal Bovine Serum (FBS; Cat # 12484-028)
- Phosphate Buffered Saline (PBS; Wisent Inc. Cat # 311-010-CL)
- Trypsin (Wisent Inc. Cat # 325-010043-EL)
- 10 cm culture dish (Cellstar. Cat # 82050-9160)
- 20-200 ul pipette (BioShop Product ID:T010.10)
- 100-1000 ul pipette (Diamed. Cat # Tec 520-1753)
- 10 ml serological pipette (BD Falcon. CA 53300-523)
- 5 ml serological pipette (BD Falcon. CA 53300-421)
- Glass Pasteur Pipettes

Procedure

1. After 48 hours of proliferation in growth medium (GM: for L6: AMEM supplemented with 10% FBS and 1% Ab-Am, for C2C12: DMEM supplemented with 10% FBS and 1% Ab-Am) in 10 cm culture dish, check the confluency of cells under the microscope. If it is 75-85% confluent, it is time to split cells.
2. Put materials (ie. Trypsin, GM, PBS) in the water bath with 37°C .
3. Turn on UV at least 15 minutes to sterile the fume hood.
4. After sterilization, clean up the cell culture hood with 70% of ethanol. Before putting sterile equipment in the cell culture hood, materials have to be cleaned up with ethanol. Hose for waste liquid and your own hand with gloves as well.
5. Take out the plates with proliferated cells from the incubator and put in the cell culture hood.
6. Aspirate GM with vacuum attached with a glass Pasteur pipettes
7. Wash the plate with 5 ml PBS to rinse off remaining GM. Use vacuum to remove PBS.
8. Put 1 mL of Trypsin and move the plate gently to spread it everywhere in the plate.
9. Place the plate in the incubator for 3 mins.
10. While waiting for 3 mins, prepare new plate(s).

    ---Put 10 ml of GM.

    ---Write info (i.e. date, passage of the cells, type of the cell, your name)

11. Take out the plate from the incubator and tap the side of the plate to make sure the all cells are lifted from the bottom of the plate.
12. Check the plate under the microscope. If the cells are not moving, tap the plate and lift the cells from the bottom of the plate.
13. Bring the plate back to the cell culture hood and add 5 ml of GM.
14. Tile the plate and pipette up and down 5 times at one side of the plate. Those action allow lifted cell and GM mix together.
15. Put the GM+cells into the new plate. The amount varies, (i.e. depends on the confluency before splitting) but it is about 800 μl-1 ml.
16. Move the new plate(s) gently back and forth to mix well the cells and GM
17. Put the new plate(s) in the incubator.
18. Clean the cell culture hood with ethanol.
19. Bring back materials in -20°C or 4°C fridge in the lab.

Harvesting Cells

Cells

L6 or C2C12 muscle cells

Materials

- Phosphate Buffered Saline (PBS; Wisent Inc. Cat # 311-010-CL)
- 20-200 μl pipette (BioShop Product ID:T010.10)
- 5 ml serological pipette (BD Falcon. Cat # CA 53300-421) or 10ml serological pipette (BD Falcon. Cat # CA 53300-523)
- 1 ml (26Gx3/8) syringe (BD. Ref # 309625)
- 1.5 ml eppendorf tube (Diamed. Tec 610-3167)
- Cell scraper (BD Falcon. Ref # 353085)
- Glass Pasteur Pipettes
- lysis buffer

Lysis Buffer

Tris Base (pH 7.5) 1M	25 mM
10% SDS	2%
0.5 M EDTA	1 mM
phosphatase inhibitor	10 μl /ml
protease inhibitor	10 μl /ml
DTT	1 μl /ml

Lysis Buffer (total volume)	10 ml	15 ml
Tris Base (pH 7.5) 1M	250 µl	375 µl
10% SDS	2 ml	3 ml
0.5 M EDTA	20 µl	30 µl
phosphatase inhibitor	100 µl	150 µl
protease inhibitor	100 µl	150 µl
DTT	10 µl	15 µl
DDH2O	up tp 10 ml	up to 15 ml

Procedure

1. Turn on UV at least 15 mins.
2. Clean up in the cell culture hood with 70% of ethanol. Before putting sterile equipment in the cell culture hood, things have to be cleaned up with ethanol. Hose for waste liquid and your own hand with gloves as well.
3. Take out the plate from the incubator.
4. Aspirate differentiation medium (DM).
5. Wash with 2 ml of PBS for each well to remove remaining DM in the 6-well plate. Gently swirl to make sure the remaining DM on the side of the well as well. Aspirate PBS.
6. Put 75 µl of lysis buffer (depend of the experiment and the amount differ) and gently move the plate to spread out the buffer throughout the well.
7. Use rubber policeman and scrub in the well.

