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Poldip2: an analysis of structure and signaling pathways to elucidate the function of a novel cell cycle regulator

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B.A., University of North Carolina at Chapel Hill, 2006

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

in

Molecular and Systems Pharmacology
Graduate Division of Biological and Biomedical Science

2013

Abstract

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Polymerase-δ interacting protein 2 (Poldip2) is an understudied protein, originally described as a binding partner of polymerase delta and proliferating cell nuclear antigen (PCNA). Numerous roles for Poldip2 have been proposed, including mitochondrial elongation, DNA replication and ROS production via Nox4. My goal is to clarify Poldip2 function by analyzing protein structure and Poldip2 regulated signaling pathways.

An *in silico* analysis of Poldip2 structure uncovered a highly conserved region on the surface of the protein that resembles a binding pocket. This region is solvent accessible, opening the possibility of Poldip2 binding an ion, small molecule or having enzymatic activity.

A gene microarray analysis comparing wild type and Poldip2 knockout samples uncovered key pathways such as circadian rhythms, cell cycle, mitochondrial function and metabolism that are affected by Poldip2 depletion. A further analysis of Poldip2 function in the cell cycle was performed.

Data presented in this study support a novel role for Poldip2 in the cell cycle. We used a *Poldip2* gene-trap mouse and found that homozygous animals die around the time of birth. *Poldip2-/-* embryos are significantly smaller than wild type or heterozygous embryos. We found that *Poldip2-/-* mouse embryonic fibroblasts exhibit reduced growth as measured by population doubling and growth curves. This effect is not due to senescence, as measured by p16 and p19 expression. There was also no change in apoptosis by Annexin V staining. Measurement of DNA content by flow cytometry revealed an increase in the percentage of *Poldip2-/-* cells in the G1 and G2/M phases of the cell cycle, accompanied by a decrease in the percentage of S-phase cells. Cdk1 and CyclinA2 are downregulated in *Poldip2-/-* cells, and these changes are reversed by transfection with SV40 large T-antigen (SV40 LTA), suggesting that Poldip2 may target the E2F pathway. In contrast, p21 expression is unaffected by SV40 LTA transfection. Overall, these results reveal that Poldip2 is an essential protein in development, and underline its importance in cell viability and proliferation. Because it affects the cell cycle, Poldip2 is a potential novel target for treating proliferative diseases such as cancer and atherosclerosis.

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Acknowledgements

I would like to thank all of the people who gave me support, feedback and encouragement during the completion of this work. Thank you to my mentor, Kathy Griendling, for always encouraging me to follow up on my ideas and focus on the "drop dead" experiments. I greatly appreciate the direction and feedback provided by my committee members Mike Hart, John Hepler and Harold Saavedra. I thank my coworkers in the lab both past and present. Having such a wonderful work environment helped keep me sane even when my experiments weren't working. I thank my trivia friends for giving me a non-science related social environment, as well as my softball team. A special thanks to my mother Susan, my father Raeford, my step-mother Cathy, my brother Michael, and my sister Olivia for always believing in me. I would also like to thank my extended family and my in-laws, who have taken me in as one of their own. Finally, I would like to thank my wife Charity Duran. We have been through a car accident, fire, ceiling cave-in and now graduate school and come out stronger because of it. Thank you for your love and support.

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List of Symbols and Abbreviations

AngII	Angiotensin II
BMP	Bone morphogenic protein
Cdk	Cyclin-dependent-kinase
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
DUOX	Dual Oxidase
FAD	Flavin adenine dinucleotide
GPCR	G protein-coupled receptor
H_2O_2	Hydrogen peroxide
HSP	Heat shock protein
LTA	Large T-antigen
MAP	Mitogen-activated kinase
MEF	Mouse embryonic fibroblast
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor κB
Nox	NADPH oxidase
$O_2^{\bullet-}$	Superoxide
OIS	Oncogene-induced senescence
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
Pold2	p50 subunit of Polymerase δ
Poldip2	Polymerase δ interacting protein 2
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
SAHF	Senescence-associated heterochromatic foci

SAM	Significance analysis of microarrays
SV40	Simian virus 40
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor β
VSMC	Vascular smooth muscle cell

Chapter 1

Introduction

Poldip2 Overview

Poldip2 Background

Polymerase delta interacting protein 2 (Poldip2, PDIP38, Mitogenin 1) is a ubiquitously expressed, 368 amino acid protein, consisting of an N-terminal mitochondrial localization sequence and two main functional domains: a conserved ApaG/F box A domain and a hemimethylated DNA binding domain called YccV (Figure 1.1). In bacteria, these domains form two independent proteins with seemingly independent functions. The two domains form a single protein in higher organisms, but the domains are highly conserved.

The function of Poldip2 has not been well studied. Much of the speculation on the functional role of Poldip2 has come indirectly from the wide range of known interacting partners (Table 1.1). These proteins are involved in DNA replication and damage repair, mitochondria function, ROS production and many other processes in which Poldip2 could take part. It has been postulated that Poldip2 may act as a scaffold protein or regulate protein trafficking due to its domain structure. No enzymatic activity of Poldip2 has been demonstrated, but the possibility cannot be ruled out without additional evidence. The following paragraphs summarize those studies in which functional outcomes and/or subcellular localization were measured.

Poldip2 was originally discovered in a yeast two-hybrid screen of the p50 subunit of DNA polymerase δ and PCNA (1). The authors of the initial study later demonstrated that overexpression of Poldip2 inhibits polymerase δ activity in HELA cells. An N-terminal 50 amino acid peptide, which is reported to contain a mitochondrial targeting sequence, is cleaved to form the mature protein (2). Poldip2 was originally thought to function primarily in the mitochondria due to colocalization with mitotracker red, though a subsequent fractionation experiment did indicate a nuclear presence as well.

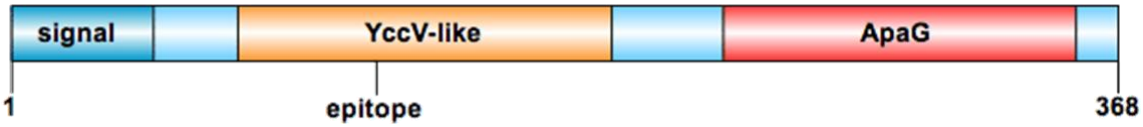

Figure 1.1. Poldip2 primary structure. Poldip2 is 368AA in human, mouse, rat and other similar eukaryotic sequences. Poldip2 is composed of a 35 amino acid signaling peptide or mitochondrial localization sequence, a YccV-like domain and an ApaG domain (DUF525 superfamily). The epitope label indicates the location of the peptide sequence used for antibody generation.

Table 1.1 Experimentally identified Poldip2 interacting proteins.

Poldip2 interacting protein	Gene symbol	Methodology	Reference
Pol δ p50 subunit	POLD2	Yeast two-hybrid, GST, co-IP	Liu et. al. (1)
Proliferating cell nuclear antigen	PCNA	Yeast two-hybrid, GST, co-IP	Liu et. al. (1)
HPV E7	N/A	GST	Xie et. al. (2)
Transcription factor A	TFAM	Co-IP	Cheng et. al. (3)
Single stranded DNA binding protein 1	SSBP1	Co-IP, crosslink IP	Cheng et. al. (3)
Leucine rich protein	LRPPRC	Crosslink IP	Cheng et. al. (3)
Lon protease, mitochondrial	PIM1	Crosslink IP	Cheng et. al. (3)
HSP90β	HS9B	Crosslink IP	Cheng et. al. (3)
HSP70, mortalin	HSPA9	Crosslink IP	Cheng et. al. (3)
Mt matrix peptidase	CLPX	Crosslink IP	Cheng et. al. (3)
HSP60, chaperonin	HSPD1	Crosslink IP	Cheng et. al. (3)
ATP synthase	ATP5B	Crosslink IP	Cheng et. al. (3)
Serine peptidase inhibitor B3	SERPINB3	Crosslink IP	Cheng et. al. (3)
Serine peptidase inhibitor B4	SERPINB4	Crosslink IP	Cheng et. al. (3)
Aspartate transaminase	GOT2	Crosslink IP	Cheng et. al. (3)
Malate dehydrogenase	MDH2	Crosslink IP	Cheng et. al. (3)
2,4-dienoyl CoA reductase	DECR1	Crosslink IP	Cheng et. al. (3)
Peroxiredoxin 3	PRDX3	Crosslink IP	Cheng et. al. (3)
ATP synthase	ATP5O	Crosslink IP	Cheng et. al. (3)
CEACAM1	CD66a	GST, co-IP	Klaile et. al. (4)
P22Phox	CYBA	Yeast two-hybrid, GST, co-IP	Lyle et. al. (5)
Polη	POLH	Yeast two-hybrid, co-IP	Tissier et. al. (6)
Rev1 polymerase	REV1	Yeast two-hybrid	Tissier et. al. (6)
Mitotic arrest deficient-like 2	REV7	Yeast two-hybrid	Tissier et. al. (6)
Phosphoinositide dependent protein kinase-1	PDPK1	Yeast two-hybrid	Vinayagam et. al. (7)
Histone deacetylase 6	HDAC6	Yeast two-hybrid	Vinayagam et. al. (7)
Activating signal cointegrator 1	ASCC2	Yeast two-hybrid	Vinayagam et. al. (7)
Selenium binding protein 1	SELENBP1	Yeast two-hybrid	Vinayagam et. al. (7)
TAK1, MAPKKK7	MAP3k7	Yeast two-hybrid	Vinayagam et. al. (7)

Elsewhere, Poldip2 has been reported to localize to the cytoskeleton (5; 8) and the plasma membrane (4). It should be noted that many of the initial experiments investigating Poldip2 localization and function were performed in cell lines: HELA, HEK293T, and MCF7. These cell lines rapidly proliferate and if Poldip2 is involved in DNA replication, its expression and localization could be influenced by the immortalization process. Additionally, Poldip2 may have distinct localizations in different cell types.

Shortly after the discovery of Poldip2 and its initial characterization, more evidence was published to support a role in the mitochondria. It was found to interact with proteins in the mitochondrial nucleoid, specifically mitochondrial transcription factor TFAM and single stranded DNA binding protein mtSSB in HELA cells (3). In C2C12 myoblast cells, Poldip2 is implicated in mitochondrial morphology, and potentially mitochondrial fusion, which occurs during the cell cycle. It was demonstrated that overexpression of Poldip2 results in increased elongation of mitochondria, and silencing of Poldip2 causes more fragmented mitochondria. Interestingly, the authors observed Poldip2 localizing to the nucleus during G2/M, which supports a role in the cell cycle (9).

More recent studies have confirmed localization in the nucleus, and also show Poldip2 in the plasma membrane and cytoplasm (4; 5). In a study including NBT-II, IEC18, RBE, and HELA cells, it was observed that Poldip2 localization is influenced by cell confluence. In subconfluent cells, Poldip2 was observed at the plasma membrane in association with the cell adhesion receptor Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 (CEACAM1). However, as the cells achieved confluence, Poldip2 translocated to the nucleus, most notably in NBT-II cells (4). This could indicate that Poldip2 links cell contact to a reduction in cell division, especially since CEACAM1 has

been previously implicated in these processes. The study also suggests that Poldip2 localization and function likely depend on tissue type.

Recent studies have implicated Poldip2 in the activity of translesional polymerases Polη, Rev1 and Rev7 (6). Depletion of Poldip2 resulted in increased Polη foci in normal conditions and reduced cell survival after UV treatment. However, another study found that Poldip2 does not associate with PCNA or Polη foci after UV treatment of cells (10). The authors propose that Poldip2 is involved in the processing of the DNA damage response protein MDM2, which may explain the reduced cell survival after UV treatment in Poldip2 depleted cells.

In vascular smooth muscle cells (VSMCs), Poldip2 has been shown to regulate Nox4 and cytoskeletal components (5). Poldip2 interacts with p22phox, the membrane component of NADPH oxidase complexes. Overexpression of Poldip2 in these cells induces production of H_2O_2 mediated by Nox4. Conversely, knockdown of Poldip2 by siRNA reduces ROS production. Poldip2 colocalizes with Nox4 in actin-associated focal adhesions and stress fibers and in the nucleus. Additionally, overexpression of Poldip2 activates RhoA, which could mean that Poldip2 is involved in actin dynamics and focal adhesion formation in these cells (5). RhoA has been previously shown to regulate mitochondrial distribution (11), which could in part explain how Poldip2 knockdown alters mitochondrial function (9).

Mice heterozygous for Poldip2 exhibit hardened aorta and reduced aortic dilatation compared to wild type mice (12). This is potentially due to increased collagen and disrupted elastic lamellae. This work represents the first demonstration of a role for Poldip2 in vivo.

No known human diseases are currently associated with Poldip2 mutations; however, extensive study has not been performed. It was determined that Poldip2 is part of 5 genes (Tmem97, Ift20, Tnfaip1, Poldip2 and Tmem199) involved in a complex sense

anti-sense architecture in which oppositely transcribed genes share loci with antisense partners. Upregulation of Poldip2 and other coregulated genes in its chromosomal vicinity correlates with poor prognosis in breast cancer (13). This could indicate that Poldip2 is involved in phenotypes related to malignancy.

Poldip2 influence on Nox protein function

Dr. Griendling's laboratory initially became interested in Poldip2 due to its interaction with P22phox, a membrane subunit of NADPH oxidase (5). NADPH oxidase (Nox) proteins are membrane-associated, multiunit enzymes that catalyze the reduction of oxygen using NADPH as an electron donor (14). Nox proteins produce superoxide ($O_2^{\bullet-}$) via a single electron reduction. The electron travels from NADPH down an electrochemical gradient first to flavin adenine dinucleotide (FAD), then through the Nox heme groups and finally across the membrane to oxygen, forming $O_2^{\bullet-}$ (15). Historically, the NADPH oxidase was known as the source of the phagocyte respiratory burst; however, in the past twenty years Nox family members and the reactive oxygen species (ROS) they produce have been identified as important contributors to many signaling pathways.

Noxes influence a variety of critical processes via their production of the reactive oxygen species $O_2^{\bullet-}$ and H_2O_2. The influence of Poldip2 on the activity of Nox proteins is just beginning to be understood. Lyle et al. (5) demonstrated that overexpression of Poldip2 increases Nox4 activity, and thereby regulates VSMC migration and cytoskeleton dynamics. Increased Poldip2 expression is associated with strengthened focal adhesions and more stress fibers. Furthermore, Nox1 and Nox4 immunoprecipitate with Poldip2 and p22phox (5), which suggests that the proteins can form a complex rather than sharing a common binding site. They also showed that an interaction between Poldip2 and Nox1 is regulated by angiotensin II. Ultimately, the interaction between Poldip2 and

p22phox could have downstream consequences on Nox related pathways. In addition to interacting with Nox1 and Nox4, p22phox is also a part of Nox2 and Nox3; however, it has not been established whether Poldip2 influences the function of these family members. For a more detailed overview of Nox protein signaling, refer to Appendix A.

