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SMURF1 as a Novel Regulator of PGC-1a

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SMURF1 as a Novel Regulator of PGC-1α

By

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Abstract

Parkinson's disease is a neurodegenerative disorder caused by the impairment and/or death of the dopaminergic neurons in the area of the brain that controls movement, and is diagnosed in roughly 60,000 Americans each year. Low levels of the protein PGC-1α have been linked to this disease, but efforts to find a chemical that causes higher production of PGC-1α. Therefore, the focus must change to determining whether or not it is possible to reduce the degradation of PGC-1α without impacting the production rate of PGC-1α, thereby increasing PGC-1α levels. Prior studies have shown that the protein CDC4 causes PGC-1α to be degraded through ubiquitination, which could make CDC4 a possible target for deletion in order to increase PGC-1α levels. However, CDC4 is a tumor suppressor gene, making it a poor target for deletion, as this could cause other problems for the patient. As a result, it has been deemed necessary to find another ubiquitin ligase protein that also happens to be an oncogene, as this would not cause as many side effects for a patient if this were deleted. Initial studies have shown that the protein SMURF1 may be a ubiquitin ligase which targets PGC-1α for degradation, and it is likely an oncogene. This is believed to be the case because an increase in SMURF1 levels in cells causes a decrease in the levels of PGC-1α within these same cells. However, it is possible that this effect is due to an increase in SMURF1 levels causing a change in the physiology of cells or some other nonspecific effect. Therefore, the next step in this study will be to determine whether or not PGC-1α and SMURF1 bind directly to each other through co-immunoprecipitation experiments.

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Introduction

PGC-1 Family of Transcriptional Coactivators

Transcriptional coactivators are proteins which increase the expression of genes, either through modifications of chromatin to increase access to DNA, or to recruit RNA polymerase II and other basal transcription machinery, thus increasing the transcription of their target genes into messenger RNA (Featherstone, 2002). One family of transcriptional coactivators is the peroxisome proliferator-activated receptor ϒ coactivator-1 (PGC-1) family. This family includes PGC-1α, PGC-1β, and PRC (PGC-1 related coactivator) (Finck & Kelly, 2006; Puigserver et al., 1998). The PGC-1 family of proteins act upon and enhance the transcription of genes like other transcriptional coactivators, but unlike other coactivator families, the PGC-1 family of coactivators is, itself, highly regulated (Lin, Handschin, & Spiegelman, 2005). This high level of regulation is unusual for transcriptional coactivators, as they do not have the same level of specificity as transcription factors, which are normally very highly regulated. This could be due to the critical tissues and pathways regulated by the PGC-1 family, particularly PGC-1α and PGC-1β. PGC-1α (798 amino acids) and PGC-1β (1023 amino acids) have similar functions, most frequently being induced in the liver, brain, skeletal muscle, cardiac muscle, and brown adipose tissue. Both PGC-1α and PGC-1β are primarily involved in the regulation of metabolic homeostasis (Liu & Lin, 2011). The third member of the PGC-1 family, PRC, while involved in the same regulatory roles as PGC-1α and PGC-1β in the same tissues, plus some others, its expression seems to be induced more strongly during cell proliferation initiation and the inflammatory response (Andersson & Scarpulla, 2001; Gelyzer & Scarpulla, 2011). This study aims to explore the regulation of one member of the PGC-1 family, PGC-1α, by the ubiquitin ligase SMURF1.

PGC-1α

PGC-1α is generally regarded as a master regulator of cellular metabolism, and as such, regulates a wide variety of metabolic genes and their pathways. The first of these metabolic pathways it regulates, and potentially the most important for its role in the pathogenesis of diseases is that it can increase the expression of oxidative stress response genes. It also regulates mitochondrial biogenesis, glucose homeostasis, and fatty acid oxidation. Its regulation of each of these pathways is brought about by a specific stimulus, producing a particular response. For example, exposure to cold external temperatures causes PGC-1α to induce ATP uncoupling in brown fat in order to increase the body's ability to regulate its temperature (Puigserver et al., 1998; Vega, Huss, & Kelly, 2000). Another external stimulus which can cause PGC-1α to activate the transcription of other genes is exercise. Exercise activates two pathways in skeletal muscle. These pathways are mitochondrial biogenesis and the formation of slow twitch muscle fibers by changing their metabolic pathways from glycolytic to oxidative (Lin et al., 2002; Scarpulla, 2011; Wu et al., 1999). Additionally, through various stimuli, including exercise, PGC-1α is capable of inducing mitochondrial biogenesis in cardiac muscle (Lehman et al., 2000). However, in terms of human disease, potentially the most important pathway regulated by PGC-1α is the protection of neurons from reactive oxygen species, chemicals known to negatively affect many molecules critical to the function of cells, through the indirect activation of antioxidant protein genes via the transcriptional coactivator activity of PGC-1α (St-Pierre et al., 2006; Valle, Álvarez-Barrientos, Arza, Lamas, & Monsalve, 2005). This increase in antioxidant proteins likely compensates for the increase in mitochondria which, by carrying out cellular respiration, produce reactive oxygen species at a much higher rate than other organelles. As such, PGC-1α not only induces responses to exercise and other stresses to the organism as a whole, but also ensures that these responses do not damage

the organism long-term. All of these pathways, when dysregulated, can lead to damage of genomic DNA, tissue dysfunction, cellular death, and possibly leading to disease. Thus the maintenance of a normal level of PGC-1α expression and activity are crucial for human health. This is particularly important because PGC-1α expression is reduced as humans age, possibly leading to some age-related conditions like Parkinson's. Ultimately, however, as PGC-1α is so highly regulated itself, efforts to ensure that it remains active in cells to reduce the impact of many diseases have proven fruitless, so our focus has now shifted to reducing its rate of degradation in order to increase its expression levels.

Implications for Human Pathology

Dysregulation of PGC-1α has been implicated in a variety of human diseases, including Parkinson's disease, diabetes, obesity, cardiomyopathy, cold sensitivity, and Huntington's disease (Crunkhorn et al., 2007; Cui et al., 2006; Finck & Kelly, 2006; Handschin & Spiegelman, 2006; Jones, Yao, Vicencio, Karkucinska-Wieckowska, & Szabadkai, 2012 ; Lehman et al., 2000; Lin et al., 2004; Patti et al., 2003; Sandri et al., 2006; St-Pierre et al., 2006; Weydt et al., 2006). Parkinson's disease is a neurodegenerative disorder caused by the impairment and/or death of the dopaminergic neurons in the area of the brain that controls movement, and is diagnosed in roughly 60,000 Americans each year ("Parkinson's disease," 2014). Parkinson's disease is a chronic, progressive condition causing tremors and difficulty in performing everyday tasks. However, unlike some other neurodegenerative diseases, both CT and MRI scans of people with Parkinson's disease often appear normal. This makes correct diagnosis of Parkinson's much more difficult, as there are other disorders which have similar symptoms but are treated much differently ("Parkinson's disease," 2014). Additionally, men are as much as 150% more likely to be

diagnosed with Parkinson's than women and roughly 13 out of every 10,000 people will end up being diagnosed with Parkinson's disease (Van Den Eeden et al., 2003).

One potential cause of Parkinson's is mitochondrial dysfunction, which could explain the fact that there is likely some genetic component to Parkinson's, as indicated by the fact that people whose parent(s) were diagnosed with Parkinson's are more likely to be diagnosed with Parkinson's themselves ("Parkinson's disease," 2014). This could, therefore, mean that a mutant gene encoding some critical function of mitochondria could be involved in the formation of some forms of Parkinson's disease. One possible mechanism by which low levels of PGC-1α could be contributing to neurodegeneration is by allowing parkin, a protein linked to Parkinson's disease, to cause the cells to destroy their own mitochondria. The cells then die due to a lack of energy (Shin et al., 2011). This lack of energy (ATP) likely affects neurons more strongly than other cell types because neurons have such a large energy demand. PGC-1α also regulates the mitochondrial antioxidant defense system within cells, alleviating the stress put on cells by their own mitochondrial byproducts (Murphy, 2009; Valle et al., 2005). Again, in cells which have high metabolic rates, such as neurons, low levels of PGC-1α could prove to be problematic because they are under more oxidative stress, and an insufficient amount of PGC-1α could make them unable to deal with this oxidative stress, causing the cells to die. Therefore, it would stand to reason that a possible treatment for one cause of Parkinson's disease could be increasing the amount of PGC-1α in these neurons. Evidence that this may be successful is the fact that restoration of PGC-1α levels has been shown to be an effective treatment for metabolic syndromes and metabolic diseases, as well as reducing the toxic effects of the protein huntingtin, the causative agent of Huntington's disease (Cui et al., 2006; Wenz, Diaz, Spiegelman, & Moraes, 2008).

Additionally, overexpression of PGC-1α in mice using adenovirus made said mice less susceptible to drug-induced Parkinson's disease.