8. Use syringe and take out all the cells and put in the eppendorph tube. In the eppendorph tube, use syringe 3 times.
9. After finishing collecting, take out the plate from the cell culture hood and put ethanol and put in the cell culture waste box.
10. Clean the cell culture hood with ethanol.
11. Close the cell culture hood, turn off the light.

Cell Fractionation

Cells

L6 or C2C12 muscle cells

(Plates for Day 0 are in GM, proliferate in 48 hours, and Day 1-6 are in DM, medium were changed every 48 hours. Days refer the time after DM was applied to cells.)

Materials

- PBS (Wisent Inc. Cat # 311-010-CL)
- Trypsin (Wisent Inc. Cat # 325-010043-EL)
- Cell scraper (BD Falcon. Ref # 353085)
- 20-200 μl pipette (BioShop Product ID:T010.10)
- 100-1000 μl (Diamed. Cat # Tec 520-1753)
- 5 ml serological pipette (BD Falcon. Cat # CA 53300-421)
- 10 ml serological pipette (BD Falcon. Cat # CA 53300-523)
- 1.5 ml eppendorf tube (Diamed. Tec 610-3167)
- Cell scraper (BD Falcon. Ref # 353085)
- 15 ml polypropylene conical tube (BD Falcon Ref # 372096)
- 50 ml polypropylene conical tube (BD Falcon Ref # 352070)
- Buffer 1
- Buffer 2
- Microcentrifuges (placed in room temperature at 4°C) (Eppendorf. Centrifuge 5415D)

- Eppendorf tube (Diamed. Tec 610-3167)
- Digital Vortex Mixer (VWR Cat # 12620-854)
- Glass Pasteur Pipettes

Buffer 1 (50 ml)

Final concentration	
10mM Tris Base (pH 7.4)	0.5 ml of 1M
10mM NaCl	0.5 ml of 1M
3 mM MgCl2	0.3 ml of 0.5M
0.5% Np-40	0.25 ml
DD H2O	up to 50 mL
protease inhibitor	10 µl /ml
phosphatase inhibitor	10 µl /ml

Buffer 2 (10 ml)

Final concentration	
50 mM Tris Base (pH 7.4)	0.5 ml of 1M
5 mM MgCl2	0.1 ml of 0.5 M
0.1 mM EDTA	2 µl of 0.5 M
1 mM DTT	10 µl of 1M
40% Glycerol	4 ml
DD H2O	up to 10 mL
protease inhibitor	10 µl /ml
phosphatase inhibitor	10 µl /ml

Procedure

1. Turn on UV at least 15 mins.
2. Clean up in the cell culture hood with 70% of ethanol. Before putting sterile equipment in the cell culture hood, things have to be cleaned up with ethanol. Hose for waste liquid and your own hand with gloves as well.
3. Take out the plate from the incubator.
4. Aspirate GM (Day 0), DM (Day 1 through Day 6).
5. Wash with 5 ml of PBS for each well to remove remaining GM or DM in the 10 cm plate. Gently swirl to make sure the remaining GM or DM on the side of the well as well. Aspirate PBS.
6. Put 1 mL of Trypsin and move the plate gently to spread it everywhere in the plate.
7. Place the plate in the incubator for 3 mins.
8. Put 5 ml of PBS in the plate and sack in and out and put in 15 ml polypropylene conical tube.
9. Put another 2 ml of PBS in the plate and take out the reminding in the plate.
10. Centrifuge (2000 RPM) in 5 mins.
11. Take out PBS and Trypsin (supernatant).
12. Add 1 ml of PBS in the tube and bring back to the lab.
13. Place the tube on the ice on the lab bench, and use pipette (100-1000 μl) and resuspend cells. Every procedure is on the ice except when you are using microcentrifuge.