Poldip2 and proliferation

Although Poldip2 may play a role in regulating Nox-related signaling processes, based on the existing literature describing other interacting partners, we must consider that Poldip2 has other essential functions in the cell. The knockout of Poldip2 has recently been demonstrated to cause perinatal lethality in mouse embryos (12), whereas Nox4 knockout mice are viable (14). This represents an obvious point of divergence of Poldip2 and Nox4. While further work is necessary to determine the cause of the Poldip2 knockout lethality, given the interaction of Poldip2 with PCNA, one possibility is a reduction in cell growth in the absence of Poldip2.

Proliferation and mitosis

One of the paramount requirements of life is the duplication of biological matter. For eukaryotic cells, this process is known as proliferation, and involves copying approximately 3.08 gigabases of nucleotides (16), splitting the nucleus and many organelles including the endoplasmic reticulum, mitochondria and the Golgi apparatus, and finally pinching the cell into two equal parts during mitosis and cytokinesis. Cellular growth is a complex and tightly regulated process, which has been organized into different phases.

Immediately after division, the cells enter the first Gap phase called G1, during which the cell contents increase in size. This phase is also called the first growth phase, and is characterized by an increase in protein synthesis. After G1, the cells undergo DNA

replication in the synthesis phase, known as S-phase. At the completion of S-phase, the cell will have duplicated each chromosome and contain twice the DNA material as prior to replication. After S-phase, the cells enter a second Gap phase, G2. During G2, the cell verifies the integrity of the DNA, and corrects errors in duplication if necessary (Figure 1.2). The cell also increases protein synthesis, and prepares for the dividing of the cell, which occurs in the next phase, mitosis. Mitosis, or M-phase is when the chromosomes segregate and the nucleus divides. Immediately following mitosis, cytokinesis occurs to divide the original cell into two daughter cells. Mitosis can be subdivided into different phases: prophase, metaphase, anaphase and telophase.

Prophase is also known as chromatin condensation. The chromatin is aided by the condensin complex in coiling into chromosomes. The nucleolus is dissolved, and centrosomes move to opposite poles of the cells. The chromosomes are then propelled by microtubule motor proteins, primarily kinesin, dynein and dynactin, toward the center of the cell (17).

Metaphase initiates with the alignment of the chromosomes in a line at the center of the cell, known as the metaphase plate, between the centromeres. Metaphase has particular importance, due to the fact that the mitotic spindle checkpoint occurs during metaphase. The anaphase promoting complex will not allow the cells to enter anaphase unless the chromosomes have been properly aligned and the kinetechores are attached to their respective bunch of microtubules.

Anaphase refers to the separation of the two sets of chromosomes into the two poles of the cell. Microtubule motor proteins act to shape the cell into an oval. Once the chromosomes have been isolated into their respective halves of the cell, nuclear envelopes form around each set of chromosomes in the final phase of mitosis, telophase. The chromosomes unwind, and the cell prepares for cytokinesis.

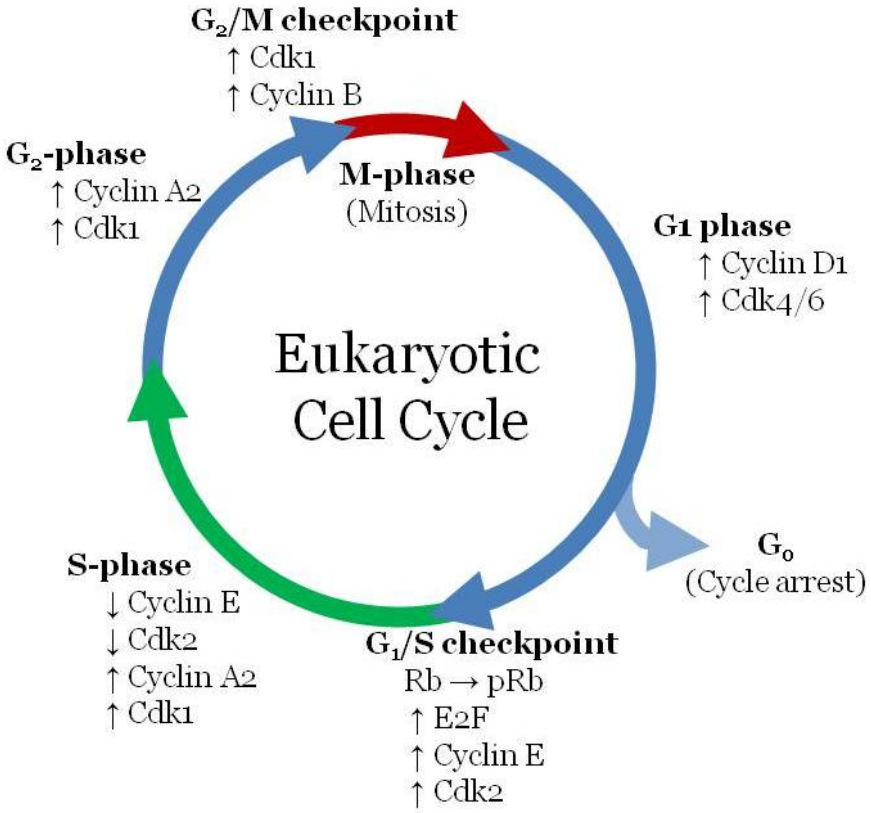

Figure 1.2. Cell cycle regulation. The eukaryotic cell cycle is made up of four main phases. Daughter cells first enter G1 with elevated Cyclin D1 and Cdk4/6, during which the cell grows in size while mRNA and protein synthesis occur. Upon sufficient growth factor and nutrient availability, the cell passes the G_1/S checkpoint by hyperphosphorylation of Rb and activation of E2F during a period of elevated Cyclin E and Cdk2. The cell then enters DNA synthesis (S-phase) with levels of Cyclin A2 and Cdk1 rising. After DNA synthesis the cell enters the second gap phase, G_2 for further growth and enters mitosis, provided DNA damage checkpoints are not activated at the G_2/M checkpoint during elevated Cyclin B and Cdk1. Mitosis divides each cellular component in two and separates DNA to opposite poles of the cell before cytokinesis occurs to split the cell in two cells which subsequently enter G_1.

After a second nucleus is formed, the cytoplasm is divided during the process of cytokinesis. A contractile ring forms between the nuclei comprised of a complex with actin and myosin II. Myosin II effectively moves along the actin filament to close the ring, which is finally pinched off and two daughter cells have been formed.

Poldip2 loss has been associated with disrupted mitotic spindle formation and reduced chromosome segregation (8). It is unclear based on previously published evidence whether this is due to a direct involvement of Poldip2 in mitosis, or if an earlier cell cycle disruption results in the observed effects. Poldip2 may influence the activity of key cell cycle regulator proteins to delay cell proliferation.

Cyclins and Cdks

Though the cell cycle can be simplified into G1, S, G2 and M phase, each of these phases represents a tightly controlled signaling system. Key regulators in the process are a class of proteins known as cyclins, which are named for their cyclic expression pattern during the cell cycle. Another level of regulation comes from the involvement of the serine/threonine cyclin-dependent-kinase (Cdk) proteins, which are often binding partners of the cyclins and regulate the activity of transcription factors, degradation pathways, and play a vital role in many cell cycle checkpoints. A total of 29 cyclins and 25 Cdks (18) have been identified in eukaryotes. An additional 9 proteins are considered to be part of the Cdk family due to similarities in domain structure. However, the function of the additional Cdk family members is still largely unknown (19).

Cyclin/Cdks can be grouped by when they are primarily active during the cell cycle in a canonical pathway. During G1 phase, Cdks 4 and 6 interact with Cyclin D to promote entry into S-phase primarily by phosphorylating Rb and the Rb-related proteins p107 and p130. In late G1 and early S phase, Cyclin E expression is elevated along with Cdk2. The Cyclin E/Cdk2 complex is activated by the licensing factor Cdc6, (20) and

promotes the synthesis of DNA in S-phase. In late S-phase, Cyclin E protein is degraded and Cyclin A expression is elevated. Initially Cyclin A complexes with Cdk2, and is involved in the activation of the Cyclin B/Cdk1 complex that is necessary for the G2/M transition to occur (21). However when S-phase concludes and G2 begins, Cyclin A begins to complex with Cdk1 to promote the G2/M transition. Cdk1 is an essential Cdk, as knockout of Cdk2, Cdk3, Cdk4, Cdk5 and Cdk6 produces viable mice (19), but the absence of Cdk1 prevents cell division entirely resulting in embryonic lethality (22).

Cell Cycle inhibitors

Additional mechanisms are in place to halt division when problems occur with DNA synthesis, cell stress, lack of nutrients and various other signals. One mechanism that halts cell division is via the upregulation of cell cycle inhibitors. These inhibitors fall into the INK4, cip/kip and Rb families.

INK4 contains four known proteins, p16INK4a, p15INK4b, p18INK4c, p19INK4d. These proteins inhibit the kinase activity of the Cyclin D complex by competitive binding with Cdk4/6. The expression of INK4 genes and their inhibition of cell growth is cell type and context dependent. The absence of pRb in a cell eliminates the inhibitory action of INK4 due to Rb inhibition being downstream of INK4 action (23).

The cip/kip family includes p21Cip1, p27Kip1, and p57Kip2. These proteins inhibit the kinase activity of the formed Cdk2 and Cdk4 complexes. P21 is transcribed by the p53 tumor suppressor (24). p21 knockout mice are viable, and gain the ability to regenerate lost appendages in an ear hole closure model (25). p21 knockout mice exhibit levels of tumor formation (26). p27 is transcribed by SP1, and is expressed in some cell types as they enter a differentiated state. Once cells are terminally differentiated, there is little need to continue cell growth, and it appears that p27 acts to slow down growth in

this situation. P57Kip2 was originally identified as a homologue of p21 and p27, and is believed to be involved in differentiation and apoptosis in various tissues (27).

The Rb family is made up of Rb, p107 and p130. The Rb family regulates the activity of E2F transcription factors. In its hypophosphorylated state, Rb binds E2F and prevents transcription of E2F target genes, which halts the cell cycle at the G1/S checkpoint. Hyperphosphorylation of Rb releases it from E2F and promotes cell cycle progression. P107 and p130, known as Rb pocket proteins, and are believed to have binding preference with different E2F family members than Rb. Rb mainly targets E2F1, 2, 3, or 4. Rb pocket proteins p107 and p130 mainly associate with E2F4 and 5. In knockout models of Rb family members, only Rb is embryonic lethal (28), suggesting that Rb can mostly compensate for the loss of p107 and p130. However, a p107/p130 double knockout exhibits neonatal lethality (29).

Senescence

A second cause of cell cycle arrest that could induce lower proliferation in Poldip2 knockout cells is senescence. Cellular senescence refers to the irreversible stalling of growth that occurs in primary cultured cells. The study of senescence has been hugely important in understanding the causes of cancers and other proliferative diseases such as atherosclerosis. It has provided a greater understanding of the cause of uncontrollable proliferation of the cells in these diseases, and provides clues on how to reverse the proliferative phenotype.

Some of the first evidence of senescence came from Hayflick et. al. (30), who made the distinction between the *cell strain*, a population of cells with a finite replicative capacity, and the *cell line*, which can proliferate indefinitely. In a later study, Hayflick observed that the lifetime of human diploid cell strains in vitro can be predicted if maintained at the same split ratio, which he believed is due to finite population

doublings of the cells (31). These observations led to the establishment of what is now called the Hayflick limit: the number of doublings that a particular cell type goes through before entering senescence. Prior to Hayflick's finding, it was believed that cells would proliferate indefinitely in culture (32).

There are some possible exceptions to the Hayflick limit, such as vascular smooth muscle cells of rodent origin. Though rat aortic smooth muscle cells are not considered a cell line, it has been reported that these cells do not undergo senescence even after hundreds of passages. Vascular smooth muscle cells of human origin do undergo senescence around passage 12 (33). This suggests that there are fundamental differences in senescence signaling between species, which will be discussed in a later section of this dissertation.

Modern senescence research recognizes that the type of senescence that Hayflick identified is replicative senescence, mainly caused by the shortening of telomeres at each doubling. Once the telomeres size is reduced below a certain length, cell cycle pathways are stalled, and the cells enter senescence (34).

Replicative senescence/telomere

The telomere consists of a tandem repeat consisting of a 6-8 base pair G-rich sequence that varies in length by species at the ends of linear chromosomes. In humans it can be several thousand base pairs long. At the end of the repeat is a shorter 3' single stranded overhang that forms a loop and pairs back into an earlier section of DNA in the telomere forming a circular structure known as the T-loop. This loop protects the overhang, and functions as a structural backbone for telomeric DNA binding proteins (35). These proteins vary by species, but in humans TRF1/2 and Pot1 seem important for maintaining the T-loop structure. Loss of TRF1 or 2 causes fused chromosomes, cell

cycle arrest, apoptosis and senescence (36). Additionally, Ku, Rif1/2, Tin2 and Tel2 are members of a telomere cap complex (35).

Though the concept of the telomere was first hypothesized by Barbara McClintock in 1941 as a method for the cell to protect the ends of chromosomes from accidental fusions (37), it was not until more recently that the telomere complex was identified. In 1972, Olovnikov and Watson predicted that chromosomes lose material at each replication (38; 39). It was a decade later before the enzyme telomerase was discovered and found to elongate the telomere sequence. A direct link was established between telomere length and senescence (40-44). These findings resulted in the awarding of the Nobel Prize for Physiology to telomere researchers in 2009. Telomerase is not typically expressed in mature somatic cells (45), which explains why they have a limited replicative capacity.

It seems, however, that the "mitotic clock" that induces senescence after cells reach their replicative capacity may not be made up of telomeres alone. After the human catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT), was cloned, many researchers overexpressed it in their cell strain of choice and tested for senescence. Many cells types such as fibroblasts, mesothelial cells, endothelial cells, T-cells and RPE cells were immortalized (46). However, mammary epithelial cells and keratinocytes were not immortalized by the expression of hTERT alone. They required the additional inactivation of $p16^{INK4A}$, which may suggest that telomere length is not the sole cause of replicative senescence (47; 48). Additionally, researchers were able to induce senescence by expression *H-ras* and *raf*. Telomerase expression does not prevent senescence induced by *H-ras* (49).