PGC-1α Regulatory Mechanisms

As mentioned previously, PGC-1α is regulated by many environmental and cellular energy stimuli in order to activate a variety of different pathways to respond to these stimuli (Cao et al., 2005; Lehman et al., 2000; Lin et al., 2002; Puigserver et al., 1998; Puigserver & Spiegelman, 2003; Rhee et al., 2003; Scarpulla, 2011; St-Pierre et al., 2006; Valle et al., 2005; Vega et al., 2000; Wu et al., 1999). In addition to responding to various environmental stimuli or energy states in the cell, PGC-1α displays high levels of post-translational modification. It has multiple ubiquitination sites, many acetylation sites, a sumoylation site, a glycosylation site, and three methylation sites that are known, though there could be many more sites that have yet to be found. Of these, some attenuate the activity of PGC-1α, either through inactivation of the protein without causing its degradation or by targeting the protein for degradation, while others increase the activity of PGC-1α (Fernandez-Marcos & Auwerx, 2011; Housley et al., 2009; Kelly et al., 2009; Olson et al., 2008; Ruan et al., 2012; Rytinki & Palvimo, 2009; Teyssier et al., 2005). One of the most important regulators of PGC-1α activity is phosphorylation, which not only increases its activity, but, under other conditions, can instead decrease its activity, depending on the kinase acting on PGC-1α and, consequently, the residue of PGC-1α which is phosphorylated (Fernandez-Marcos & Auwerx, 2011; Wei, Pan, Mao & Wang, 2012). For example, AMPK and p38 MAPK both enhance mitochondrial oxidative metabolism through the phosphorylation of PGC-1α and that Akt and Clk2 both reduce hepatic gluconeogenesis through the reduction of PGC-1α activity caused by their phosphorylation of PGC-1α different sites from AMPK and p38 MAPK (Jäger, Handschin, St-Pierre & Spiegelman, 2007; Li, Monks, Ge, & Birnbaum, 2007; Puigserver et al.,

2001; Rodgers et al., 2005). Of these post-translational modifications, ubiquitination may be the most promising as a target for the treatment of diseases which result from reduced levels of PGC-1α, since ubiquitination often results in degradation of the protein, either through the proteasome or the lysosome (Xu et al., 2009). One protein which has been found to specifically target PGC-1α for degradation through the addition of ubiquitin chains is CDC4 (Olson et al., 2008). Ideally, CDC4 would then be targeted for inactivation to increase the PGC-1α expression levels within the cells by slowing its degradation, but, unfortunately, CDC4 not only targets PGC-1α for degradation, presumably by the addition of K48-linked ubiquitin chains, but it also happens to be a tumor suppressor gene, making the inactivation of CDC4 a risky proposition (Mao et al., 2004; Rajagopalan et al., 2004; Spruck et al., 2002). Therefore, another ubiquitin ligase which targets PGC-1α for degradation must be found. Fortunately, PGC-1α has been observed not only with the K48-linked ubiquitin chains added by CDC4, but also with K29-linked ubiquitin chains, which have also been shown to target other proteins for degradation, indicating that another ubiquitin ligase is acting on PGC-1α (Figure 1).

E3 Ubiquitin Ligase SMURF1

Our lab has found that an increase in the expression of one K29 ubiquitin ligase results in the reduction of PGC-1α expression levels. This ubiquitin ligase is known as Smad ubiquitination regulatory factor 1, or SMURF1. Unlike CDC4, which would likely be problematic if inhibited due to its tumor suppressor activity, SMURF1 is an oncogene, and one of the few ubiquitin ligases known to covalently attach K29-linked ubiquitin chains to its target (Bernassola, Karin, Ciechanover, & Melino, 2008; Fei et al., 2013; Kwei et al., 2011). Since it is an oncogene, the problem with pursuing CDC4 inactivation as a therapeutic method, namely the fact that this would mean the inactivation of a tumor suppressor gene, would be avoided. SMURF1 belongs to the C2-

WW-HECT subfamily of HECT E3 ubiquitin ligases and is essential in the pathways of bone morphogenic protein, Wnt-PCP, Transforming Growth Factor-β, and MEKK-JNK through its promotion of the degradation of their key regulators (Narimatsu et al., 2009; Suzuki et al., 2002; Yamashita et al., 2005; Zhu, Kavsak, Abdollah, Wrana, & Thomsen, 1999). At the N-terminus of SMURF1 is a C2 domain and the HECT domain is at the C-terminus, with two WW domains between them (Bernassola et al., 2008). Normally, a WW domain binds to a PY motif on the target molecule, making this the canonical SMURF1-substrate interaction site (Suzuki et al., 2002; Yamashita et al., 2005). C2 domains are generally about 130 amino acids long, and bind calcium molecules. They typically cause proteins to be membrane-bound. They are also involved in the recognition of substrates, though these are mostly lipid molecules. It is beginning to be understood, however, that these domains are used by ubiquitin ligases, particularly SMURF1 for target recognition (Corbalan-Garcia & Gomez-Fernandez, 2014; Fei et al., 2014; Lu et al., 2011; Tian et al., 2011). WW domains are characterized by two tryptophan residues roughly 20-22 amino acids apart from one another, and consist of 40 amino acids folded into a stable, triple-stranded β-sheet. These WW domains also tend to be used as substrate recognition sites in proteins, recognizing proline-rich regions on their substrates (Macias, Wiesner, & Sudol, 2002; Suzuki et al., 2002; Yamashita et al., 2005). This proline-rich region is not found in PGC-1α, however, indicating that there may be some other SMURF1-substrate interaction site. Finally, the HECT domain is the active site of an E3 ubiquitin ligase which uses a cysteine to receive an activated ubiquitin monomer from an E2 protein and covalently attaches it to the target protein of the E3 ubiquitin ligase (Metzger, Hristova, & Weissman, 2012). SMURF1 uses the C2 and WW sites for substrate recognition, and subsequently its HECT domain covalently attaches the ubiquitin molecules to the target protein. This is supported by the fact that, in some instances, the C2 domain of SMURF1 is

involved in substrate interaction rather than the WW domains (Fei et al., 2014; Lu et al., 2011; Tian et al., 2011).

Identification of K29 Ubiquitin Ligases in the Regulation of PGC-1α

As shown in Figure 1, an in vivo ubiquitylation assay using ubiquitin mutants that are restricted to forming only K29 or K48 chains demonstrated that both K29 and K48-linked ubiquitin chains are added to PGC-1α, and since it is already known that CDC4 is the ligase which adds the K48-linked ubiquitin chains, it must be determined which ubiquitin ligase is targeting PGC-1α through the addition of K29-linked ubiquitin chains. In order to determine this, PGC-1α and the indicated K29 ubiquitin ligases were co-transfected into HEK293T cells. The four K29 ubiquitin ligases were chosen due to the fact that they were the only four K29 ubiquitin ligases known at the time. As shown in Figure 2, only SMURF1 produced a decrease in the expression levels of PGC-1α, identifying it as a potential K29 ligase which targets PGC-1α. Further evidence that this may be true is indicated in Figure 3; when equal amounts of PGC-1α and GFP are transfected into cells along with increasing amounts of SMURF1, an increase in SMURF1 levels produces a decrease in PGC-1α levels.

Aim of this Research

The aim of this study is to examine the interaction between the transcriptional coactivator PGC-1α and the E3 ubiquitin ligase SMURF1 in order to elucidate potential novel therapies for metabolic disorders. In particular, inactivation of SMURF1 could decrease the degradation rate of PGC-1α in vivo, increasing PGC-1a levels in the hope that this could be protective against a variety of diseases, and possibly restorative for some metabolic disorders.

Approaches

In order to investigate the interaction between PGC-1α and SMURF1, three approaches were utilized. First, co-immunoprecipitation experiments were conducted in order to determine whether or not SMURF1 and PGC-1α bind to one another, again using the transient transfection of HEK293T cells to produce the proteins. Second, to assess whether or not a reduction in SMURF1 levels produced an increase in PGC-1α levels, SMURF1-targeting short interfering RNAs (siRNAs) were co-transfected along with PGC-1α. Third, to determine if a knockout of SMURF1 caused the cessation of K29 ubiquitination of PGC-1α, CRISPR/Cas9 knockout of SMURF1 was employed. In all of these approaches, cellular lysates were resolved using SDS-PAGE and analyzed through the use of Western blotting.

Short Interfering RNAs

Short interfering RNAs (siRNAs) are double-stranded pieces of DNA usually between 19 and 21 base pairs in length which consist of a guide and passenger strand (Elbashir et al., 2001). These short pieces of RNA can be used to activate an endogenous, conserved eukaryotic response to knock down specific protein expression (Agrawal et al., 2003). This knockdown can then be used to assess the effects the loss of one protein has on others. In order to use these dsRNA pieces to knock down the expression of a specific gene, the siRNAs are transfected into cells along with any other appropriate genes needed for analysis. After these siRNAs make it into a cell, the RISC (RNA induced silencing complex) is formed, and this complex separates the two strands of the siRNA and uses them as ‘guides’ to find other, complementary sequences in order to destroy them by endonuclease activity or bind to them and block translation (Agrawal et al., 2003; Hammond, Bernstein, Beach, & Hannon, 2000). The two strands in the siRNA will bind to a specific target mRNA through complementary base pair binding when a part of the RISC, which makes the design

of the siRNAs important, as any nonspecific knockdown must be avoided if possible to minimize confounding variables (Leuschner, Ameres, Kueng, & Martinez, 2006). Therefore, siRNAs are designed to be perfectly complementary to their target gene and have as many differences a possible (no less than 2) with every other known gene in the target organism.