14. Put in the mixture of pellet and PBS in the 1.5 ml of eppendorf tube and centrifuge 2300 RPM for 3 minutes at 4°C in a microcentrifuge.
15. Remove PBS. Five hundred μl of Buffer 1 is added to the pellet and resuspended.
16. The samples were put on ice and vortex for 15 sec every minute for 5 mins at low speed.
17. From the resulting lysate, 100 μl aliquot was put in eppendorf tube.
18. Centrifuge the eppendorf tube 2300 RPM for 3 minutes at 4 °C in a microcentrifuge. After centrifugation, the supernatant is collected as cytosol.
19. To make sure there was no contamination, wash 2 times with 200 μl of Buffer 1.
20. Resuspend the pellet with 150 μl of Buffer 2. This was the nuclear fraction.
21. Place tube in -80°C.

^{35}S-Methionine Pulse-Chase and Immunoprecipitation experiments

Cells

L6 muscle cells

Materials

- Growth Medium (GM) with antibiotics: AMEM (Wisent Inc. Cat # 310-010-CL) supplemented with 10% FBS (Cat # 12484-028) and 1% Ab-Am (Wisent Inc. Cat # 450-115-EL)
- Growth Medium (GM) without antibiotics: AMEM (Wisent Inc. Cat # 310-010-CL) supplemented with 10% FBS (Cat # 12484-028)
- Differentiation Medium (DM): (AMEM (Wisent Inc. Cat # 310-010-CL) supplemented with 2 % Horse Serum (HS: cat # 26050-088) and 1% Ab-Am (Wisent Inc. Cat # 450-115-EL))
- ^{35}S-Methionine/Cystine (American Radiolabeled Chemicals, Inc)
- Sterile L-Met/L-Cycteine 300 mM
- PBS (Wisent Inc. Cat# 311-010-CL)
- Microcentrifuges (placed in room temperature and 4°C)
- 15 ml polypropylene conical tube (BD Falcon Ref # 372096)
- 50 ml polypropylene conical tube (BD Falcon Ref # 352070)
- 5 ml serological pipette (BD Falcon. Cat # CA 53300-421)
- 10 ml serological pipette (BD Falcon. Cat # CA 53300-523)
- Eppendorf tube (Diamed. Tec 610-3167)

- Disposable Pasteur pipettes 5 3/4" (Fisher Scientific Cat # 12-678-6G)
- Bench-coat
- CHAPS buffer (see the recipe below)
- CHAPS (solid) (sigma-Aldrich C5070)
- High Salt buffer (HSB) (see the recipe below)
- Low Salt Buffer (LSB) (see the recipe below)
- Protein G (BioMag cat # 311812)
- DynaMag™-2 Magnet (life technologies: cat # 12321D)

CHAPS Buffer Recipe:

- *CHAPS Buffer Recipe* – 500 mL

Component	Calculated Weight	Measured Weight
40mM HEPES [pH 7.5]	4.765 g	4.777 g
120mM NaCl	3.507 g	3.509 g
1mM EDTA	1mL of 0.5M solution	1 mL of 0.5M solution
10mM Pyrophosphate	1.110 g	1.107 g
10mM Glycerophosphate	1.08 g	1.082 g
50 mM NaF	1.05 g	1.051 g
0.5mM Orthovandate	1.25 mL of 200mM solution	

  0.3 % CHAPS added before use

- Store in 4 degree fridge

- *CHAPS Buffer with CHAPS*

Component	Proportion
CHAPS Buffer w/o CHAPS	-
CHAPS (solid)	0.30%
1 mM DTT	1 µl/ml
0.5 mM Nav	2.5 µl/ml
1 mM Benz	5 µl/ml
Protease Inhibitor	10 µl/ml
Phosphatase Inhibitor	10 µl/ml
6.25 mM NEM	

High Salt Buffer	
50 mM TrisHCl	3.14 g
500 mM NaCl	11.68 g
5mM EDTA	0.744 g
1% Triton X-100	4 ml
0.5% sodium deoxycholate	2 g
0.1% SDS	0.4 g
0.04% β-mercaptoethanol	0.16 ml

pH 7.4 at 4°C

final volume is 1liter

Low Salt Buffer	
20 mM TrisHCl	3.15 g
150 mM NaCl	8.76 g
5mM EDTA	1.86 g
0.5 % Triton X-100	5 ml
0.1 % β-mercaptoethanol	1 ml

pH 7.4 at 4 °C

final volume is 1liter

*^{35}S-Methionine/Cystine Pulse Period (*24 hour after seeding cells in 21-10cm plates, cells are labelled with ^{35}S-Methionine/Cystine.)