Though the cause of senescence by the shortening of telomeres is not completely understood, a lot of progress has been made. It is believed that once the telomere is shortened to a certain length, the T-loop is disrupted and the protein structure that

protects the end of the chromosome is unable to bind. This disruption induces proteins that are involved in the DNA damage response. Key indicators of this are γ-H2AX positive foci and the activation of ATM and ATR. These kinases activate CHK1 and CHK2, which promote the phosphorylation of p53 and cdc25. Additionally, $p16^{INK4A}$ and Rb are upregulated in replicative senescence. Recent evidence links telomere dysfunction to activation of p53 and repression of peroxisome proliferator-activated receptor gamma (PGC-1α and PGC-1β) in the mitochondria. This ultimately leads to increased ROS and cardiovascular defects in telomerase deficient mice (50).

Redox/stress-induced senescence

A great deal of research has linked oxidative stress to senescence. H_2O_2 induces senescence in a wide variety of cell types, and induces similar signaling pathways to replicative senescence. Activation of p53, p21, and various other cell cycle regulators has been shown in H_2O_2-induced senescence. Additionally, due to recent evidence that replicative senescence causes increased ROS production in the mitochondria, it is possible that oxidative senescence is downstream of replicative senescence (51; 52).

In the field of aging, redox-induced senescence has been a topic of great interest. It is believed that the many of the phenotypes associated with aging are due to senescence or cell death caused by long term oxidative DNA damage. Due to this theory, antioxidant therapy has been pursued as a way to slow or prevent aging. Unfortunately, it does not appear that ubiquitous use of antioxidants is the fountain of youth that it was originally believed to be. In fact mortality may increase with the long-term use of certain antioxidants such as Vitamins A and E (53). This could be due the lack of specificity of most antioxidants, or compensation by the body's own antioxidant systems to balance the increased level of exogenous antioxidants. Evidence from studies in the

cardiovascular system demonstrates the requirement for reactive oxygen species in maintaining normal physiological function.

Although much of the literature concentrates on senescence induced by oxidative stress, it appears that blocking H2O2-based signaling can also cause senescence (54). This suggests that too high or low levels of hydrogen peroxide can lead to senescence. Since Poldip2 regulates ROS production via Nox4, there is a potential role for Poldip2 in senescence.

Oncogene-induced senescence (OIS)/Tumor suppressor loss senescence

Senescence induced by oncogenes or tumor suppressor loss has been proposed as a mechanism to prevent primary cells from becoming stem cells. OIS occurs in vivo in tumors, potentially to restrict the growth of benign tumors (55). It shares the SASF phenotype with redox-senescence possibly due to activation of NF-κB (56). One of the primary oncogenes involved in OIS is Ras via Raf-MEK-ERK activation (57). Though OIS is currently considered unique from other types of senescence, it has been hypothesized that OIS occurs in response to DNA damage caused by ROS or other sources (58). Indeed many of the same cell cycle checkpoint pathways are activated, including ATR/ATM and p53 (59).

Immortality

MEFs

The development of NIH3T3 cells provided a means for advancing knowledge on overcoming senescence in somatic cells. The cells enter a growth phase for several passages following initial preparation from embryos. Around passage 5-6, mouse embryonic fibroblasts (MEFs) enter a senescent phase. If they are continuously

passaged, they will have a second growth phase, at which point they are immortalized. It should be noted that mouse cells express telomerase and maintain long telomeres in culture, so MEF senescence is independent of the telomere. It is hypothesized that MEF senescence may be a result of oxidative stress (34). Since the cells reliably spontaneously immortalized after being passaged for a set number of doublings, it was clear that a subpopulation of cells exhibited renewed growth (60). A number of proteins have been identified as responsible for the immortalization of MEFs, but primarily mutations in Ras, mortalin (Hsp70) or p53 are responsible for MEFs spontaneously exiting senescence (61). Many of the same mutations have subsequently been identified in cancers, providing a greater understanding of how point mutations can lead to unrestricted cell growth.

Biomarkers of Senescence

For researchers, it is important to have reliable methods to quantitatively measure senescence. The most obvious is lack of growth; however, this is not specific to senescent cells. Morphological changes can be observed; senescent cells tend to be larger and flatter than growing cells (62).

One of the first reliable *in vivo* assays for senescence to be identified was the SA-β-gal assay (63). Cells are incubated in a pH 6.0 buffer in the presence of Xgal, which is cleaved to produce an insoluble blue compound, visible by light microscopy. It is believed that the activity of β-gal at pH 6 in senescent cells is caused by the increased size of the lysosomal compartment in senescent cells. Due to the role of autophagy in senescence, the lysosome becomes enlarged and *GLB1* mRNA and protein is increased (64).

An alternate method of identifying senescence is by Senescence-associated heterochromatic foci (SAHF). This can be visualized in cells by staining for methylated

Lys 9 of Histone H3. In senescent cells, the total level will decrease and become a punctuate pattern. Another marker is HP1γ, which translocates to promyelocytic leukemia (PML) bodies in the nucleus during senescence (65).

Microarray studies of senescent cells have shown an induction of many inflammatory genes. As a result of this inflammatory response, a variety of cytokines are secreted. This phenotype is described as the senescence-associated secretory phenotype (SASF). Senescence-associated secreted factors are IL6, IL8, IGFBP7, MMP3 (66).

Senescence and the cell cycle

Though senescence seems similar to quiescence with respect to the cell cycle, there is a distinct difference. Quiescent cells will readily enter a growth phase with the introduction of a growth factor, whereas senescence is considered an irreversible process. It has been suggested that cellular senescence is protective to prevent unlimited cell proliferation (34). Although we can experimentally overcome senescence by manipulating gene expression of various factors, there is no obvious physiological role for a reversal in senescence (67). Senescent cells, however, share many cell cycle characteristics with quiescent cells, which can make distinguishing them experimentally challenging. Both are delayed at the G1/S transition of the cell cycle. Due to these similarities, it is conceivable that Poldip2 could be implicated in cellular senescence.

Proliferative disease

Numerous diseases are caused by excess cell proliferation including cancer, atherosclerosis, post-angioplasty restenosis, and autoimmune disease. Other conditions such as organ rejection after transplant are caused by proliferation of immune cells. Here, I will discuss the proliferative diseases with the highest rates of mortality: cancer, atherosclerosis and restenosis. Considering the potential role of Poldip2 in proliferation,

inhibition of Poldip2 function could reduce the progression of the diseases discussed in this section.

Cancer, or malignant neoplasm, is the collective term for diseases that cause unrestricted cell growth. Generally, cells begin to divide and form tumors, which can metastasize and invade other parts of the body. If the cell growth is not controlled, it will cause death. The prevalence of cancer in the US according to the latest NCI statistics (2008-2010) is that 41% of men and women born today will be diagnosed with some form of cancer in his/her lifetime, with an overall 5-year survival rate of 66% (68). The causes of cancer are extremely diverse and still not well understood.

Hereditary cancers can typically be traced to a particular inherited genetic defect. Environmental and chemical factors such as radiation, tobacco and alcohol use, and carcinogenic substances can increase cancer risk by causing DNA mutations or by stimulating cell growth. Additionally, certain viruses such as HPV can cause cancer by integrating into cellular DNA within protective genes, eliminating their activity (69). Dietary and lifestyle factors such as maintaining a healthy weight, eating fruits and vegetables and avoiding refined foods can be protective. Although the mechanism is not completely understood, it potentially occurs by strengthening the immune system and avoiding carcinogenic compounds in foods (69).

Atherosclerosis and the resulting coronary artery occlusion is one of the leading causes of death worldwide. It is characterized by narrowing of arteries caused endothelial cell damage which results in the exposure of VSMCs to platelet derived growth factor (PDGF) causing excess growth into the intima (70). Cholesterol plaques that form in atherosclerosis also contain oxidized LDL, which has been demonstrated to promote VSMC proliferation (71). Plaques can block blood vessels over time, and in some cases rupture causes embolisms that can block blood flow to the heart or brain causing stroke or myocardial infarction, respectively (72).

There are several key risk factors for atherosclerosis, including high blood pressure, high cholesterol, diabetes and smoking. Recent evidence suggests that the formation of atherosclerosis is tied to an autoimmune response to endothelial cell stress (73). Macrophages are recruited to the site and attempt to destroy the deposited cholesterol, forming foam cells in the processes. Smaller, less mature plaques can be stabilized by aggressive cholesterol control and medication. Treatment of a mature plaque is generally mechanical in nature, either angioplasty or coronary bypass. A certain percentage of angioplasty patients experience a recurrence of the original blockage, which is called restenosis.

Restenosis is typically caused by damage to the endothelium during angioplasty, and can result in additional lipid buildup, thrombus formation and proliferation of VSMCs (74). Recent evidence suggests that drug eluting stents that locally target antithrombolitic or antiproliferative drugs to the affected area can dramatically reduce restenosis rates (75).

Pharmacological inhibitors of the cell cycle

The ultimate goal for research on potential cell cycle regulators such as Poldip2 is the development of anti-proliferative therapies. The cell cycle is an attractive target for pharmacological intervention to treat proliferative diseases such as cancer and atherosclerosis. Additionally, such drugs can be used to prevent tissue rejection after transplantation by reducing the proliferation of immune cells.

The earliest chemotherapy drugs were derived from chemicals that were found to be effective cancer treatments before their mechanism was well known. The earliest effective cancer therapeutics, the alkylating agents, were derived from the chemical weapon mustard gas by Goodman and Gillman (76). It is now known that alkylating agents damage DNA, particularly the N-7 position of guanine, which results in DNA

crosslinking (77). This ultimately results in cell cycle arrest and apoptosis. Many nitrogen mustard analogues have been derived including chlorambucil, cyclophosphamide, and mechlorethamine. Many analogues also exist such as platinum analogues like cisplatin. Once the cell cycle based mechanisms of these drugs became clearer, more targeted approaches to drug design were initiated.

One of the earliest examples of a cell cycle pathway being targeted experimentally is folic acid. Folic acid analogues were designed to disrupt the function of enzymes that require folic acid after it was recognized to be important for cell proliferation (78; 79). These antimetabolite drugs interfere with DNA or RNA synthesis, which ultimately blocks cell growth in the S-phase. Methotrexate binds dihydrofolate reductase, which prevents nucleoside production (80). Later, purine and pyrimidine antagonists such as 6-MP and 5-FU were developed to prevent nucleotide production. These drugs prevent the synthesis of necessary components for the replication of DNA (81).

Later efforts discovered that plant alkaloids such as taxane analogues from yew trees and vinca analogues from periwinkle interfere with microtubule dynamics (82). This results in mitotic defects due to the lack of chromosome segregation and cytokinesis.

Certain types of antibiotics have proven effective as cancer therapeutics. Anthracyclins blocks DNA synthesis and causes DNA damage partly by inhibiting topoisomerase II, and bleomycin causes DNA strand breaks by an unknown ROS-dependent mechanism.

Modern efforts at chemotherapy drug discovery have focused more specifically on cell cycle signaling. Blocking growth receptor signaling pathways such as the MAP kinase cascade has the advantage of potentially inhibiting multiple cancer processes: proliferation, invasion/metastasis, cell differentiation and promoting cell death (83). Although mTor inhibitors such as rapamycin and sirolimus are primarily used to prevent

transplant rejection by inhibiting the cell cycle in T-cells, there is evidence of efficacy in cancer treatment.

Kinase inhibitors have also been attractive targets for chemotherapy; imatinib, a small molecule inhibitor of BCR-Abl, has great efficacy due to its specificity for cancer cells and growth pathways targeting. Several Cdk inhibitors are currently in clinical trials as anti-cancer agents. (84). The E2F pathway has also been targeted to prevent cell replication, although no drug has yet been approved (85).

Having a better understanding of the structure and function of cell cycle proteins is key in targeted pharmaceutical development.

Dissertation Aims

In this introduction, I have described the current state of knowledge on Poldip2. Although we originally identified Poldip2 as a p22phox-interacting protein that activates Nox4, it is likely that Poldip2 plays a larger role in cellular processes than the regulation of Nox alone. The objective of this work is to increase our understanding of Poldip2 structure and function. To accomplish this goal, I performed *in silico* analysis of Poldip2 structure to identify potential interacting domains. I then performed microarrays to characterize downstream gene changes associated with loss of Poldip2. Based on pathway analysis, I hypothesized that Poldip2 regulates cell cycle progression, and performed a detailed analysis of cell cycle signaling in MEFs lacking Poldip2. In this dissertation, I identify two primary growth-related signaling pathways regulated by Poldip2 and describe a new role for Poldip2 as a cell cycle regulator. This work provides a foundation for future research into its potential as a therapeutic target for proliferative diseases.

Chapter 2

Poldip2 Structure

Introduction

Poldip2 has been demonstrated to play diverse biological roles including DNA replication and repair (6; 8), mitochondrial function and elongation (3; 9), downstream signaling of a cell adhesion receptor (4), cytoskeletal reorganization and regulation of reactive oxygen species production (5), and maintenance of vascular structure and function (12). To better understand how Poldip2 mediates such diverse functions, we set out to investigate Poldip2 structure.

As described below, the *Poldip2* gene product is composed of two functional domains based on homology: at the N-terminus is the YccV domain, and at the C-terminus is the ApaG domain. In bacteria, ApaG and YccV are two independent proteins. This raises the possibility that the structural domains that make up Poldip2 may have independent functions. Though little is known about the 3D structure of the Poldip2 protein, the structures of ApaG (86) and a YccV-like protein (87) have been solved. Using modern folding algorithms, we can more accurately predict the structure of Poldip2 using the solved structures of the domains that make up the full-length protein. Given the assumption that the 3D structure of these domains should also be conserved even though these two independent proteins have become a single protein, we have undertaken to study the structure of Poldip2 *in silico*. Our goals are to identify potential surface phosphorylation sites on Poldip2, and to identify potential protein-protein interaction sites or other surface features that are not obvious based on primary sequence analysis.

The YccV-like domain is located on the N-terminal half of the full-length Poldip2 protein, AA 74-200. Little is known about the function of the YccV protein (also called HspQ) for which the domain is named. The structure of the YccV protein is composed of a beta sheet structure with a terminating alpha helix (87). A study in *E. coli* found that YccV binds to hemimethylated DNA at the bacterial origin of replication, *oriC* (88). This

results in a decrease in the expression of *dnaA*. Additionally, YccV knockout resulted in overexpression of *dnaA*. DnaA is a protein responsible for the initiation of DNA replication in prokaryotes by promoting the unwinding of DNA (89). Interestingly, eukaryotic mitochondria have similar *ori* sequences. In humans they are known as *oriH* and *oriL*. A second study implicates YccV as a heat shock factor, designated HspQ (90). The authors propose that YccV alters the stability of DnaA, rather than regulating the transcription of the *DnaA* gene. The authors did not observe YccV binding to *ori* sequences, indicating that some controversy still exists about the binding and function of YccV/HspQ.