CRISPR/Cas9

CRISPR/Cas9 is a novel genome-editing technology capable of specifically knocking out a gene or set of genes in mammalian cells. This technology takes advantage of what has been described as a likely adaptive immune system for *E. coli*, where the bacteria are able to recognize viral DNA and cleave it using the endonuclease Cas9 in order to prevent any bacteriophages which are able to infect the cell from being able to replicate and kill the bacteria (Bhaya, Davison, & Barrangou, 2011; Deveau, Garneau, & Moineau, 2010; Horvath & Barrangou, 2010; Makarova et al., 2011). Cas9 is a nuclease which cuts DNA at PAM sequences within DNA, and it is guided to these sequences using a guide RNA which, through complementary binding, recognizes its complementary sequence in genomic DNA, allowing Cas9 to cut accurately (Cong et al., 2013; Mali et al., 2013; Ran et al., 2013). When Cas9 cuts DNA, it creates a double-stranded break with no 'sticky ends,' which causes a eukaryotic cell to repair this break using the error-prone method of non-homologous end joining (Harms et al., 2014). Since this repair mechanism is so error-prone, the reading frame of the gene being cut is often disrupted in such a way that a stop codon will be produced much earlier than normal, stopping the translation of the full, functional gene. As such, expansion of cells which have been transfected with CRISPR/Cas9 DNA and/or RNA must then be clonally expanded in order to ensure that only cells whose genomes have been successfully edited will be used to start a new culture of cells.

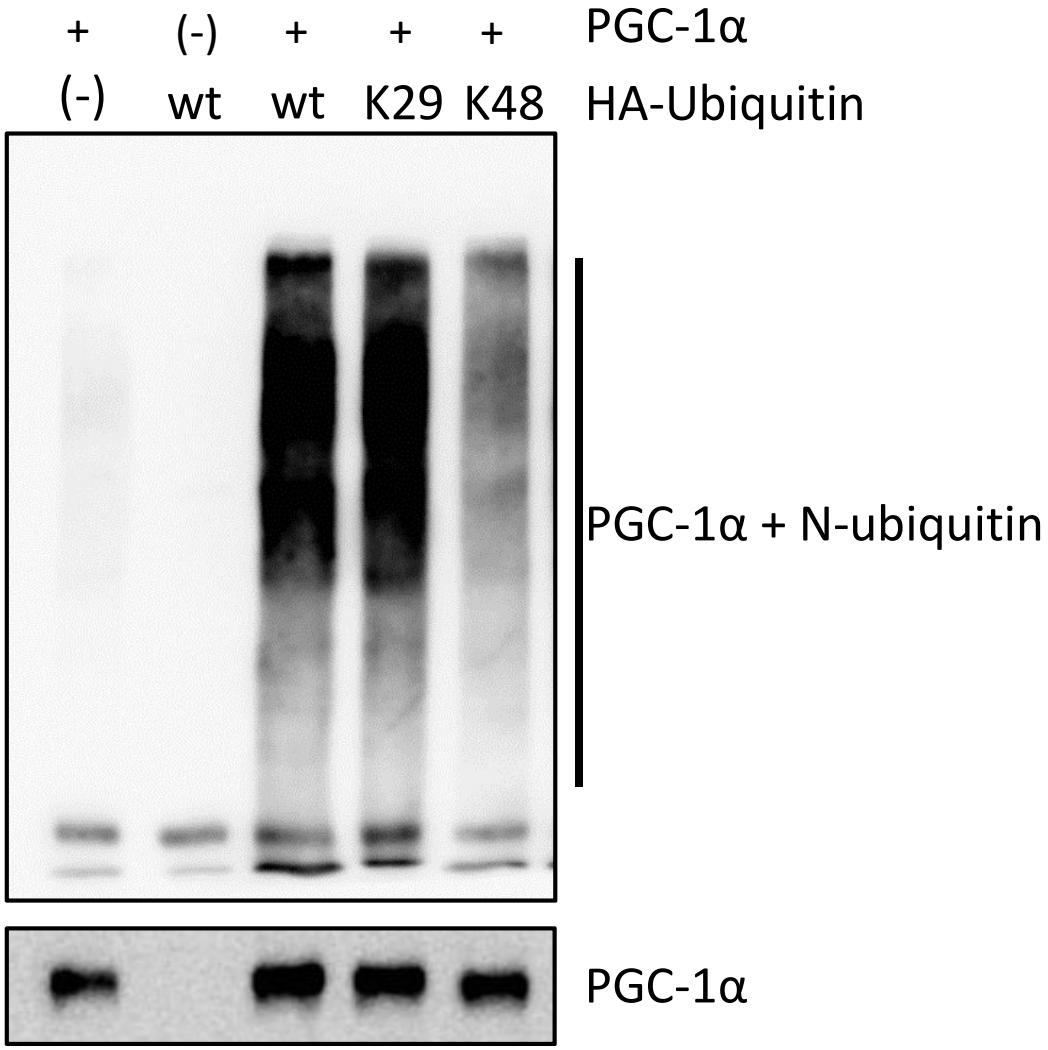

Figure 1. PGC-1α has been observed with two types of ubiquitin chains, namely K48 and K29-linked chains. Flag-tagged PGC-1α, in addition to the indicated HA-tagged mutants of ubiquitin, were transfected into HEK293T cells and the flag-tagged PGC-1α was purified through immunoprecipitation. The mutant forms of ubiquitin replaced all but either the lysine at the 29th or 48th amino acid position with arginine. After separation by SDS-PAGE, samples were analyzed using Western blot to detect HA-tagged ubiquitin or Flag-tagged PGC-1α, and both K48 and K29-linked chains were observed bound to PGC-1α.

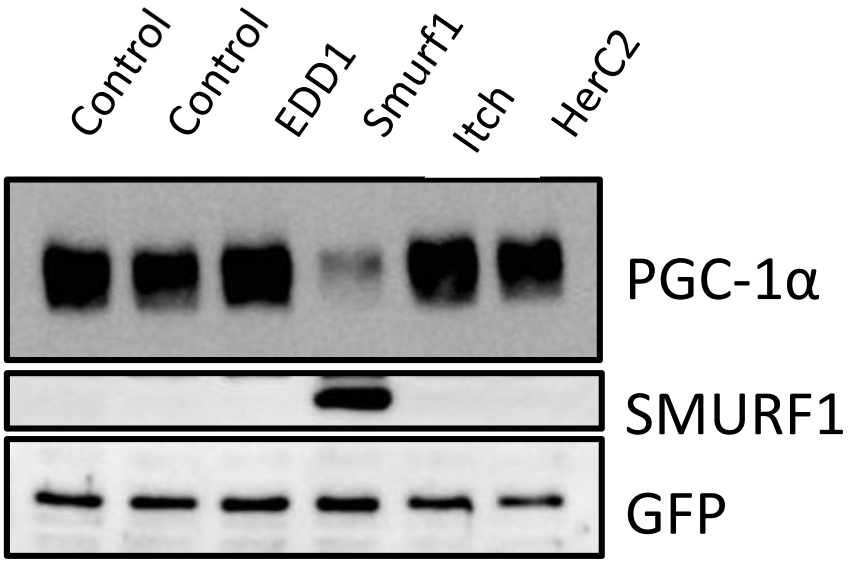

Figure 2. SMURF1 Reduced the Quantity of PGC-1α. HEK293T cells were co-transfected with PGC-1α, GFP, and the indicated K29 ubiquitin ligases. Lysates were separated by SDS-Page and analyzed using Western blot. Of the various ubiquitin ligases known to add K29-linked ubiquitin chains, only SMURF1 produced a decrease in PGC-1α levels.

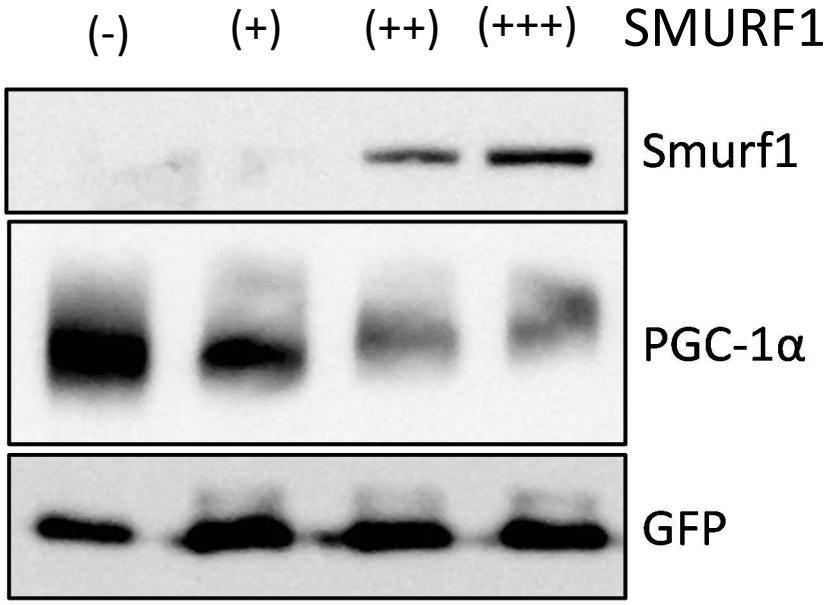

Figure 3. An increase in SMURF1 levels produced a progressive decrease in PGC-1α levels. HEK293T cells were co-transfected with PGC-1α, GFP, and increasing amounts of SMURF1. Lysates were separated by SDS-Page and analyzed using Western blot. As SMURF1 levels increased, a progressive decrease in PGC-1α levels was observed.