1. Take out ^{35}S-Methionine/Cystine in -80°C, 30 mins before conducting this protocol to thaw.
2. Make medium with 4 mL of GM and 30 μCi per plate in 50 mL tubes.
3. Take out plates from the incubator and aspire GM in the plates.
4. Add GM+^{35}S Methionine/Cystine
5. Incubate 24 hours

Chasing Period

Since I used radioactive material, I cannot vacuum medium in the wasting flask. Therefore, prepare radioactive wasting container in the cell culture hood, split bench-coat in the cell culture hood and turn on UV at least 15 minutes.

1. Make DM with extra methionine and cysteine (2 mM).
2. Take out 18 plates from the incubator (3 of them are harvesting, so leave in the incubator in this point)
3. Take out medium in the plate by using a gun pipette (instead of vacuuming) and put in the radioactive wasting container. Compared with vacuuming with glass Pasteur pipettes, we cannot aspirate medium well. Therefore, use 1000 μl pipette and remove the rest of the medium in the plates.
4. Put 5 ml of PBS in the plates. Swirl and remove PBS with reminding medium ^{35}S-Methionine/Cysteine with the same manner as mentioned in step 2. Repeat twice.
5. Add DM with 2 mM of methionine/cysteine in the plates. Since I change every 2 hours at the beginning, add 2 ml of them. Before leaving the lab (longer hour incubating, add 5 ml).

Harvesting and IP

First day

1. Prepare ice in the big container in radioactive area in the lab.
2. Bring back 3 plates to the lab.
3. Do the same things as step 3, 4 in the previous section: remove GM ^{35}S-Methionine/Cysteine with ice cold PBS twice. Put plates on ice.
4. Put CHAPS buffer with CHAPS (450 μl/plate) and spread it with scraper gently.
5. Wait 20 minutes.
6. Put the lysate in the eppendorf tube (LOAD).

7. Prepare eppendorf tubes for IP with 400 µl of CHAPS buffer, 3 µl of PDCD4 antibody, 300 µl of lysate.
8. Bring IP tubes to the cold room. It is ideal to have rotating equipment, however we don't have it, so I put on the tube rack and set those sample rotate as much as possible on the rocker for 20-24 hours.

Second day (after 20-24 hours incubation)

prepare Low Salt Buffer (LSB) with milk and High Salt Buffer (HSB). They should be on ice.

Low Salt Buffer with milk

Component	Proportion
Low salt Buffer	-
1mM DTT	1 µL/mL
0.5 mM NaV	2.5 µL/mL
1mM Benz	5 µL/mL
Protease Inhibitor	10 µL/mL
Phosphatase inhibitor	10 µL/mL
6.25 mM NEM	
milk	0.10%

High Salt Buffer

Component	Proportion
High salt Buffer	-
1mM DTT	1μL/mL
0.5 mM NaV	2.5 μL/mL
1mM Benz	5 μL/mL
Protease Inhibitor	10 μL/mL
Phosphatase Inhibitor	10 μL/mL
6.25 mM NEM	

1. The next day (20-24 hours later), prepare magnetic beads.

    - Pick up magnetic beads from 4°C fridge. Before taking out magnetic beads, put the bottle up and down to make pullets are resuspended.
    - Prepare 1.5 ml eppendorf tube and set on the magnetic rack.
    - Put 300 μl (100 μl/sample) of resuspended magnetic beads in an eppendorf tube (enough for 3 samples).
    - Wait 1-2 minutes.
    - Take out the supernatant.
    - Take out the tube from the magnetic rack and put 500 μl LSB without additive (original bottle in the fridge), resuspend by hand (not by pipette). Gently shake the eppendorf tube up and down.
    - Put the eppendorf tube back on the magnetic rack and wait 1.5 minutes

- Take out supernatant (pipette tip should touch the other side of the tube. Never touch the magnetic beads).
- Repeat those washing process 3 times.
- Put 320 μl of LSB with milk (recipe is p.117) (since we need at least 100 μl/sample, make sure I have enough).
- Resuspend the eppendorf tube with moving gently up and down.