There is evidence of YccV domain proteins functioning in the mitochondria. A study of MUS-10, a protein with a C-terminal YccV-like domain in the eukaryotic bread mold *Neurospora crassa,* reports altered mitochondrial morphology, instability of mtDNA and cell growth defects associated with senescence (91).

The structure of the bacterial protein homologue of the second Poldip2 domain, ApaG, has been solved in the bacteria *Xanthomonas axonopodis* pv. *Citri*, *Vibrio cholera, Shewanella oneidensis, and Bordetella pertussis* (86). The authors also identified a potential ATP or GTP binding motif GXGXXG, but were not able to show binding *in vitro*. All four ApaG homologues share a structure containing 7 antiparallel β-strands, which form 2 β-sheets, one with 3 strands and one with 4 strands. This β-sandwich structure resembles the fibronectin type III (Fn3) fold, which is reported to function as a protein interaction domain or a cell adhesion domain on extracellular proteins (92). A recent study found that an Fn3 domain protein interacts with actin and maltos binding protein in bacteria (93).

Other clues about the function of the ApaG domain come from CorA, a bacterial ApaG domain protein (94). CorA has been established as a divalent cation transporter of Mg2+ and Co2+ (95). In bacteria, CorA forms a pentamer gated funnel that crosses the

cell membrane (96). The ApaG domain section of the protein is completely internal in the case of CorA, suggesting that it plays a role in protein-protein interactions between the units of the pentamer.

Due to the similarity with bacterial homologues, we believe an analysis of the relationship between structure and function in human Poldip2 based on the solved crystal structures of the bacterial YccV and ApaG proteins could identify important regions for future study. Based on the high level of homology between the ApaG domain and bacterial ApaG, we hypothesize that some of the highly conserved residues in this domain are important for the function of Poldip2. We report here on an *in silico* analysis of Poldip2 homologues, a hypothetical 3D structure of each domain and full-length Poldip2, and identify residues for mutation that may disrupt Poldip2 function.

Methods

Sequence alignment

A total of 51 different Poldip2 sequences were compared for structural similarities using the ClustalW-based Multiple Sequence Viewer in Maestro (Schrödinger, LLC). An identity threshold of 75% was used to visualize the degree of conservation of Poldip2 residues. The following species *Poldip2* sequences were compared to the query sequence, *Homo sapiens* (NP_056399.1), listed in order of their sequence similarity to the rat sequence, with corresponding accession number: *Pan paniscus* (XP_003812711.1), *Pongo abelii* (XP_002827205.1), *Macaca mulatta* (NP_001248642.1), *Nomascus leucogenys* (XP_003277155.1), *Oryctolagus cuniculus* (XP_002718977.1), *Sus scrofa* (XP_003358222.2), *Pteropus alecto* (ELK01827.1), *Canis lupus familiaris* (NP_001240832.1), *Bos Taurus* (NP_001091572.1), *Otolemur garnetti* (XP_003797211.1), *Loxodonta Africana* (XP_003416901.1), *Rattus norvegicus* (ACN54045.1), *Mus musculus* (NP_080665.1), *Monodelphis domestica*

(XP_001368593.1), *Saimiri boliviensis boliviensis* (XP_003931567.1), *Papio Anubis* (XP_003912542.1), *Bos grunniens mutus* (ELR47964.1), *Equus ferus caballus* (XP_001918039.2), *Ailuropoda melanoleuca* (XP_002912422.1), *Cricetulus griseus* (XP_003495855.1), *Taeniopygia guttata* (NP_001232073.1), *Mustela putorius furo* (AES04410.1), *Ovis aries*, *Xenopus tropicalis* (NP_001017098.1), *Felis catus* (XP_003996568.1), *Xenopus laevis* (NP_001085182.1), *Sarcophilus harrisii* (XP_003770085.1), *Oryzias latipes* (XP_004075778.1), *Takifugu rubripes* (XP_003968776.1), *Danio rerio* (NP_997879.1), *Myotis davidii* (ELK26922.1), *Pan troglodytes* (XP_003315593.1), *Gorilla gorilla gorilla* (XP_004042115.1), *Salmo salar* (ACN60338.1), *Caligus rogercresseyi* (ACO10246.1), *Acromyrmex echinatior* (EGI61673.1), *Camponotus floridanus* (EFN64395.1), *Harpegnathos saltator* (EFN89414.1), *Ixodes scapularis* (XP_002405939.1), *Megachile rotundata* (XP_003700238.1), *Nasonia vitripennis* (XP_001599719.1), *Apis Florea* (XP_003690564.1), *Bombus terrestris* (XP_003394842.1), *Bombus impatiens* (XP_003485599.1), *Apis mellifera* (XR_014973.2), *Metaseiulus occidentalis* (XP_003742125), *Culex quinquefasciatus* (XP_001863572.1), *Anopheles gambiae* (XP_322007.4), *Acyrthosiphon pisum* (XP_001948226.2), *and Drosophila melanogaster* (NP_649540.1). Additionally, a sequence alignment was performed comparing the *Homo sapiens* ApaG domain with 15 species of bacteria, *A. ferrooxidans* (B7J8G8), *B. bacteriovorus* (Q6MK56), *D. aromatica* (Q47AB8), *E.coli* K12 (P62672), *E. tasmaniensis* (B2VGP5), *M. magneticum* (Q2VZE7), *O. anthropi* (A6WVX4), *R. etli* (Q2KCU6), *S. dysenteriae* (Q32K44), *S. choleraesuis* (Q57TH1), *S. enteritidis* (B5R1S7), *S. oneidensis* (Q8EB92), *T. denitrificans* (Q3SGR3), *X. axonopodis citri* (Q8PP26), and *Y. pestis* (Q1C0H6).

in silico **Protein Folding**

The I-TASSER server (97) was used to make 3-dimensional models of Poldip2 domains based on sequence similarity to ApaG and YccV. The Poldip2 domains were individually submitted to the I-TASSER server in order to use the existing data for the ApaG and YccV structures in the calculation of the Poldip2 structure. The full length Poldip2 structure was modeled using the Phyre2 server (98). Maestro (Schrödinger) and Pymol (Schrödinger) were used to visualize and analyze the modeling data. Solvent accessibility was determined using a 1.4Å sphere as a representation of a water molecule rolled over the molecular surface of the protein.

Site directed mutagenesis

Site directed mutagenesis was performed using the Agilent Technologies Quickchange Lightning Kit. Five Poldip2 mutants were generated by single mutations to the Myc-Poldip2 template described in Lyle et al.(5). The following amino acid changes were made: G300L, T238A, T292A, T295A, T292E.

Experimental Results

Poldip2 Domain Structure

To study the primary structure of Poldip2, we performed a protein BLAST search using the human amino acid sequence. As noted above, two functional domains were reported in Poldip2, corresponding to a YccV-like domain at the N-terminus and an ApaG domain at the C-terminus of the protein (Figure 2.1). In order to gain insight into which residues are conserved across species we performed a sequence alignment of Poldip2 homologues using the ClustalW algorithm (99) (Figure 2.2).

A total of 51 different primary sequences from mammals, insects, fish and birds were aligned with the human Poldip2 sequence to identify evolutionarily conserved regions and residues. Sixty-five residues out of 368 total amino acids were identical

across all 51 sequences; 46 of those were within the 121 AA that make up the ApaG domain.

The YccV also showed a high degree of conservation overall: 78 AA out of 126 AA that make up the YccV domain show 80% similarity among the 51 sequences aligned. Furthermore, 10 AA were identical across all 51 species.

The sequences with the lowest degree of similarity to the human sequences were from insects. Much of the difference occurs within the first 50 amino acids, which has been proposed to function as a mitochondrial targeting sequence or signaling peptide in the mammalian Poldip2 (2; 3). This sequence is cleaved, lowering the molecular weight of Poldip2 from 42 kDa to 37 kDa. The functional protein length has not been measured in insect cells, but insect Poldip2 sequences lack the signaling peptide altogether. Additionally, the *D. melanogaster* Poldip2 sequence contains a 74 AA insertion within the YccV domain that is not present in any other species. However, the *drosophila* sequence comes from a protein prediction based on the full genome sequencing and has not been verified, so we cannot rule out the possibility that the full length Poldip2 protein in *drosophila* does not contain this insertion.

In silico Poldip2 modeling: YccV domain

Though no crystal structure of Poldip2 has yet been solved, both the YccV-like domain and the ApaG domains that make up the protein have homologues that have been crystallized (86; 87). Furthermore, since the sequences alignments indicate that these domains have a high degree of conservation, we believe that the domains will also be conserved in their 3-dimensional structure. In order estimate the tertiary structure of Poldip2, we used the I-TASSER protein folding server (97) to predict the folding of the N-terminal YccV domain and the C-terminal ApaG domain independently.

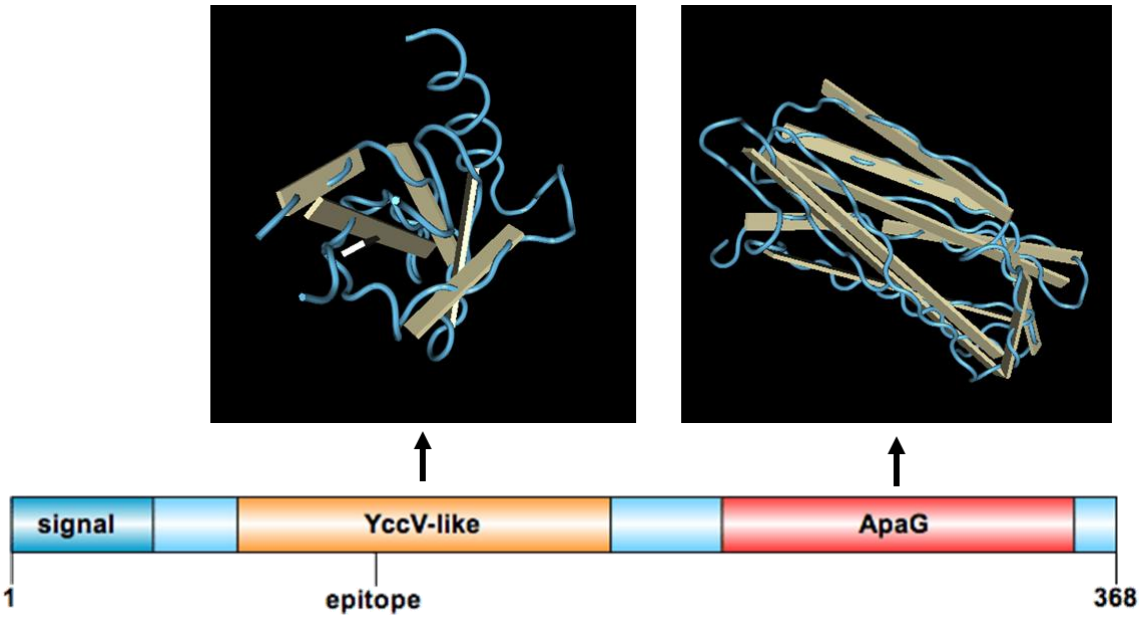

Figure 2.1. Poldip2 structural domains. Poldip2 is composed of a 35 amino acid signaling peptide or mitochondrial localization sequence, a YccV-like domain and an ApaG (DUF525 superfamily) domain. The structure of the YccV-like protein is composed of β-sheets with a terminating alpha helix. The ApaG domain is made up of 7 β-strands that form a fibronectin type III –like fold.

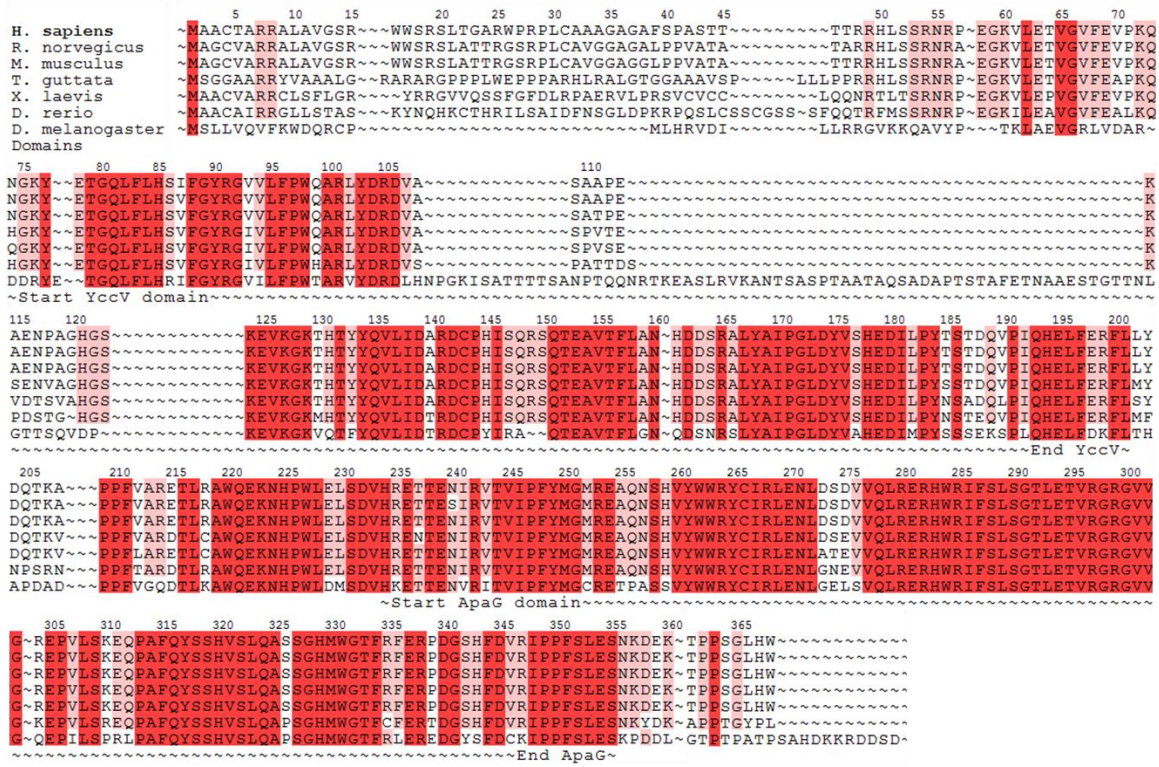

Figure 2.2. Poldip2 Sequence alignment. Poldip2 homologues from 51 different species were compared using the ClustalW algorithm. Representative examples were chosen for visualization. The residues are shaded by their degree of similarity in the full 51 species. The lightest shad of pink corresponds to 80% of that amino acid being identical across all species. The darkest red corresponds to 90% conservation across all sequences.