Materials and Methods

Cell Culture

HEK293T and DLD1 cells were grown in an Eppendorf CO_2 incubator at 5% CO_2 and 37° C in Dulbecco's modified Eagle's medium (DMEM; Corning Cell Grow) supplemented with 5% newborn calf serum, 5% fetal bovine serum, and 100 units/mL penicillin, 2mM glutamine, and 100 □g/mL streptomycin (PSG; Invitrogen). The cells were grown in 100 mm diameter and 12-well plates, with 10 mL of medium per plate for the 100 mm plates and 1 mL of medium per well for the 12-well plates. Cultures were allowed to grow to 90-100% confluency then passaged 1:5-1:7 utilizing 0.05% trypsin.

Transient Transfection

HEK293T cells were transfected using the calcium phosphate precipitation method, Lipofectamine 2000, or Lipofectamine 3000. The calcium phosphate precipitation method was used for the K29 ubiquitin ligase panel and co-immunoprecipitation experiments. The Lipofectamine 2000 method was used for the overexpression of SMURF1 experiments. The Lipofectamine 3000 transfection method was used to transfect HEK293T cells for the short interfering RNA experiments and to transfect DLD1 cells for the CRISPR/Cas9 knockout of SMURF1. Transfection of the cells was conducted 4-6 hours after re-plating cells into the appropriate cell culture plates for the experiment in order to give the cells ample time to re-attach to the plates prior to subjecting them to the stress of transfection.

Plasmid DNAs

- pEBB Flag-tagged wildtype PGC-1α (500ng/μL)
- pEBB wildtype PGC-1α (500ng/μL)

- pCMV5B SMURF1 (500ng/µL)
- pCMV5B SMURF1 Flag C699A (500ng/µL)
- pcDNA3 GFP (500ng/µL)
- Myc (500ng/□L)
- pCINeo-myc-Itch (500ng/µL)
- pcDNA5 EDD (500ng/µL)
- pcDNA3 HerC2 (500ng/µL)

Buffers

Lysis buffers:

- 1x NETNF (100mM Tris-HCl at pH 8.0, 100 mM NaCl, 5 mM EDTA, 5% glycerol, 50mM NaF, and 0.1% NP-40), supplemented with protease inhibitors: 100 µM PMSF (Sigma), 1x Protease Arrest cocktail (G-Biosciences)
- 1x RIPA (1x PBS [137mM NaCl, 12mM phosphate, 2.7mM KCl, pH 7.4), 1% NP-40, 0.05% SDS, 0.05% sodium deoxycholate]
- 1x RIPA High [1x PBS (137mM NaCl [supplemented with extra NaCl to make a final concentration of 337mM], 12mM phosphate, 2.7mM KCl, pH 7.4), 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate]
- 1x RIPA Low [1x PBS (137mM NaCl, 12mM phosphate, 2.7mM KCl, pH 7.4), 1% NP-40, 0.05% SDS, 0.25% sodium deoxycholate]
- 1x SDS Lysis Buffer: 1:1 ratio of 1x SDS loading buffer (below) to 1x NETN lysis buffer (above).

- SDS loading buffer: 2x (0.5M Tris pH 6.8, 20% SDS, 50% glycerol, bromophenol blue dye) with DTT (1:5)

Co-Immunoprecipitations

HEK293T cells grown in an incubator set at 37°C and 5% CO_2 were transfected using the calcium phosphate method, using an equal amount of flag-tagged SMURF1 C699A and PGC-1α in all but one plate, where SMURF1 was omitted and replaced with an empty PCDNA3 vector. Flag-tagged SMURF1 C699A is a mutant form of SMURF1 in which the 699th amino acid of SMURF1, a cysteine, was mutated to an alanine, and the Flag epitope tag has also been added to the protein. This mutation causes the SMURF1 to be able to bind to its substrates, but to be unable to add ubiquitin chains. These cells were allowed to grow for 48 hours, after which they were lysed using ice cold 1x RIPA buffer. Three hours prior to lysis, however, cells were treated with MG132, a proteasome inhibitor, to prevent unwanted protein degradation. This lysate was allowed to rock for one hour at 4°C in order to homogenize the lysate, then the insoluble fraction was removed by centrifugation. The cleared lysates were then added to Anti-Flag beads which had been equilibrated in the same RIPA buffer and allowed to rock overnight at 4°C to encourage binding of the flag tags to the anti-flag beads. The next day, the beads were washed four times, the first two washes in ice cold low-salt RIPA buffer, and the last two in ice cold high-salt RIPA buffer. After the last wash, roughly 50μL of beads and buffer combined remained, and an equal amount of 2x loading buffer was added before the samples were separated by size using SDS-PAGE and analyzed using western blotting.

- Anti-Flag (DYKDDDDK) Affinity Gel beads purchased from: biotool.com.

Transient Knockdown of SMURF1 by Short Interfering RNA (siRNA)

In order to find out if knockdown of the detectable endogenous SMURF1 produced an increase in PGC-1α levels from a transfection which delivered the same amount of PGC-1α to cells, HEK293T cells grown at 37°C and 5% CO_2 were transfected using the Lipofectamine 3000 method. The short interfering RNAs (siRNAs) were designed using GE Dharmacon's online siRNA design software, and candidate siRNAs were chosen based on their dissimilarity with PGC-1α, as any unintended knockdown of PGC-1α by siRNAs intended to only reduce SMURF1 levels would produce erroneous results. SMURF1 siRNA1 and siRNA 2 were both the default siRNA produced by GE Dharmacon, while SMURF1 siRNA 3 was ordered with ON-TARGETplus modifications to increase its specificity and reduce off-target effects (GE Dharmacon). In order to determine if an increasing knockdown of SMURF1 produced a similar increase in PGC-1α levels, increasing amounts of siRNA were used, and matched with the same amount of Control 5 siRNA in another culture of cells to ensure that any effects observed were due to a change in SMURF1 levels rather than being transfected with siRNA. A further control against this possibility was that one culture was given no siRNA when transfected and another was transfected with no siRNA or PGC-1α to ensure that a baseline level of SMURF1 expression was shown. Two days after transfection, cells were lysed using loading buffer lysis buffer and proteins were separated by size using SDS-PAGE and analyzed using Western blotting.

Target Sequences Ordered from GE Dharmacon:

- Control 5 siRNA 5' UGGUUUACAUGUCGACUAA 3'
- SMURF1 siRNA 1 5' GCACUAUGAUCUAUAUGUU 3'
- SMURF1 siRNA 2 5' AAAGAGAUCUAGUCCAGAA 3'

- SMURF1 siRNA 3 5’ GGGAAAACGGAUUCGAUAA 3’

CRISPR/Cas9

Guide-it™ CRISPR/Cas9 system (Green) ordered from Clontech

Target sequence oligonucleotides:

- Forward: 5'-CCGGTGGCACATAACACTGTCAG-3'
- Reverse: 5'-AAACCTGACAGTGTTATGTGCCA-3'

CRISPR/Cas9 target sequences were chosen using the MIT CRISPR Design Tool (crispr.mit.edu), and the appropriate overhangs were added to ensure that the oligonucleotides chosen would properly ligate into the pGuide-it-ZsGreen vector. These complimentary oligonucleotides were then annealed to each other, cloned into the pGuide-it ZsGreen vector, transformed into Stellar Competent E. coli cells, and the resulting plasmid was purified from a liquid culture of these cells according to the protocol outlined in the Clontech Guide-it CRISPR/Cas9 Systems User Manual (Clontech, 2015). DLD1 cells, grown in complete DMEM at 37^{o}C and 5% CO_2, were transfected with the Guide-it™ vector complete with annealed target oligos using the Lipofectamine 3000 method. Immediately before transfection, complete DMEM was exchanged for DMEM with half the serum of complete DMEM and no PSG to encourage proper formation of DNA-containing micelles and to avoid the influx of a deleterious amount of penicillin or streptomycin from these same micelles, as the first few attempts to knock out SMURF1 through this method resulted in cell death. Next, cells were trypsinized and serially diluted in a 96-well plate in order to select several transfected cells, chosen by the fluorescence of the protein ZsGreen which is expressed by the vector along with the guide RNA and Cas9 enzyme, in order to start cultures which will be analyzed to verify SMURF1 knockout. In order to verify

that one of the resulting cultures successfully knocked out the SMURF1 gene, a Western blot will be performed in order to show that no SMURF1 remains, and PCR amplification and sequencing of the SMURF1 gene will be performed in order to show the change in the gene, explaining any knockout results shown by Western blotting.