2. Add 100 μl of magnetic beads to each sample and incubate samples in the cold room 1-2 hours (the same as the first day step 8, rotating on the rocker. To be sure about the sample rotation, check the sample every 20 minutes)
3. Bring back samples to the lab and put on the magnetic rack.
4. Take out supernatant and put on ice.
5. Put 500 μl of LSB with milk/tube and resuspend.
6. Wait 1 minute and invert the rack (because magnetic beads may remain on the cap). Wait another 30 seconds.
7. Take out supernatant (2 times washing).
8. Put 1 mL of HSB and resuspend.
9. Wait 1 minute and invert the rack and wait another 30 seconds.
10. Take out supernatant.
11. Take out samples from the magnetic rack and put 100 μl of 1X SB (we usually store 4X SB in the lab, therefore dilute with double distilled water). Try to shoot the magnetic beads with 1X SB and let the beads come down to the bottom of the tubes.
12. Vortex with gentle speed (3-4).

13. Boil samples for 5 minutes at 95°C.

14. Vortex for 3-5 seconds.

15. Centrifuge for 2 minutes at 13,000 g force.

16. Place samples on the magnetic rack and wait 2 minutes.

17. Transfer supernatant to new eppendof tubes (Eluate).

RNAi

Materials

- Opti-MEM (Life technologies: cat # 31985-070)
- Lipofectamine RNAiMAX reagent (Life technologies: cat # 13778-150)
- siRNA scramble or PDCD4 #1 or #2 (Sigma Aldrich)
- 15 ml polypropylene conical tubes (BD Falcon Ref # 372096)
- Growth Medium (GM) without antibiotics (AMEM (Wisent Inc. Cat # 310-010-CL) supplemented with 10% FBS (Cat # 12484-028))
- Growth Medium (GM) (AMEM (Wisent Inc. Cat # 310-010-CL) supplemented with 10% FBS (Wisent Inc. Cat # 12484-028) and 1% Ab-Am (Wisent Inc. Cat # 450-115-EL))
- 6 well plates (Cellstar: cat # 657160)

	component	amount	Website
	cells	250000/well	250000/well
A	Opti-MEM Medium	118 μl/well	150 μl/well
	Lipofectamine RNAiMAX reagent	7.07 μl/well	9 μl/well
B	Opti-MEM Medium	121.4 μl/wel	150 μl/well
	siRNA (10μM)	3.54 μl/well	9 μl/well

*"amount" is the one I modified from the website one. Since I need to add 250 μl/well, the "Website" one is a lot of wasting. Most important thing is to have 30nM of RNAi.

This info is older version in Life Technologies, therefore, please check the current one on the website.

1. In the cell culture hood, prepare 4 of 15 ml tubes and label as Optimum, Lipofectamine (for making "A") PDCD4 (for making part of "B-1") and Scramble (for making part of "B-2").
2. Pour Optimum from the bottle to the 15 ml-Optimum tube (Not to touch the mouth of the bottle, not to use pipette gun).
3. Make A in Lipofectamine tube.
4. Make B-1 and B-2 in the PDCD4 and Scramble tubes.
5. Put A into B-1 and B-2 and gently move the tube and mix well.
6. Wait at least 5 minutes.
7. While waiting, add 1 mL/well of GM without antibiotics, and add cells/well.
8. Add 250 μl/well of diluted lipofectamine RNAiMAX Reagent
9. Next day, add 1 mL/well of GM (with antibiotics).

mRNA Experiment

RNAi treatment

Cells

- 250000 cells/well and 2 wells of sample put in one tube
- 2 samples/day/treatment (scramble as control or PDCD4 knock-down)

Protocol

Refer the mRNA section

RNA extraction

Materials

TRIzol Plus RNA Purification Kit (Life technologies Cat # 12183555)

Check the website and obtain the manual of TRIzol Plus RNA Purification Kit.