Since Poldip2 has a cleaved N-terminal signaling peptide or mitochondrial targeting sequence in all the cell types that we have tested, we have removed this sequence from the input sequence to more closely resemble the fully processed Poldip2 protein. Additionally, we did not include the linker region between the YccV domain and ApaG domain in our domain-specific predictions. The input sequence of AA 74-200 of the human Poldip2 sequence represents the N-terminal YccV domain peptide used for folding (Figure 2.3A). The Poldip2 YccV domain model was superimposed on the crystallized model of the YccV domain crystallized from *E. coli* (Figure 2.3B). Both models share similar β-sheet structures, but the generated model for Poldip2 lacks a C-terminal alpha-helix that appears in the *E. coli* YccV structure. The conserved residues from the sequence alignment were mainly internal residues, though there was a region of surface similarity that may correspond to a binding pocket (Figure 2.3C). The generated model is scored on multiple parameters. The C-score is a confidence score based on simulation algorithms that ranges from [-5-2], with 2 being the highest degree of confidence that the model has the same structure as the predicted protein. The TM-score is a scale for measuring the similarity between two proteins, in this case the modeled protein and the template used in the model. The score ranges [0-1], where zero is no similarity and one is identical. It is generally accepted that a score of >0.5 indicates correct topology (97).

In silico Poldip2 modeling: ApaG domain

The C-terminal sequence that was used for modeling was AA 234-355, which contains the entirety of the ApaG domain, but does not contain the linker region between the two domains (Figure 2.4A). Superimposing the generated model with the crystallized ApaG domain reveals a high level of structural similarity (Figure 2.4B). Both structures are composed of seven β-strands which form two β-sheets, one with three β-strands and one

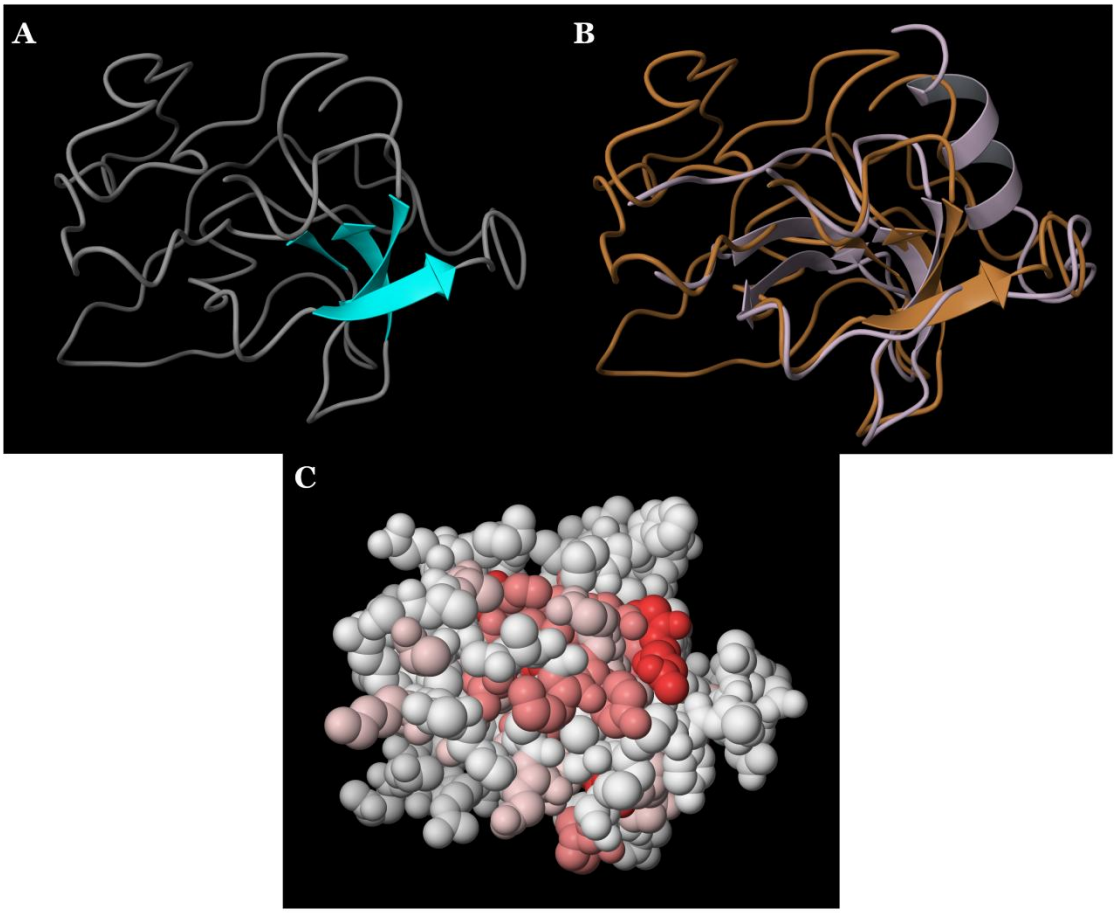

Figure 2.3. Human Poldip2 YccV domain model. The YccV domain from human Poldip2 (AA 74-200) was used to generate a hypothetical 3-dimensional structure. (A) The Poldip2 YccV domain is displayed as a ribbon model. C-score=-1.20. (B) The Poldip2 model (brown) is overlaid on top of the crystallized structure of the hypothetical YccV protein from *E. coli* (grey). TM score = 0.56±0.15. (C) A space-fill model was generated and colorized based on 80% sequence similarity between the human Poldip2 YccV domain and the amino acid sequences of 5 bacterial YccV proteins.

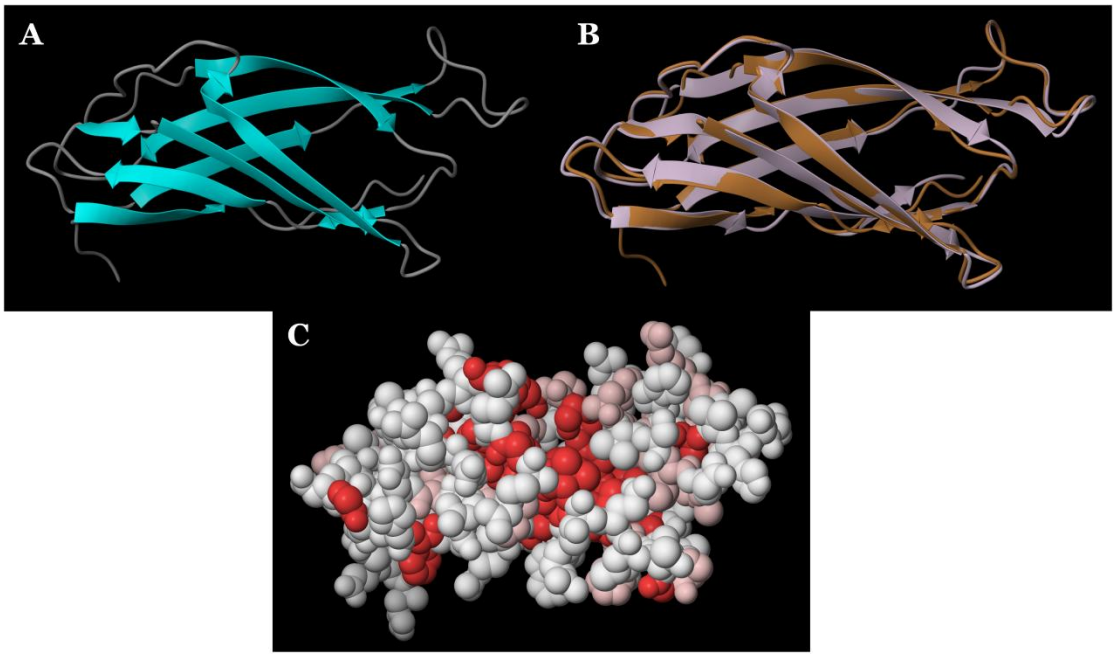

Figure 2.4. Human Poldip2 ApaG domain model. The ApaG domain from human Poldip2 (AA234-355) was used to generate a hypothetical 3-dimensional structure. (A) The Poldip2 ApaG domain is displayed as a ribbon model. C-score=1.68, TM-score 0.95±0.05 (B) The Poldip2 model (gold) is overlaid on top of the crystallized structure of the ApaG protein from *X. citri* (white). (C) A space-fill model was generated and colorized based on 80% sequence similarity between the human Poldip2 ApaG domain and the 51 sequences displayed in the sequence alignment (Figure 2.2).

with two β-strands. The high TM-score indicates a high level of confidence that the modeled structure represents the true molecular structure of the ApaG domain.

One interesting feature that the 3D model of Poldip2 reveals is the presence of hydrophobic residues on the surface of the protein, particularly in the region between the two β-sheets. These hydrophobic residues are among the most highly conserved residues in the protein. Typically hydrophobic residues are protected internally to avoid contact with the aqueous environment in cells. Due to the high level of conservation seen in the sequence alignment and evidence in the literature that ApaG proteins are involved in protein-protein interactions, we predict that the conserved, hydrophobic surface residues are important for protein interactions.

in silico Poldip2 modeling: full length Poldip2

The full length of human Poldip2 (368AA) was used for the combined model (Figure 2.5). Though 55% of residues were modeled at >90% confidence, the majority of the region with high confidence was located in the C-terminal ApaG domain. The combined structure resembles the two independently folded domains connected by a linker region.

The mitochondrial targeting sequence/signaling peptide at the N-terminus of Poldip2 folds into two α-helix structures. The linker region between the two protein domains also forms α-helixes.

The YccV domain in the full model more closely resembles the solved YccV structure than the Poldip2 YccV domain model, containing a βsheet and terminating α-helix. The highly conserved hydrophobic residues in the ApaG domain are left exposed, supporting their potential role in Poldip2 function.

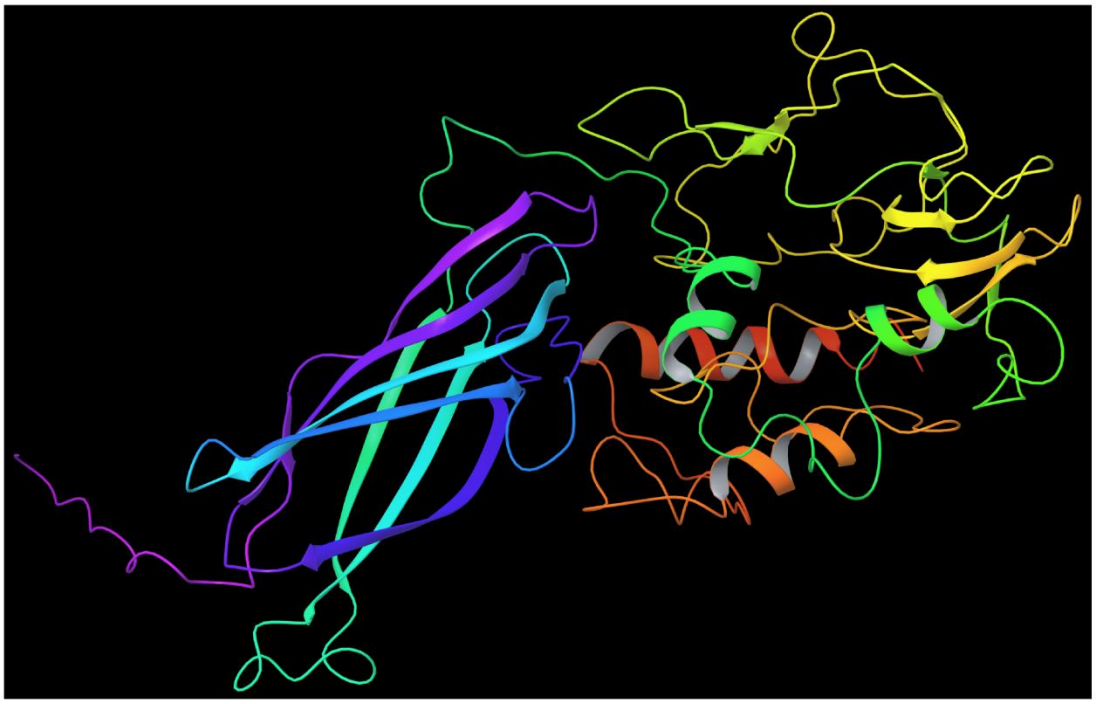

Figure 2.5. Model of full length Poldip2. The full length of Poldip2 was modeled using the Phyre2 folding server. The color corresponds to the amino acid location in the protein sequence from N-terminus (red) to C-terminus (purple).

Sequence and Structural comparison between bacterial and human ApaG

The high level of conservation in the ApaG domain of higher organisms led us to hypothesize that the bacterial ApaG would also contain conserved residues that could be important for protein function. A total of 15 ApaG proteins from different species of bacteria were aligned with human Poldip2 (Figure 2.6A). A number of identical residues are seemingly spread out across the primary sequence of the ApaG protein; however, when looking at conserved regions on the ApaG model, they are primarily located in a single binding region or pocket in 3-dimensional space (Figure 2.6B,C). The most highly conserved residues are clustered around a dimple in the protein surface (Figure 2.7A). By applying a solvent accessibility surface to the molecule, an accessible pocket is revealed among the highly conserved, hydrophobic residues (Figure 2.7B). This pocket is large enough for an ion, or would allow close association with an amino acid in another protein or other unidentified molecule. It is located at the site of the potential ATP binding site motif GXGXXG, which is 100% conserved across all 51 higher organisms and all 15 species of bacteria.