SDS-PAGE

Cell lysate samples were prepared for separation by SDS-PAGE electrophoresis by boiling at 100^{o} C for 7-10 minutes before loading 20 µL into the wells in an 8% or 12% gel, depending on the size of the protein of interest for the individual gel. Gels were run at 80V until the sample made it past the stacking gel, then 120V for roughly 1.5 hours. Precision plus Protein Standard (Bio-Rad) was used as a sizing standard.

Western Blot

The proteins contained in the completed SDS-PAGE gels were transferred to nitrocellulose membranes at either 350mA for 3 hours or 80mA overnight in a wet transfer module. Next, a 10% solution of milk in 1x TBST (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) was used to block the membrane, preventing nonspecific antibody binding to the membrane. Excess milk was rinsed off of the membrane using 1x TBST. Membranes were then incubated at 4^{o} C with primary antibody diluted into 1x TBST, 0.5% BSA, and 0.1% Sodium Azide overnight on a rocker. Next, the primary antibodies were re-collected into their storage tubes and placed back in a 4^{o} C refrigerator for reuse and the membranes were washed briefly in a solution of the appropriate secondary antibody diluted 1:10,000 into 1xTBST, 1% milk as described below for one hour on a rocker at room temperature. Secondary antibody solution was disposed of and membranes were

washed for one hour in enough TBST to cover them, with the TBST being exchanged every 5 minutes prior to development of the blots using a Kodak imager.

Antibody Probes

- Anti-Flag:
    - Primary (Mouse), 1:1000 dilution, purchased from Sigma Life Science M2 antibody
    - Secondary (anti-mouse) 1:5000 dilution, purchased from Santa Cruz Biotechnology
- Anti-SMURF1:
    - Primary (Mouse) 1:1000 dilution, purchased from Abnova
    - Secondary (anti-mouse) 1:5000 dilution, purchased from Santa Cruz Biotechnology
- Anti-GFP:
    - Primary (Rabbit) 1:1000 dilution, purchased from Santa Cruz Biotechnology
    - Secondary (anti-rabbit) 1:5000 dilution, purchased from Santa Cruz Biotechnology
- Anti-PGC-1:
    - Primary (Rabbit) 1:1000 dilution, purchased from Santa Cruz Biotechnology
    - Secondary (anti-rabbit) 1:5000 dilution, purchased from Santa Cruz Biotechnology
- Anti-β-Actin:
    - Primary (Rabbit) 1:1000 dilution, purchased from Santa Cruz Biotechnology
    - Secondary (anti-rabbit) 1:5000 dilution, purchased from Santa Cruz Biotechnology

All primary antibodies were dissolved in a solution of 9mL 1x TBST, 1mL 5% BSA, and 20µL 10% sodium azide.

All secondary antibodies were dissolved in a solution of 9mL 1x TBST and 1mL 10% powdered milk dissolved in 1x TBST.

Results

PGC-1α copurifies with SMURF1

In order to gain support for the hypothesis that SMURF1 is directly binding to and targeting PGC-1α, we tested whether or not SMURF1 and PGC-1α bind to one another. This was determined through the use of co-immunoprecipitation experiments in which an equal amount of PGC-1α was transfected into HEK293T cells and either a control DNA or Flag-tagged SMURF1 C699A, a mutant form of SMURF1 where the Cysteine at the 699th amino acid position has been mutated to an Alanine, making a form of SMURF1 which binds to target proteins but will not covalently attach ubiquitin monomers, was co-transfected with PGC-1α. Next, flag affinity beads were added to purify proteins with the flag epitope tag and samples were analyzed before and after purification with antibody-bound beads. PGC-1α only appeared in samples which also contained Flag-tagged SMURF1 C699A, indicating that PGC-1α and SMURF1 bind to each other (Figure 4). This result supports the hypothesis that SMURF1 is directly binding to PGC-1α which suggests that it causes the degradation of PGC-1α through the addition of K29-linked ubiquitin chains.

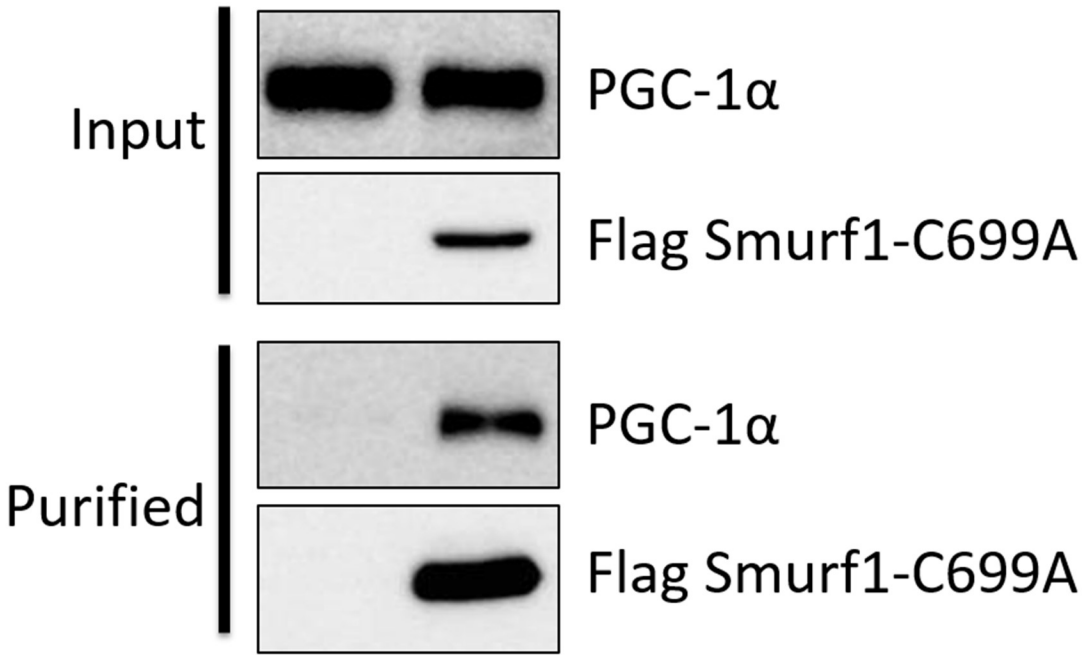

Figure 4. PGC-1α co-purifies with SMURF1. HEK293T cells were co-transfected with PGC-1α, GFP, and Flag SMURF1 C699A. Lysates were mixed with flag-affinity beads to purify SMURF1, all proteins not bound to the beads were washed away, and samples were separated by SDS-Page and analyzed using Western blot. After purification, PGC-1α was only observed in samples containing flag-tagged SMURF1, indicating that SMURF1 and PGC-1α bind to one another.

SMURF1-targeting siRNA #1 and SMURF1-targeting siRNA #2 not only knock down SMURF1 but also PGC-1α, suggesting off-target effects of these siRNAs

To determine if SMURF1 could be a viable target for inactivation to increase PGC-1α levels therapeutically, siRNA was used to transiently knock down SMURF1 in HEK293T cells. SMURF1-targeting siRNA #1, when compared to an equal amount of control siRNA#5, which our lab has shown in prior experiments to have no effect on PGC-1α expression levels knocked down both SMURF1 and PGC-1α (Figure 5). SMURF1-targeting siRNA #1 was purchased from GE Dharmacon's library of premade siRNA products guaranteed to knock down a specific gene. However, they are not guaranteed to have no effect on other human genes, as observed here. In fact, the knockdown of PGC-1α was stronger than the knockdown of SMURF1, indicating either an off-target effect of the siRNA on PGC-1α is causing this knockdown or that the effect of

SMURF1 on PGC-1α is the opposite of our prediction. However, this second possibility is likely not the case because, as shown in preliminary experiments, overexpression of SMURF1 causes such a dramatic decrease in PGC-1α expression levels within HEK293T cells (Figure 2 & 3). Attempts were made to alter the transfection conditions and the amounts of siRNA and PGC-1α transfected in order to find an amount of siRNA which made it into cells to knock down SMURF1 expression while not targeting PGC-1α. However, none of these attempts were successful in finding a condition which knocked down SMURF1 while leaving PGC-1α unaffected. Additionally, a second SMURF1-targeting siRNA (SMURF1-targeting siRNA #2) was ordered from the same library of premade siRNA sequences. Unlike SMURF1-targeting siRNA #1, we were unable to produce any interpretable results using SMURF1-targeting siRNA #2.