Follow the protocol.

RNA concentration determination

Materials

- Smart Spec^{TM} Plus (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group)

- TrUView disposable Cuvette (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group. Cat # 170-2510)
- Buffer EB (QIAGEN. cat # 19086)

How to use Smart Spec™ Plus (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group)

- Flick the power button behind Smart Spec™ Plus.
- Smart Spec™ Plus start beeping and start blinking.
- Press "DNA RNA" button.
- Choose Nucleic RNA and the setting should be 1.0 = 40 μg/ml
- Put 297 μl of EB buffer in Cuvette and press "Read Blank"
- Take out Cuvette from Smart Spec™ Plus and discard the content and put 297 μl of EB buffer and 3 μl of RNA and press Read Sample". Write down absorbance of 260, RNA concentration. Press A260:A280 and record the ratio.

How to obtain cDNA

Materials

- iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group, cat#1725038)
- Autoclaved 1.5 ml eppendorf tubes

- PCR 8-cap strips (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group, cat # TCS 0803)
- PCR tubes (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group, cat # TLS 0851)
- 5345 PCR Mastercycker, Gradient Thermal Cycler (eppendorf)

Protecol

Follow the company's protocol. Except the "Reaction Protocol" For reverse transcription, I did 60 minutes instead of 30 minutes at 42°C.

qPCR

Materials

- SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group, cat#172-5271)
- cDNA (obtained previous section)
- Primers (forward)
- Primers (reverse)
- Autoclaved double distilled water
- PCR 8-cap strips (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group, cat # TCS 0803)
- PCR tubes (Bio-Rad Laboratories ((Canada)) Ltd Life Science Group, cat # TLS 0851)

- VWR Galaxy mini

Protocol

- Mix the following compoents. cDNA add the last.

Component	
SsoAdvance	10 μl/tube
Primer (F)	1.25 μl/tube
Primer (R)	1.25 μl/tube
DD water	6 μl/tube
Diluted cDNA	1.5 μl/tube

- Centrifuge 2 seconds.

qPCR cycle

1. 95 °C: 2 mins
2. 95 °C: 10 sec
3. 57 °C: 20 sec
4. Plate read
5. Go to 2, 40 times
6. 95 °C: 10 sec
7. 65 °C: 31 sec
8. 65 °C: 5 sec

9. Plate read
10. Go to 8, 60 times

Overexpression Experiment

Cells

L6 muscle cells (160000 cells/well)

Materials

- Lipofectamin 2000 (Life technologies: cat # 11668-019)
- Opti-MEM (Life technologies: cat # 31985-070)
- 6-well plates (Cellstar: cat # 657160)
- 2.5 μg plasmid DNAs
- Autoclaved Eppendorf tube (Diamed. Tec 610-3167) or (if the amount of total mixture is more than 1.5 ml, use 15 ml polypropylene conical tube (BD Falcon Ref # 372096)

Procedure

- Seed cells to be 70-75% confluency in 24 hours.

24 hours later:

- Turn on UV at least 15 mins
- Clean up in the cell culture hood with 70% of ethanol. Before putting sterile equipment in the cell culture hood, things have to be cleaned up with ethanol. Hose for waste liquid and your own hand with gloves as well.

- Keep OPTIMEM, Lipofectamine 2000 and DNA on ice.
- Make mixture of A

mixture A	per well
OPTIMEM	125 ul
Lipofectamine 2000	5 ul

- Make mixture of B

mixture B	per well
OPTIMEM	125 ul
DNA	2.5 ug

- Put mixture A into mixture B. The amount has to be equal amount. Since the amount of mixture B depends on the DNA, calculate and make sure the total amount of mixture A is more than total amount of mixture B (considering the pipetting error).
- Wait at least 5 minutes.
- While waiting, wash plates with AMEM (without any supplementation)
- Add 1 mL AMEM (without any supplementation) per well.
- Add 250 µl of DNA lipid complex per well.
- After 2-3 hours later, add 10% of FBS (since 1,25mL of AMEM+DNA lipid complex are in a well, add 125 µl of FBS should be added per well).