Investigating Poldip2 function and binding to p22phox

Due to the Fn3 fold-like structure in the C-terminal ApaG domain, we believe the C-terminal portion of Poldip2 is more likely to be involved in protein-protein interactions. Therefore, we concentrated on the C-terminal model to identify key surface residues (Figure 2.4C) to mutate to attempt to disrupt binding between Poldip2 and its identified binding partner, p22phox (5). We have initially focused mainly on potentially phosphorylation sites, due to preliminary data generated in the lab suggesting Poldip2 phosphorylation (data not shown). Looking specifically for residues on the surface of the protein that are conserved, we chose to make the following point mutations: G300L,

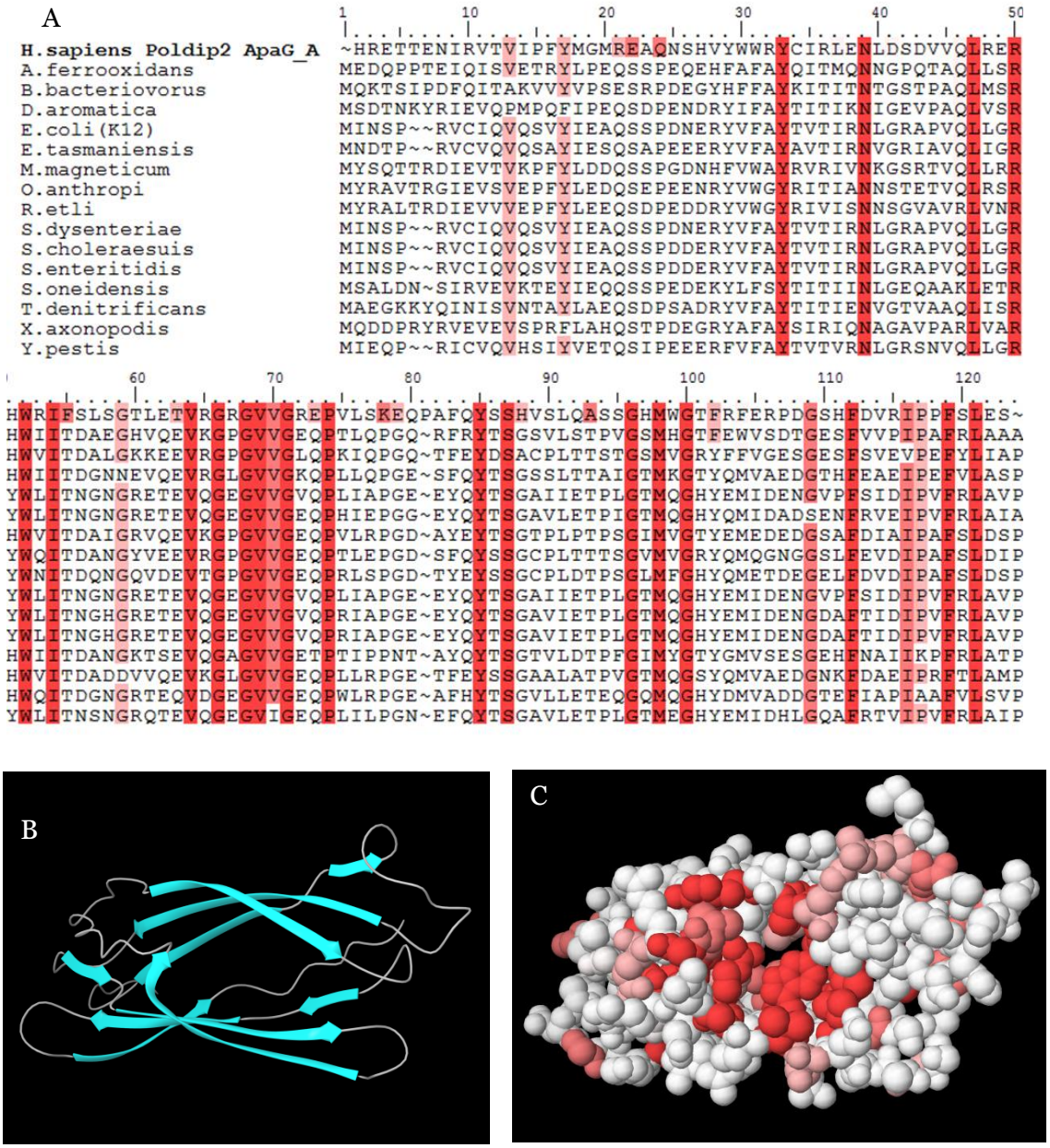

Figure 2.6. The human Poldip2 ApaG domain has a high degree of sequence similarity to bacterial ApaG proteins. (A) The human Poldip2 ApaG (AA234-355) domain was aligned with 15 bacteria ApaG protein sequences using the ClustalW algorithm. The similarity color threshold was set to 80%: 100% identical residues are displayed red, 90% similar is pink and 80% similar is light pink. (B) A ribbon model is shown. The coloration was applied to the model generated of the ApaG domain and displayed as a (C) space fill model.

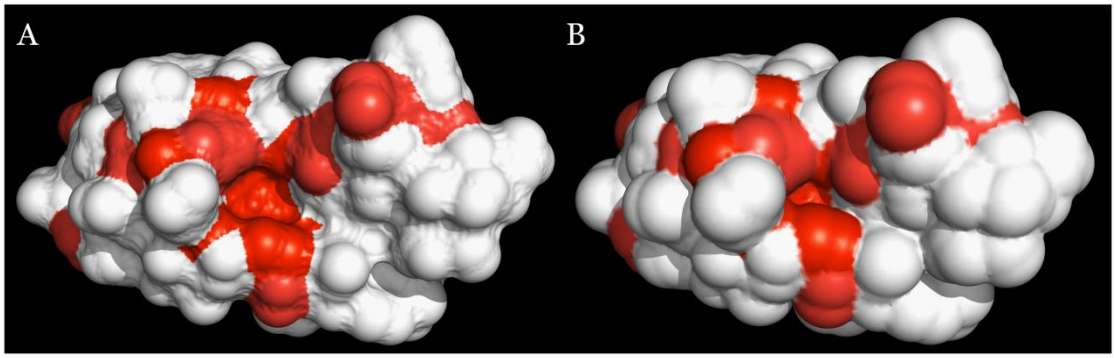

Figure 2.7. ApaG surface structure and solvent accessibility. (A) A surface model was generated of the human Poldip2 ApaG domain using Pymol. (B) The surface was analyzed for solvent accessibility using a 1.4Å threshold to correspond to a water molecule. Red corresponds to 100% conserved residues from the Figure 2.5 alignment, pink corresponds to 80% conserved residues.

T238A, T292A, T292E, T295A, S319A and S319E (Figure 2.8). G300 is one of the glycine residues in the potential ATP binding motif, and was chosen to potentially disrupt Poldip2 protein-protein binding or other function. These mutated proteins will be tested in future experiments.

Conclusions

Ultimately, understanding the function of Poldip2 will require study in the context of a biological system. However, we can gain enormous insight into the function of poorly studied proteins such as Poldip2 by investigating structural similarities to other better studied proteins, and identifying residues that have been well conserved. Though Poldip2 does not appear to be a member of a protein family, its two main domains can help us understand and classify Poldip2. In this study, we have analyzed the overall sequence of Poldip2, generated hypothetical structures of the YccV-like domain and the ApaG domain, predicted binding sites on the surface of ApaG and engineered point mutations in a Poldip2 plasmid that could influence its function.

Little is known about the function of the YccV protein in bacteria; however, two studies have proposed that YccV (HspQ) may bind DNA or regulate the expression of DnaA, which is involved in prokaryotic DNA replication. Due to the structural similarities between the YccV-like domain of *Poldip2*, and the YccV protein in *E. coli*, and the evidence suggesting that Poldip2 can be localized to the mitochondria, one of its functions could be promoting mitochondrial DNA replication. There is evidence for Poldip2 interacting with DNA binding proteins in the mitochondria such as mtSSB and TFAM (3). Though mitochondrial replication is a regulated part of the eukaryotic cell cycle involving mitochondrial elongation and fragmentation, the DNA replication machinery in mitochondria more closely resembles prokaryotic machinery compared to that of eukaryotic cells. However, since DNA is replicated in both cases, we cannot

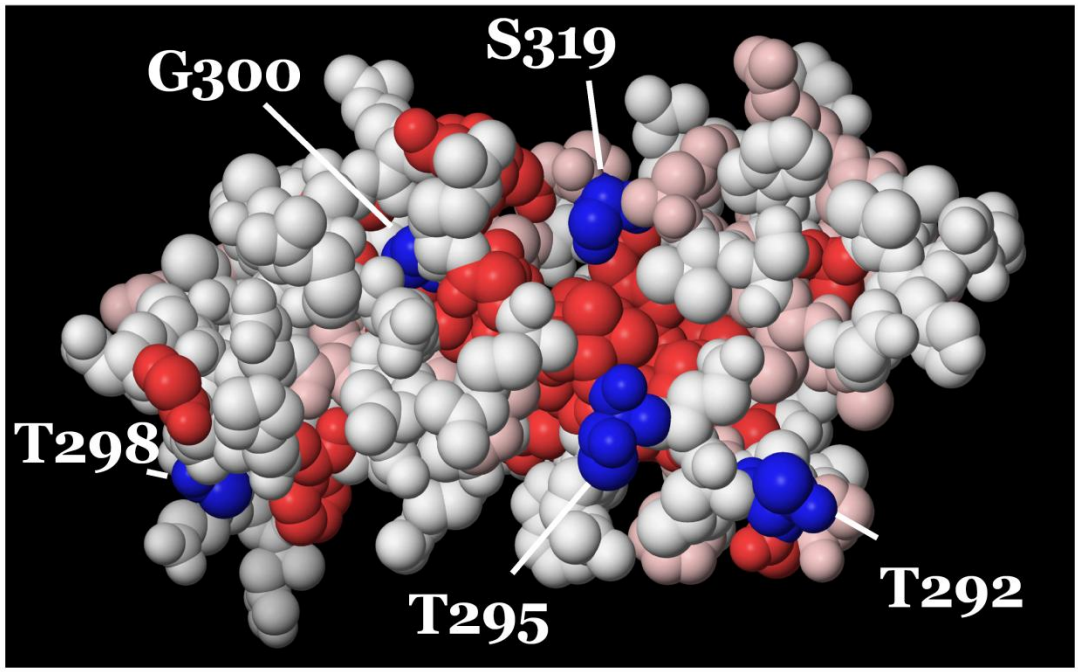

Figure 2.8. Mutated residues in Poldip2 ApaG domain. Residues targeted for mutation are highlighted in blue on the space fill model of the human Poldip2 ApaG domain.

dismiss the possibility that Poldip2 is involved in mitochondrial genome replication as well as DNA duplication. Mitochondria replication is known to be synchronized to the cell cycle, presumably to prevent ROS produced during oxidative phosphorylation from damaging chromosomal DNA (100). Poldip2 could be a link between mitochondrial replication and nuclear replication. Overexpression of Poldip2 results in increased elongated mitochondria whereas Poldip2 silencing increases fragmented mitochondria (9), perhaps due to effects on mitochondrial DNA duplication.

The protein MUS-10 from the bread mold N. *Crassa* contains a C-terminal YccV domain. Knockout of MUS-10 causes mitochondria defects similar to those shown in Poldip2 knockdown cells, including defects in mitochondrial morphology (9; 91). Though the YccV-like domain makes up only a small portion of the MUS-10 protein, the functional similarities between knockout of both proteins may suggest YccV plays an important role in the mitochondria.

The model of the ApaG domain of Poldip2 shares a high degree of structural similarity to the ApaG protein. The bacterial ApaG containing protein CorD is the best studied; it has been identified as a Mg^{2+}/Co^{2+} transporter. One of the cell compartments with the greatest concentration of magnesium is the mitochondria. Additionally mitochondria uptake cobalt; however, cobalt can be toxic above low quantities. Magnesium is an essential ion in both DNA duplication and ATP production. In DNA duplication, magnesium is important for DNA stability and necessary for the activity of many enzymes involved in DNA duplication. ATP must contain a bound magnesium ion to be biologically active. Though Poldip2 lacks the structural elements that make CorD a channel, it could retain divalent ion binding capabilities. Bound Mg^{2+} is known to play a role in ATP binding in kinases (101) and is necessary for the activation or activity of many enzymes (102; 103).

The Poldip2 ApaG domain contains an ATP/ADP-binding motif GXGXXG adjacent to a solvent-accessible structure that resembles an active site. This binding motif is also known as the G-loop, and is known to be conserved in many kinases to correctly orient ATP for phosphate transfer (104). Though an *in vitro* experiment has been performed to assess the binding of ATP or GTP to the bacterial ApaG protein, the authors did not identify any measurable binding (86). There remains the possibility that ATP binding only occurs after activation of ApaG by post-translational modification or binding to an ion or other molecule. The G-loop raises the possibility that Poldip2 may act as a kinase. Binding to a variety of proteins involved in DNA duplication and mitochondrial dynamics has been demonstrated, but phosphorylation of these proteins by Poldip2 has not been assessed.

Another possibility is that Poldip2 has ATPase activity, which would generate measurable ADP and inorganic phosphate. The released energy could drive motion along actin fibers, phosphorylate proteins or transport ions across membranes.

Mitochondria are the primary producers of ATP in eukaryotic cells. Since YccV has been shown to influence the activity of DnaA, and CorA with an ApaG domain regulates magnesium transport, which also occurs in the mitochondria, the two proteins may act in similar cellular compartments. The combined protein Poldip2 could be involved in magnesium regulation and DNA/ATP synthesis in both the mitochondria and the nucleus of eukaryotic cells. If Poldip2 is a kinases or other ATPase, the mitochondrial localization sequence at the N-terminus could send the protein to the mitochondria to bind ATP before it exits and is active elsewhere.

In order to test the involvement of Poldip2 in ATP activity or binding, we plan to use radiolabeled ATP to measure binding to Poldip2 in the presence or absence of Mg^{2+} or various other ions. We will also measure ATP binding in Poldip2 mutants, which could mimic the conformation change that occurs during activation. If warranted, we

could also perform a kinase assay using one of Poldip2's binding partners as a substrate; however, it is still unclear what signal activates Poldip2.

Another interesting aspect of the ApaG domain structure is the presence of the Fn3-like fold. If the Fn3-like fold causes binding to actin or another cytoskeletal protein, it would explain how Poldip2 localizes to focal adhesions in vascular cells as reported by Lyle et al. (5). Due to the high usage of ATP/ADP in cytoskeletal force generation and muscle movement, Poldip2 could be localized to the cytoskeleton to provide active Mg-bound ATP to these processes.

We have previously shown that the binding of Poldip2 to p22phox increased Nox4 activity. In order to quantitatively measure the binding between P22phox and Poldip2, we will employ in vitro transcription and translation of both proteins. The purified proteins will be combined and immunoprecipitated using epitope tags, V5 for p22phox and myc for Poldip2. We hypothesize that some of the point mutations will reduce or eliminate the binding with p22phox.

Given that Poldip2 contains two domains with seemingly independent functions in prokaryotes, it is likely that the Poldip2 protein has multiple roles in the eukaryotic cell. The degree of conservation of the amino acids that make up these domains suggests a functional importance that has been maintained during evolution. Though the ApaG domain has a high accuracy confidence, the YccV domain is less conserved and contains more disordered structures that lead to uncertainty. Ultimately, the full tertiary structure of Poldip2 will not be known with confidence until the protein is crystallized.