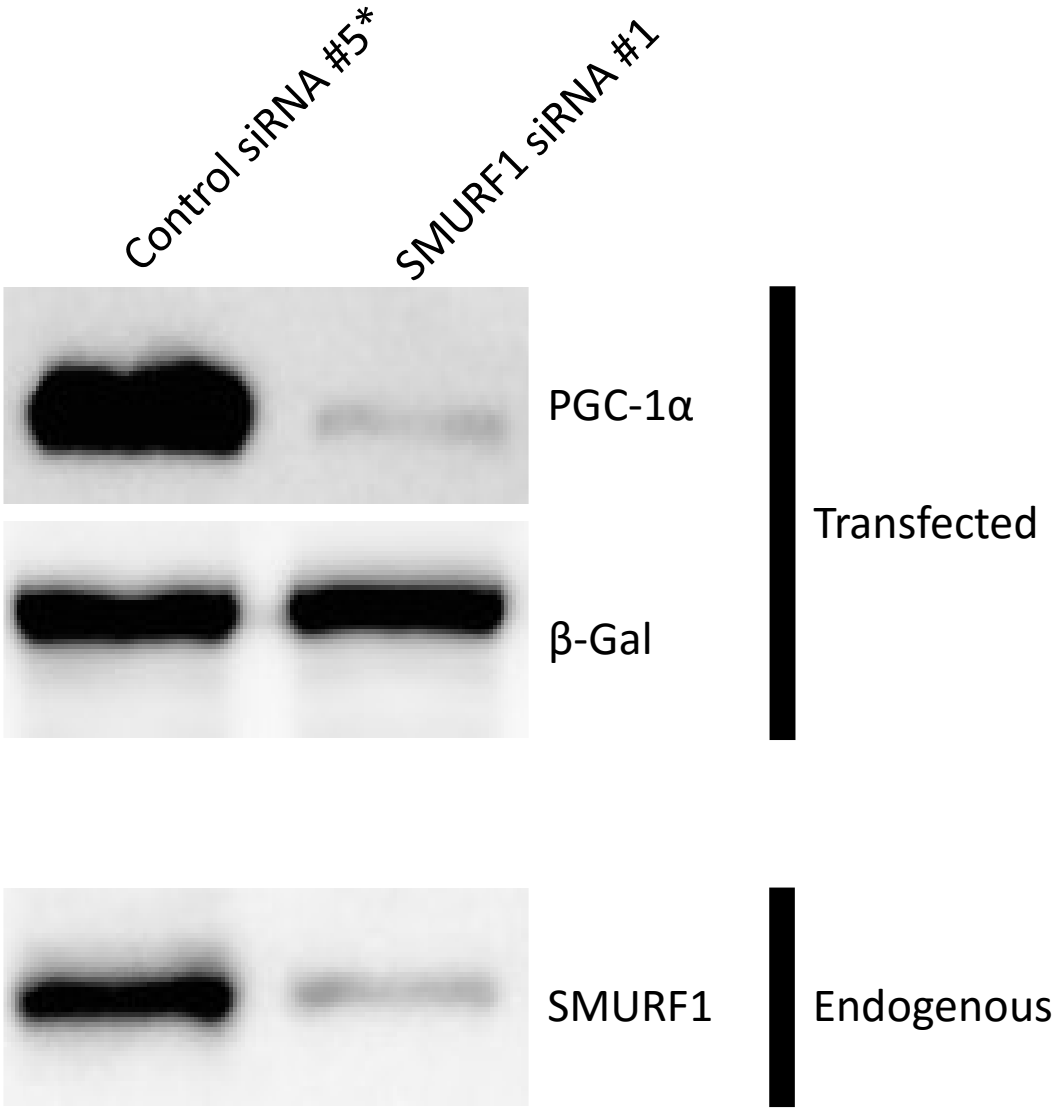

Figure 5. Smurf siRNA #1 produced a knockdown of SMURF1 and PGC-1α levels. HEK293T cells were co-transfected with PGC-1α, GFP, and 100 ng of Control siRNA #5 or Smurf siRNA #1 in a 12-well cell culture plate. Lysates were separated by SDS-PAGE and analyzed using Western blot. The addition of SMURF1 siRNA #1 produced a knockdown of SMURF1 and PGC-1α levels compared to cells transfected with Control siRNA #5, indicating that the SMURF1 siRNA #1 was having an off-target effect on PGC-1α expression levels. (*=siRNA with ON-TARGETplus modification)

SMURF1-targeting siRNA #3 knocks down SMURF1, which produces an increase in PGC-1α levels

In order to determine if the effect seen in SMURF1-targeting siRNA #1 was caused by the siRNA or the reduction in SMURF1, the siRNA was redesigned, producing SMURF1-targeting

siRNA#3. First, the coding sequence for SMURF1 was entered into the GE Dharmacon design tool, which determines the siRNA sequences which should produce a knockdown of their target gene, in our case, SMURF1. This tool also predicts the likelihood of off-target effects. Of these, we selected the siRNA sequence which had the best chance of knocking down SMURF1 while also having the least off-target effects. Additionally, since one of the main sources of off-target knockdown is homology of the seed regions (the first and last 5-6 nucleotides) of the sense and antisense strand of siRNA, we chose siRNA with the least homology to PGC-1α within its seed regions and requested the addition of the ON TARGETplus modification to the siRNA which reduces off-target effects. The ON TARGETplus modification combines the inactivation of the sense strand with a 2'-O-methyl ribosyl substitution at position 2 in the guide strand, which is within the seed region (Jackson et al., 2006). This modification should eliminate at least half of the off-target effects of a given siRNA. When cells transfected with an equal amount of PGC-1α and GFP as well as Control siRNA #5 or SMURF1-targeting siRNA #3 were compared, a knockdown of SMURF1 was observed. Importantly, PGC-1α levels were dramatically increased (Figure 6), indicating SMURF1 inactivation could be a viable therapeutic treatment in the future.

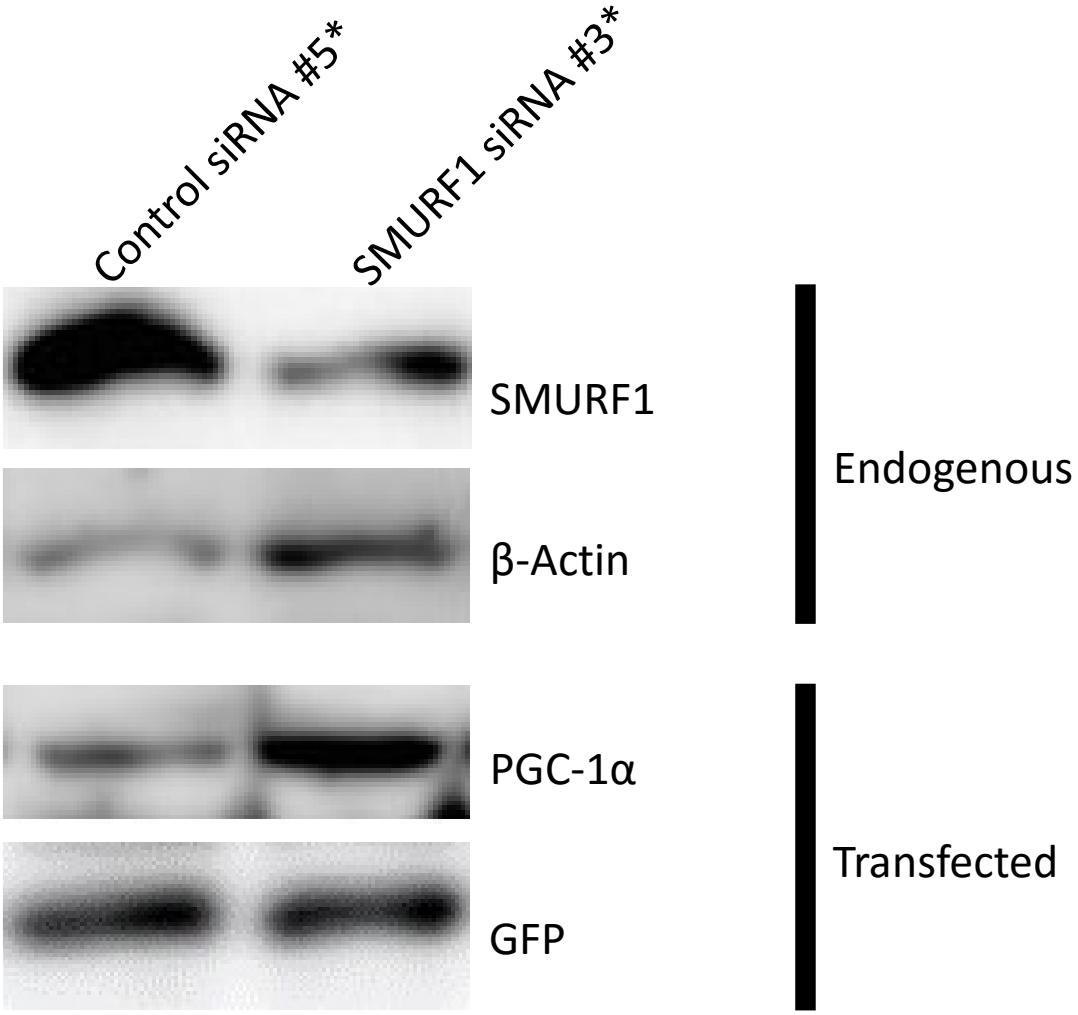

Figure 6. SMURF1 siRNA #3 produced a knockdown of SMURF1 and a subsequent increase in PGC-1α levels. HEK293T cells were co-transfected with PGC-1α, GFP, and 100ng of Control RNA #5 or SMURF siRNA #3 in a 12-well plate. Lysates were separated by SDS-PAGE and analyzed using Western blot. The addition of SMURF1 siRNA #3 produced a knockdown of SMURF1 and an increase in PGC-1α levels compared to cells transfected with Control siRNA #5. (*=siRNA with ON-TARGETplus modification)

CRISPR/Cas9 causes a decrease in SMURF1 expression levels in a heterogeneous population of cells

Finally, CRISPR/Cas9 was used to edit the genome of DLD1 cells in order to produce a SMURF1 knockout cell line. DLD1 cells, human colorectal adenocarcinoma cells, were chosen because they have a relatively normal karyotype, with 86% having the normal 46 chromosomes. However, many of these with a normal number of chromosomes have a high incidence of translocation mutations, and roughly 17% of the cells exhibit polyploidy. It is important to use cells which are mostly diploid when conducting CRISPR/Cas9 experiments to avoid errors caused by excess copies of a gene within each cell preventing complete knockout of said gene. This cell line could then be used to determine if PGC-1α is still modified with K29-linked ubiquitin chains in the absence of SMURF1. To accomplish this, either the pGuide-it ZsGreen (CRISPR/Cas9) plasmid with a guide sequence specific to SMURF1, or a control plasmid, were transfected into DLD1 cells. Next, a guide RNA was designed in order to target the Cas9 enzyme to cleave DNA at a specific sequence, particularly the SMURF1 gene, oligonucleotides for the specific guide sequence for SMURF1 were ordered, and ligated into the pGuide-it ZsGreen plasmid. Sequencing was conducted and, as shown in Figure 7, all but one of the four attempts to isolate a correctly ligated plasmid were successful. As shown in Figure 8, a sequence at the beginning of the coding sequence for SMURF1 was chosen in order to encourage a stop codon closer to the beginning of SMURF1 than the end to be brought into frame with the start codon. After cleavage by guide RNA-aided Cas9, a double-stranded break is repaired using the host cells' own error-prone DNA repair mechanism non-homologous end-joining, which can bring any of the stop codons highlighted in Figure 8 into frame with the start codon, preventing the SMURF1 gene from making a functional protein. As such, it is important to not guide the Cas9 enzyme to a site late in the gene, as this could allow enough of the gene, in this case SMURF1, to be made to produce a

functional or partially functional product. After transfection of 6 plates of DLD1 cells with the pGuide-it ZsGreen plasmid with the SMURF1-targeting insert, all but one of each culture was frozen for future clone selection. A fluorescence microscope was utilized to assess transfection success, and roughly 10% of the cells were observed to be transfected, as shown in Figure 9. The true percentage of cells transfected could actually be higher, but, as the fluorescence microscope available was not optimized for ZsGreen, our ability to detect all of the cells transfected was limited. The two cultures not chosen for cryopreservation were analyzed using Western blotting to give an initial indication that the knockout of SMURF1 via CRISPR/Cas9 was successful. As shown in Figure 10, SMURF1 expression levels in cells treated with CRISPR/Cas9 were lower than cells treated with control DNA, indicating that our guide RNA and Cas9 enzyme were truly knocking out SMURF1 in a percentage of the cells, and the control protein, β-actin, confirmed this observation, since a higher amount of β-actin was present in cells treated with CRISPR/Cas9. This confirms the observation because every cell should have roughly the same amount of β-actin. As such, this would indicate that, if anything, more cells were lysed in the CRISPR/Cas9 treated group, but despite the fact that there were more cells, less SMURF1 was observed. It is also possible that there is more β-actin in cultures which have a reduction in SMURF1 levels because SMURF1 is causing a reduction in the amount of β-actin within the cells. This is likely the case because SMURF1 has been shown to be involved in the degradation of proteins related to β-actin, particularly axin.

Figure 7. Sequence data following ligation of guide RNA sequence into pGuide-it ZsGreen vector. In each of the four attempts, the expected, designed guide RNA sequence was found, though an apparent mutation or sequencing error was detected in sample 2-1, which is likely due to the inaccuracy of next-generation sequencing when dealing with repeated sequences. As such, sample 2-2 was chosen for the continuation of the experiment, as it also produced the highest yield from a mini prep kit.

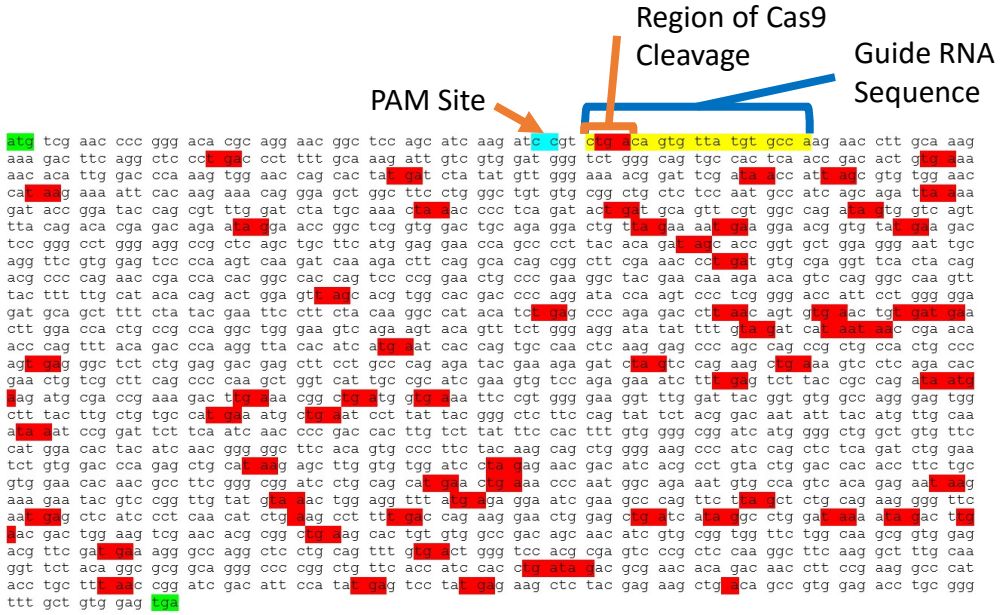

Figure 8. SMURF1 coding sequence, PAM site, and guide RNA sequence used to target the Cas9 enzyme to the SMURF1 gene. The PAM site is a sequence of any nucleotide followed by two guanines (NGG), and is the site recognized by the Cas9 enzyme. Possible stop codons which may be brought into the same reading frame through non-homologous end-joining after CRISPR/Cas9 manipulation of the genome of DLD1 cells are highlighted in red.

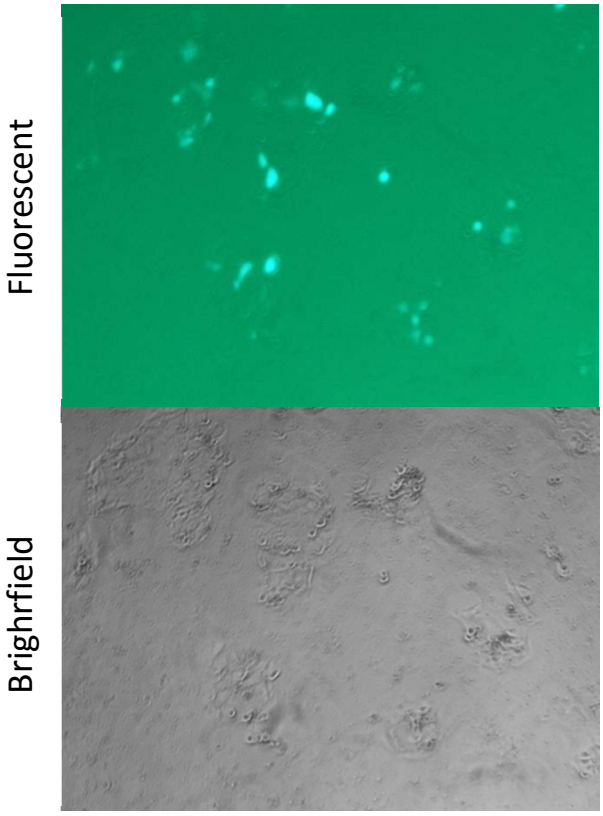

Figure 9. Lipofectamine 3000 transfection of DLD1 cells with pGuide-it ZsGreen plasmid with SMURF1 guide RNA insert shows roughly 10% efficiency. Forty eight hours after transfection of DLD1 cells with pGuide-it ZsGreen plasmid with SMURF1 guide RNA insert, cells were observed using a fluorescence microscope, and roughly 10% of the cells were transfected with enough of the plasmid to be detectible, as indicated by ZsGreen fluorescence.