Chapter 3

Poldip2 gene arrays and pathway analysis

Introduction

Since its discovery, Poldip2 has been functionally classified by its binding partners and subcellular localization. The identified binding partners are involved in a variety of cellular processes. These binding partners fit into a number of pathways, including DNA replication and repair (Polδ, Polη, PCNA, HDAC6), mitochondria function (mtSSB, Hsp90β, ATP5β, LonM), reactive oxygen species generation (p22phox) and cell surface receptors (CEACAM1) (Table 1.1). Though protein binding can be indicative of function, it is an indirect measure. Here, we investigate gene expression changes induced by Poldip2 downregulation further clarify the function of Poldip2.

In order to obtain a global perspective of the genes that Poldip2 regulates, we chose to use large-scale cDNA microarrays. We performed arrays using RNA extracted from the aortas of Poldip2+/- mice to investigate the effect of Poldip2 loss in the vasculature. Additionally, we performed arrays using Poldip2-/- mouse embryonic fibroblasts to investigate gene expression changes in a cell model lacking Poldip2. A pathway analysis was performed to make connections between affected genes in the two arrays.

Methods

Preparation of cDNA from mouse aortas

Male mice were euthanized using CO2 and perfused at physiological pressure from the left ventricle with 26 mM sodium citrate in 0.9% sodium chloride (pH 7.4) to clear blood from the aorta. Whole thoracic aortas were quickly removed and placed in RNA later solution (Ambion) at 10°C overnight. Adherent fat was carefully removed and aortas were homogenized with a motorized rotor/stator device. Total RNA was purified using the RNeasy kit (Qiagen). Following reverse transcription (RT) with 15mer random

primers using Superscript II enzyme (Invitrogen), cDNA was purified with the QIAquick kit (Qiagen).

Preparation of cDNA from mouse embryonic fibroblasts

Pregnant females were euthanized with CO2 13.5 days post-conception. Embryos were isolated, with head and developed organs removed. The embryos were mechanically dissociated and seeded on gelatin coated plates. After two passages, total RNA was isolated using the RNeasy kit (Qiagen). Following reverse transcription (RT) with 15mer random primers using Superscript II enzyme (Invitrogen), cDNA was purified with the QIAquick kit (Qiagen).

Gene array

Three Poldip2+/+ and three Poldip2+/- aorta total RNA samples were hybridized to a Mouse6v2 bead array (Illumina). Four Poldip2+/+ and four Poldip2-/- MEF total RNA samples were hybridized to a Mouse8v2 array (Illumina).

Statistics: Significance Analysis of Microarrays

Gene arrays were analyzed using Significance Analysis of Microarrays (SAM) as previously described (105). This method incorporates the q-value (106) into microarray significance analysis to reduce the false discovery rate that occurs with methods designed for smaller sample sets.

Results

Array Results

After performing Significance Analysis of Microarrays (SAM) using a false discovery rate of less that 5% and a minimum fold change of 1.5, we found 17 genes were

upregulated in Poldip2+/- aorta samples and 13 genes were downregulated (Table 3.1). The Poldip2-/- MEF array identified 6 significantly upregulated genes and 6 downregulated genes (Table 3.2). To elucidate Poldip2 function, we used both sets of data to find common signaling pathways with multiple genes that were affected by Poldip2 knockdown. There were some genes that were only changed in one tissue, likely due to the mixed cell population in aortas, changes induced by placing cells in culture or fundamental difference between vascular cells and MEFs.

Circadian clock genes

A group of 5 key genes involved in regulating the circadian clock (*Npas2*, *Clock*, *Dbp, Tspan4,* and *Tef*) were significantly altered in the aorta samples (Table 3.1), but were unchanged in the MEF samples. Poldip2-/- MEF samples did, however, exhibit higher expression of Ccrn4l, a poorly studied circadian gene which closely resembles nocturnin (107). Other circadian genes showed differences as well, but did not meet the statistical cutoff for significance (*Per3* = -3.49, *Per2* = -1.72, *Rora* = -2.60, *Rorc* = -1.67, *Nr1d1* = 1.49, *Nr1d2* = -1.49). Interestingly, the paralogs *Npas2* and *Clock* displayed increased expression with the loss of Poldip2, but *DBP*, *Tspan4*, and *Tef* had decreased expression (Figure 3.1). This difference supports the circadian clock pathway being regulated in aorta by Poldip2 expression. Previously published evidence in aorta suggests that *Npas2* and *Clock* share the same Circadian period (6.0); however, *Dbp* (16.5), *Tspan4* (18.0) and *Tef* (18.0) display opposite periods of expression (108; 109). Additionally, the hormone Leptin has been demonstrated to have rhythmic expression (110-112), and although it did not meet our false discovery statistical cutoff, the expression of *Leptin* (Lep = -6.65) was lower in *Poldip2+/-* mouse aortas, further supporting altered circadian rhythms in these animals.

Gene Name	Definition	Fold Change
NPAS2	Neuronal PAS domain-containing 2	14.52
LYZL4	Lysozyme-like 4	2.94
RASL11A	Ras-like, family 11, member A	2.58
MTHFD2	Methylenetetrahydrofolate dehydrogenase	2.47
LEO1	RNA polymerase-associated protein	2.34
SLC45A3	Solute carrier, family 45, member 3	2.10
4933400N17RIK	Regulatory factor X 8 (Rfx8)	1.83
IRF2BP2	Interferon regulatory factor 2 BP 2	1.80
NFKBIA	Nuclear factor κB inhibitor (IκBα)	1.77
GADD45G	Growth arrest and DNA-damage-inducible protein 45γ	1.74
TMEM181	Transmembrane protein 181	1.71
KREMEN	Kringle containing transmembrane protein	1.68
E230024B12RIK	Unknown gene	1.64
CLOCK	Circadian locomotor output cycles kaput	1.62
MKKS	McKusick-Kaufman syndrome	1.60
MAPKBP1	Mitogen-activated protein kinase BP 1	1.59
CCRK	Cell cycle related kinase	1.54
EEF2K	Eukaryotic elongation factor-2 kinase	-1.52
2310036D04RIK	pyridoxal (vitamin B6) kinase (Pdxk)	-1.55
DBP	Albumin D-box binding protein	-1.57
TSPAN4	Tetraspanin 4	-1.78
SMPDL3B	Sphingomyelin phosphodiesterase 3B	-1.94
MREG	Melanoregulin	-1.99
LONRF1	LON peptidase N-terminal domain and ring finger 1	-2.09
HOMER2	Homer protein homolog 2	-2.16
POLDIP2	Polymerase δ-interacting protein 2	-2.27
TEF	Thyrotroph embryonic factor	-2.31
BMP3	Bone morphogenic protein 3	-2.41
LOC100041516	FAM205A-like protein	-2.43
GM129	Predicted gene 129	-2.85

Table 3.1. Aortic genes regulated by Poldip2 depletion. Three Poldip2+/+ and three Poldip2+/- aorta total RNA samples were hybridized to a Mouse6v2 bead array. All values shown were significant by significance analysis of microarrays (SAM). Fold changes shown in green are fold higher than control, red represents lower than control.

Gene Name	Definition	Fold Change
Ivl	Involucrin	1.80
Gabrb1	GABA A receptor, beta 1	1.61
Ccrn4l	Carbon catabolite repression 4-like	1.60
Tgm2	Transglutaminase 2	1.57
Parm1	Prostate androgen-regulated mucin-like protein 1	1.54
Angptl4	Angiopoietin-like 4	1.52
Tmem199	Transmembrane protein 199	-1.63
Medag	mesenteric estrogen-dependent adipogenesis	-1.64
Pde1a	Phosphodiesterase 1A	-1.65
Pdk1	Phosphoinositide-dependent kinase-1	-1.73
Mgarp	Mitochondria-localized glutamic acid-rich protein	-1.92
Poldip2	Polymerase δ-interacting protein 2	-94.2

Table 3.2. Genes regulated by Poldip2 deletion in MEFs. RNA was isolated from MEFs generated using four independent Poldip2+/+ and Poldip2-/- embryos at passage 2. cDNA was hybridized to a Mouse8v2 array. All values shown were significant by significance analysis of microarrays (SAM). Fold changes shown in green are fold higher than control, red represents lower than control.

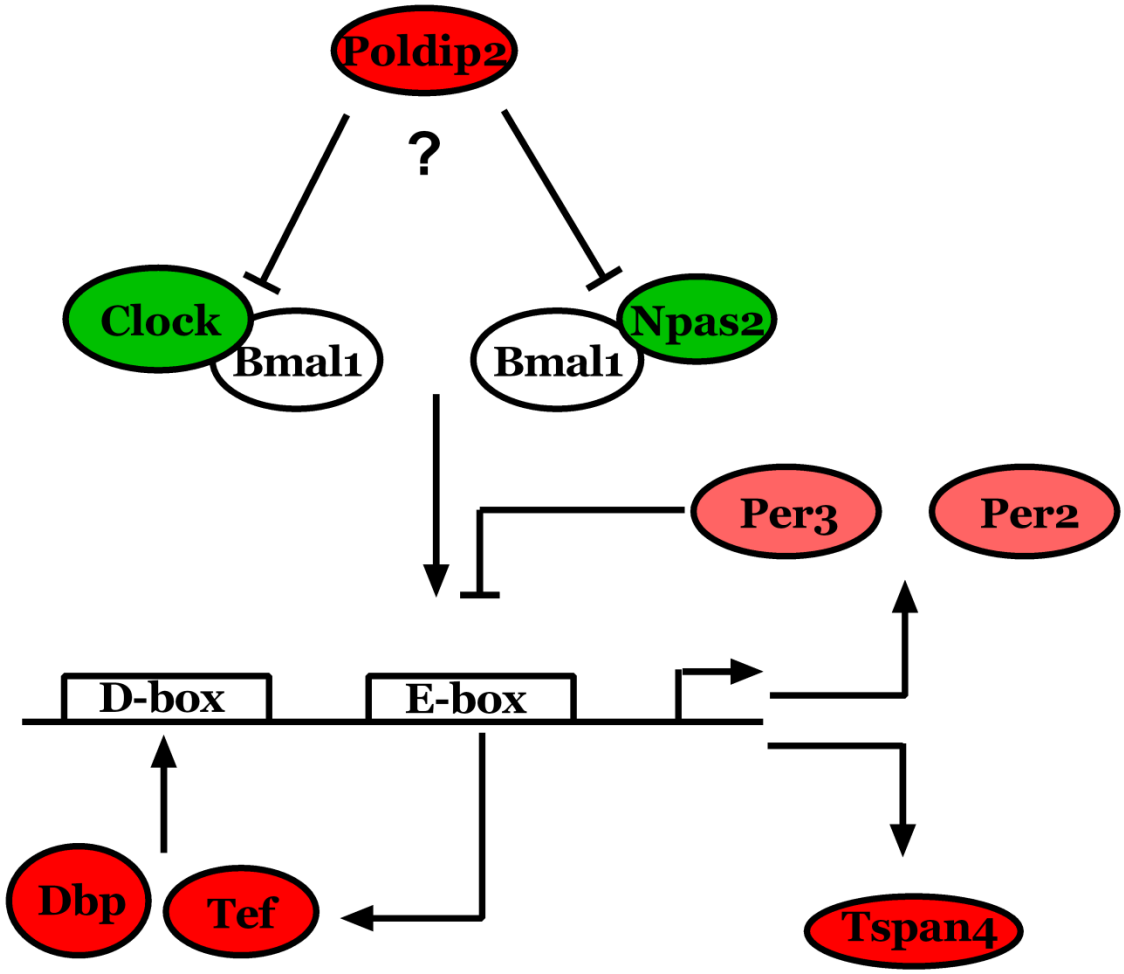

Figure 3.1. Hypothetical Poldip2 regulation of the circadian clock. Decreased expression of *Poldip2* in mouse aorta causes upregulation of PAS domain genes *Clock* and *Npas2*. E-box regulated genes *Per2/3*, *Dbp*, *Tef*, and *Tspan* are decreased by *Poldip2* downregulation. Two D-box binding transcription factors, *Dbp* and *Tef* , were downregulated in aorta samples lacking *Poldip2*. The clock-regulated *Tspan4* was also decreased. Green shading represents increased expression compared to Poldip2+/+ samples. Red shading represents significant downregulation, pink represents a downregulation that is not significant.

Cell cycle genes

A number of cell cycle and growth related genes were altered in the arrays (Figure 3.2). In the aorta, BMP3 (-2.41), a member of the TGFβ family of proteins, was reduced in Poldip2+/- samples. BMP3, unlike other BMPs is thought to act as an antagonist to the BMP receptor (113). Additionally, the GPCR HOMER2 (-2.16) has been suggested to be involved in suppression of tumor growth (114).

Two key proteins in cellular growth, E2F4 (-1.1 MEF) and CCRK (-1.2 MEF, 1.54 Aorta) were altered in Poldip2 -/- MEFs. E2F4 is known as a repressor E2F, and functions by blocking activating E2F family members from progressing the cell cycle (115). CCRK is an activator of the Cdk proteins which promote cell cycle progression (116) and cell survival (117).

A number of proteins involved in DNA damage responses were upregulated in Poldip2 deficient cells. In MEFs, Hist1H1C (1.24), which encodes a HistoneH1 protein involved in nucleosome structure was upregulated. Hist1H1C is translocated to the cytosol in the event of DNA damage and activates apoptosis via activation of Bak on the mitochondria outer membrane (118). Top1 (1.26) codes for topoisomerase, a protein involved in cutting a single strand of DNA to allow it to unwind during transcription. Upregulation of Top1 has been implicated in DNA breaks which can ultimately lead to activation of DNA damage pathways such as p53, and ultimately apoptosis (119). Though, Top1 (1.26) did not meet our fold change cutoff of 1.5, the difference was statistically significant, and its possible role in DNA damage pathways warrants further study.