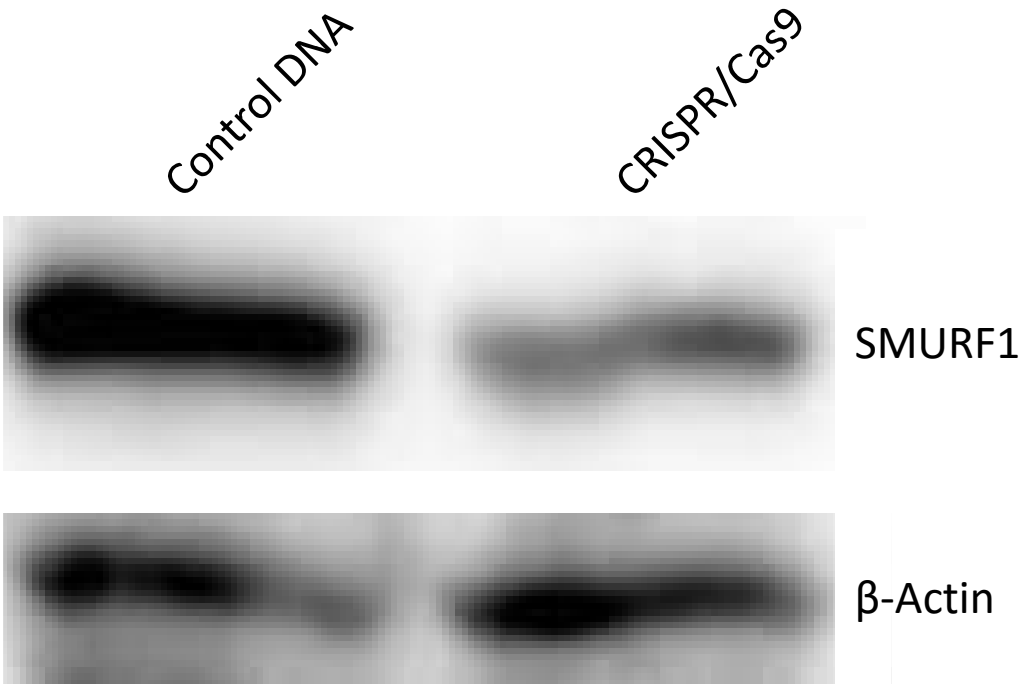

Figure 10. Transfection of DLD1 cells with a plasmid containing the Cas9 enzyme and a guide RNA sequence specific to SMURF1 produced a knockdown of SMURF1 expression levels. DLD1 cells were transfected with either the CRISPR/Cas9 plasmid or a control DNA. These cells were then lysed and separated by SDS-PAGE. Compared to the cells transfected with control DNA, cells which were transfected with the CRISPR/Cas9 system exhibited far less SMURF1 in their heterogeneous culture, indicating success in knockout of the SMURF1 gene in at least a portion of the cells.

Discussion

Since PGC-1α is such a critically important transcriptional coactivator for cellular metabolism, it is important to determine how it is regulated. It has been implicated in many human pathologies, most notably Parkinson's disease. Our lab is particularly interested in the regulation of PGC-1α through ubiquitination, as the inhibition of ubiquitination could be a viable method for treating Parkinson's by increasing the levels of PGC-1α. Prior studies have shown that PGC-1α is ubiquitinated by CDC4, a ubiquitin ligase known to attach K48-linked ubiquitin chains to its targets, causing them to be degraded by the lysosome. However, inhibition of CDC4 would be risky, as it is a tumor suppressor gene. As such, another ubiquitin ligase must be found which targets PGC-1α for degradation. Fortunately, as shown in Figure 1, K29-linked chains of ubiquitin are also added to PGC-1α, and in higher quantities than the K48-linked chains. This indicates that another ubiquitin ligase is adding K29-linked chains to PGC-1α. Figures 2 and 3 show that our initial study has identified SMURF1 as a likely candidate for the ubiquitin ligase which adds these K29-linked chains to PGC-1α. This identification is due to the fact that, as shown in Figure 2, only SMURF1 produces a dramatic reduction in PGC-1α levels when compared to other K29 ubiquitin ligases. Additionally, as shown in Figure 3, progressively increasing the amount of SMURF1 in cells produces a similar, progressive reduction in PGC-1α levels. Another fortunate characteristic of SMURF1 is that it is an oncogene, meaning that its potential inactivation as a treatment for Parkinson's and other diseases would avoid the likely problem with this same approach for CDC4. This treatment would likely be either siRNA or a drug given to patients which would inactivate SMURF1 or disrupt its interaction with PGC-1α to prevent SMURF1-mediatede degradation of PGC-1α.

Simply increasing the amount of SMURF1 in cells and observing a decrease in PGC-1α levels is not enough to definitively show that SMURF1 is the protein which is causing the degradation of PGC-1α levels through its ubiquitin ligase activity, however. This effect could be due to another characteristic of SMURF1 which, through some other mechanism, causes a decrease in the amount of PGC-1α in cells through a change in cell physiology due to the increase in the amount of SMURF1. As such, co-immunoprecipitation experiments were conducted in order to determine if PGC-1α co-purifies with SMURF1. As Figure 4 shows, PGC-1α co-purifies with flag-tagged SMURF1, though it must be said that this does not definitively prove that the two are directly bound to one another. In order to determine this, mutations of PGC-1α must be produced and a similar experiment must be conducted in order to determine the region or residues on PGC-1α to which SMURF1 binds, which will also demonstrate that this is a specific interaction, rather than a non-specific artifact of overexpression of PGC-1α and SMURF1.

Next, short interfering RNA (siRNA) experiments were conducted in order to determine if the inactivation of SMURF1 to increase PGC-1α levels may work. This is important because, since PGC-1α is so highly regulated itself, it could be the case that an increase in SMURF1 produces a decrease in PGC-1α levels, but, due to some other regulatory mechanism, a reduction in SMURF1 levels may not produce an increase in PGC-1α expression levels. Our first attempt to knock down SMURF1 levels through the use of siRNA were met with unexpected results, as have other siRNA experiments conducted with PGC-1α in the past. Figure 5 shows that Smurf-targeting siRNA #1 not only knocks down SMURF1, but also produces an unintended, stronger knockdown of PGC-1α. This has been observed in the past with control siRNAs which were supposed to leave all human genes unaffected. The most likely reason this is occurring is twofold, most notably the instability of PGC-1α and the frequent homology with siRNA seed regions on PGC-1α. The

instability of PGC-1α is important as a factor which may cause siRNA off-target effects to efficiently knock down PGC-1α expression levels because, as PGC-1α expression is so sensitive, any small disruptions of its expression will be quickly seen as a strong knockdown of the gene, while other genes may be able to overcome these relatively infrequent off-target effects. These off-target effects are likely not because PGC-1α is being knocked down by the intended mechanism of siRNA knockdown but because of homology with the seed regions of siRNA guide RNAs. This incomplete homology causes the guide RNA in the RNA-Induced Silencing Complex to block translation of PGC-1α, rather than causing the degradation of PGC-1α mRNA. As such, new siRNA was designed with particular care to avoid homology with PGC-1α in the seed region of said siRNA, and this new siRNA, SMURF1-targeting siRNA #3, was shown to knock down SMURF1, producing an increase in PGC-1α levels (Figure 6). However, it is unclear it this extra accumulation of PGC-1α is active or not, which will need to be determined in order to verify if this siRNA-induced increase in PGC-1α levels would be therapeutically viable.

Next, CRISPR/Cas9 knockout of SMURF1 was pursued, as the ability to analyze knockout cells and determine if the lack of SMURF1 causes the cessation of K29-linked ubiquitin chain addition to PGC-1α would be an extremely powerful tool. Initial attempts, when analyzed with Western blot, show that cultures of cells which have not been selected for SMURF1 knockout still show a strong knockdown of SMURF1 expression in the heterogeneous culture (Figure 10). However, attempts to select single cells transfected with CRISPR/Cas9 have been met with difficulty, as cells treated with CRISPR/Cas9 detach from the cell culture plate and die when diluted in a 96-well plate. This cell death could mean a variety of things, ranging from sensitivity to dilution and lack of ability to survive when DLD1 cells are too sparse, to SMURF1 deletion being lethal to these cells. In order to determine this, coating of the cell culture plates with

extracellular matrix proteins may be necessary, or Fluorescence Activated Cell Sorting may be required as the method of selecting CRISPR-treated cells. Once a SMURF1 knockout cell line is established, the effect on PGC-1α by this deletion should be analyzed to further elucidate the relationship between SMURF1 and PGC-1α.

Future Directions

In the future, efforts to determine whether SMURF1 causes the degradation of PGC-1α via SMURF1 ubiquitin ligase activity should focus on finding a binding site for SMURF1 on PGC-1α. This will be accomplished by determining if mutant forms of PGC-1α exist which are immune to SMURF1 in order for these to function as specificity controls, verifying that the effects we have observed are due to SMURF1 ubiquitin ligase activity rather than physiological changes resulting in changes in PGC-1α levels or some other nonspecific effect due to the overexpression of PGC-1α and SMURF1. Additionally, a successful clone selection of CRISPR/Cas9 knockout cultures should be completed in order to obtain complete knockout cultures of SMURF1. These complete knockout cultures will be useful in determining whether or not the deletion of SMURF1 in DLD1 cells impacts the ability of these cells to add K29-linked ubiquitin chains to PGC-1α. Should this be found, it could be claimed that, at the very least, SMURF1 is involved in the addition of these K29-linked chains to PGC-1α. Ultimately, the goal of this research will be to develop a therapeutic approach to inhibit SMURF1 in order to restore PGC-1α levels in patients, though this will also require the determination as to whether or not any additional PGC-1α which accumulates through SMURF1 inactivation or inhibition is active through the measurement of genes whose transcription is regulated by PGC-1α. Finally, research of the regulation of PGC-1α and the other members of the PGC-1 family must continue and expand, as dysregulation of PGC-1 coactivators is involved in many diseases, particularly diseases which typically have poor outcomes for patients.

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