The aorta samples also exhibited activation of genes associated with DNA damage/senescence. LEO1 (2.34) is an RNA polymerase-associated protein that may play a role in senescence. Expression of a Leo1-like domain induced senescence in 2BS fibroblasts (120). Gadd45g (1.74) was upregulated in Poldip2 deficient aortas. In

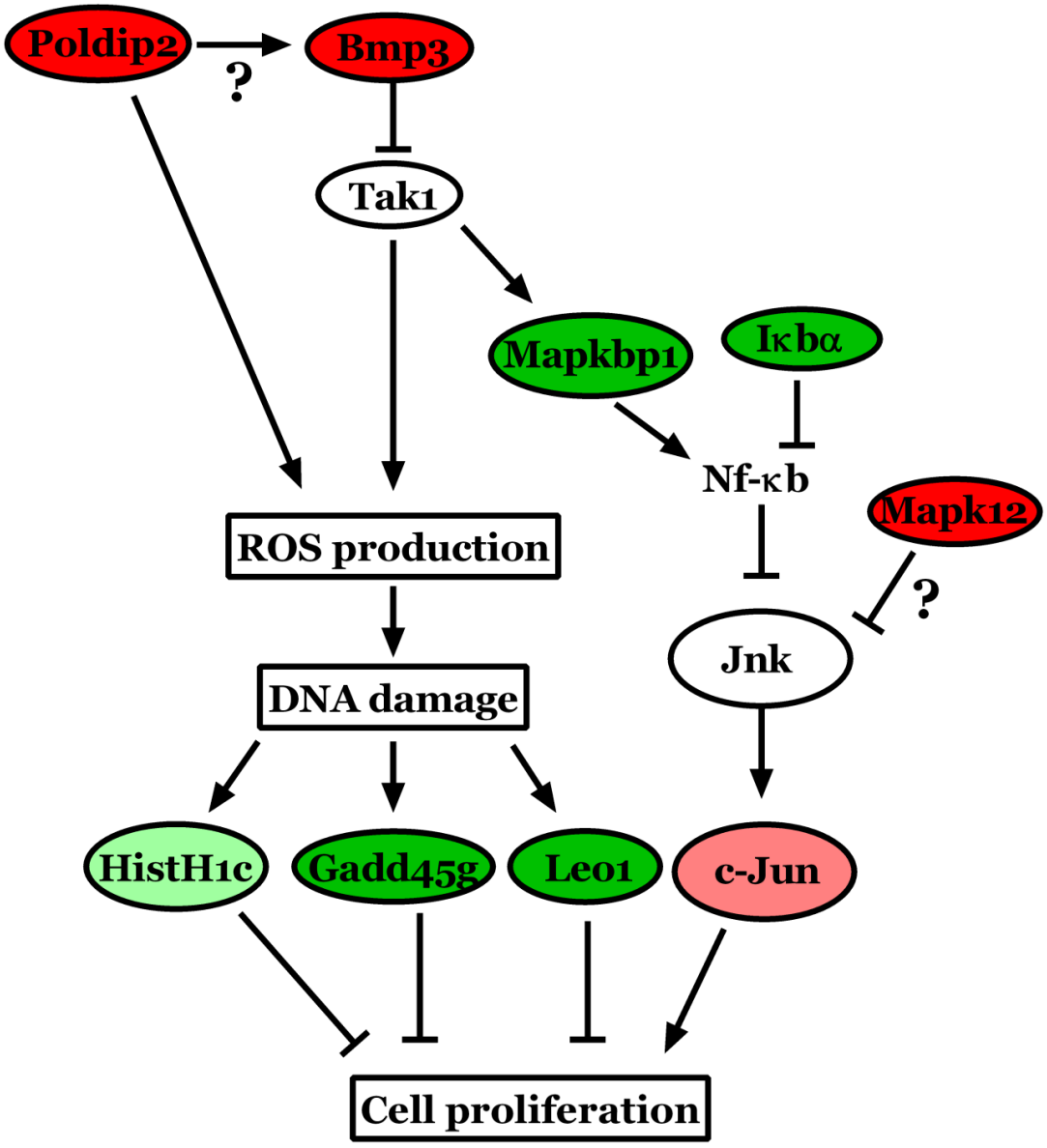

Figure 3.2. Hypothetical regulation of cell cycle genes by Poldip2. Reduced Poldip2 results in reduced BMP3 gene expression, which is an inhibitor of TAK1. TAK1 and Poldip2 both influence ROS production potentially causing DNA damage. HitH1c, Gadd45g and Leo1 genes are all elevated in Poldip2 knockdown which could contribute to reduced cell proliferation. Additionally Mapkbp1 and Iκbα RNA are elevated which could ultimate reduce c-Jun expression via Nfκb, which would also reduce cell proliferation.

response to DNA damage, Gadd45 induces growth arrest and repair pathways including activating p21, inhibiting Cdc2/CyclinB and interacting with PCNA (121-124).

Another stress response gene upregulated in aorta is Mapkbp1 (1.59), also-known-as JNK-binding protein 1. Mapkbp1 has been proposed to act as a scaffold that promotes the phosphorylation of JNK by MEKK1 and TAK1 (125). Additionally Mapkbp1 enhances the activation of the stress response transcription factor NF-κB by TAK1 and TRAF2 (125). The NF-κB regulator IκBα, NFκBIA (1.77), was also upregulated in the aorta samples. IκBα inhibits NFκB by masking its nuclear localization sequence, keeping it localized to the cytoplasm (126).

Poldip2 contains a mitochondrial localization sequence, and knockdown has been demonstrated to alter mitochondrial morphology (9). Poldip2 has been implicated in associating with proteins in the mitochondria such as TFAM and mtSSB (2; 3; 9). Beyond these interactions little is known about the function of Poldip2 in the mitochondria. Poldip2 knockout in MEFs caused downregulation of Mgarp (HUMMR) and Pdk1 genes (Table 4.1). Pdk1 is responsible for the inactivation of pyruvate kinase by phosphorylation. This reduces the conversion of pyruvate in acetylcoA in the cytosol, resulting in preferential conversion of pyruvate to lactose in the cytosol.

Mgarp (HUMMR) is a mitochondrial glutamic acid-rich protein that is upregulated by HIF1α during hypoxia (127). In neurons, knockdown of Mgarp results in altered mitochondrial transport (128). Interestingly, Mgarp expression is known to greatly vary during development, and could indicate that the MEFs produced from Poldip2 knockout embryos are at a different phase of development from their wild type siblings.

In aorta, Poldip2 downregulation resulted in an increase of Methylenetetrahydrofolate dehydrogenase (MTHFD2) (Table 4.2). This mitochondrial

Mg^{2+} and NAD-dependent enzyme is part of the glycine and serine synthesis pathway (129).

Metabolism

Beyond the mitochondrial proteins mentioned in the previous section, downregulation of Poldip2 caused a reduction in several key metabolic enzymes. In the MEFs, Phosphodiesterase 1A (Pde1a) was reduced, and in aorta both pyridoxine (vitamin B6) kinase (Pdxk) and Sphingomyelin phosphodiesterase, acid-like 3b (Smpdl3b) were reduced (Tables 3.1 and 3.2).

Pde1a regulates the levels of cAMP and cGMP in the cell by hydrolysis to 5-prime monophosphates, AMP and GMP (130). Pdxk phosphorylates vitamin B6 to form pyrodoxal phosphate (PLP), which is a coenzyme in a variety of transamination reactions. PLP is considered the active form of vitamin B6. Vitamin B6 deficiency has been linked to a variety of dermatological and neurological disorders as well as glucose intolerance. Smpdl3b is a hydrolase of the DNase I family. It catalyzes the breakdown of sphingomyelin into phosphocholine and ceramide (131). Phosphocholine is an important player in mitochondrial respiration.

Conclusions

By investigating genes that are altered in Poldip2 deficient cells and tissue, we have identified several key common pathways. Though there were few common genes identified in both microarrays, the pathways that were affected are remarkably consistent. The differences in specific gene expression could be due to variances in pathway functionality in adult aorta tissue, which is composed of multiple cell types, compared with isolated embryonic fibroblasts. It is well established that cell growth and apoptosis pathways have unique characteristics in different tissues (132; 133).

In this study, we identified multiple genes altered by *Poldip2* reduction in circadian rhythm, cell cycle, mitochondrial and metabolic pathways. There are several possible explanations for how Poldip2 could be regulating such a wide variety of cellular pathways; although more experimentation is clearly indicated to determine the mechanism of how Poldip2 might regulate these pathways, one can speculate about possible scenarios.

The two main functional domains that form full length Poldip2, YccV and ApaG, could impart Poldip2 multiple distinct functions. This could be due to binding sites for multiple proteins existing on the surface of the Poldip2 protein. Alternatively, proteins regulated by Poldip2 could contain a binding motif that binds a common region on the Poldip2 structure. Poldip2 has been observed in multiple cellular compartments including the mitochondria, nucleus, cytoplasm and stress fibers (2-5; 8). Poldip2 could have distinct binding partners that control its localization or activity in certain regions of the cell. This seems to be the case with CEACAM1, which can control Poldip2 localization to the plasma membrane in certain conditions (4). Poldip2 could regulate also protein levels by post-translational modification, trafficking, stabilization or forming protein complexes that ultimate alter the activity of transcription factors.

Although the pathways control distinct cellular processes, there are key common links between these pathways. The cell cycle, for instance, exhibits cross talk between circadian rhythm, metabolic and mitochondrial pathways. The cell cycle is a tightly regulated process that is delayed when the cell senses a lack of some essential component necessary for the completion of the processes.

The circadian clock genes regulate the expression of many genes based on light signals or metabolic signals from digesting food (134). Recent evidence has implicated the Bmal1/Clock complex in regulating the expression of Wee1 kinase, which inhibits the activity of the Cdk1/CyclinB1, thereby regulating the G2/M transition of the cell cycle

(135). CyclinD1 and c-Myc have also been shown to be regulated in a circadian manner. In addition to cell cycle regulation, there is evidence of circadian control of apoptosis. The circadian proteins Per1 and Tim have been found to complex with Atm/Chk2 and Atr/Chk1, respectively, suggesting possible involvement in DNA damage sensing or apoptosis (134).

Mitochondrial pathways also share key cross talk with the cell cycle. Mitochondria produce energy from oxidative respiration. As a consequence of respiration, a large amount of ROS are produced around the mitochondria. Presumably due to the possibility of ROS causing DNA damage, there is reduced respiration rate during DNA duplication even in the presence of excess oxygen; instead the cell relies on glycolysis (Warburg effect). It has been proposed that the lowered respiration rate increases the activity of proliferative kinases such as ERK1/2 and Akt (136). In situations of mitochondrial stress, there is activation of JNK and p38, which can cause growth arrest and apoptosis (100).

Many of the metabolic proteins that were altered in Poldip2 deficient cells are implicated in mitochondrial function. The Vitamin B6 that is produced in part by the activity of Pdxk is important for the synthesis of CoA, which assists the transport of fatty acids from the cytoplasm to the mitochondria. One function of Smpdl3b is the conversion of sphingomyelin to phosphocholine, an important component of mitochondrial respiration (137) Pde1a regulates cAMP, which has profound effects on mitochondrial biogenesis via regulation of glutathione redox pathways (138). cAMP also represents another link between mitochondria and cell cycle. Elevated cAMP and cGMP levels regulate cyclin dependent kinase activity, which can ultimate arrest the cell cycle (139) in addition to affecting michochondria metabolism.

A limitation of this study lies in the fact that gene arrays provide a single snapshot of equilibrium conditions. We cannot rule out the possibility that many of the

gene expression changes that we have observed are compensatory, and not a direct result of Poldip2 knockdown. However, in the context of multiple proteins affected in larger pathways, compensatory responses can give us clues about the protein function. One would expect proteins with similar functionalities to Poldip2 to compensate for loss of Poldip2 function. Additionally, future studies of mRNA and protein expression in a transient knockdown of Poldip2 will improve our understanding of these pathways.

From these two gene microarrays, we have identified several pathways that loss of Poldip2 alters. These data can be used to guide future experimentation regarding Poldip2 function, as well as to identify potential diseases to which Poldip2 misregulation may contribute. While we do not yet know the mechanism of how Poldip2 alters gene expression, the identification of multiple genes from several key pathways including cell cycle, circadian rhythm, mitochondria function and fundamental cell metabolic processes provides a clue to its functions.

Chapter 4

Poldip2 in the cell cycle

Introduction

Poldip2 (also known as PDIP38 or mitogenin 1) was originally identified as a binding partner of polymerase-δ and Proliferating Cell Nuclear Antigen (PCNA) (1). Subsequent research has implicated Poldip2 in DNA replication and repair (6; 8), mitochondrial function and elongation (3; 9), and downstream signaling of a cell adhesion receptor (4), as well as cytoskeletal reorganization and regulation of reactive oxygen species production (5). The diversity of cellular processes in which Poldip2 participates and their relevance to cell division suggests that it plays an essential role in cell biology, particularly in cell growth.

The cell cycle is made up of four main phases: G1, S, G2 and M phases. Key cell cycle proteins such as cyclins and cyclin-dependent kinases (Cdks) exhibit altered expression or activity during different phases of the cell cycle. During late G1 and early S, CyclinD1 complexes with Cdk4, and Cdk2 complexes with CyclinE to hyper-phosphorylate Rb, releasing its inhibition of the transcriptional activators E2F1, E2F2 and E2F3a which promote cell cycle progression (140). The cell cycle inhibitors p16, p19, p21, and p27 can inhibit the activity of cyclin-kinase complexes such as Cdk4/CyclinD1 and Cdk2/CyclinE/CyclinA to arrest the cell cycle in G1 by preventing phosphorylation of Rb. After G1, the cells undergo DNA replication (also known as the synthesis phase, S-phase). During S-phase and early G2, Cdk2 associates with CyclinA and phosphorylates proteins related to transcription factor activity, DNA damage repair and cell cycle checkpoints (19). In mid S phase, CyclinA associates with Cdk1, which phosphorylates many of the same targets as the CyclinA/Cdk2 complex, but also regulates cytoskeletal changes to prepare for chromosome segregation and cytokinesis. CyclinA begins to be degraded and replaced by CyclinB in G2 (19). During mitosis, or M-phase, chromosomes segregate and the nucleus divides. Numerous tumor suppressor proteins, such as p53, act throughout the cell cycle in response to DNA damage, oncogene activation, and other

types of stress. p53 activation results in different outcomes such as senescence, cycle arrest and apoptosis depending on activated binding partners. Notably, p53 transcribes p21 which can inhibit many stages of the cell cycle by blocking cdk/cyclin complex activity (19).

Several papers describe a possible role for Poldip2 in DNA replication/repair (1; 2; 6) or mitosis (8), that occur during S-phase and M phase, respectively. However, much of the current understanding of Poldip2 function stems from speculation based on the known functions of Poldip2 binding partners. The known Poldip2 interacting proteins with roles in DNA duplication/repair are PCNA, the p50 subunit of polymerase δ, and Polη. Poldip2 has been demonstrated to reduce polymerase δ activity in vitro (2). The precise role of Poldip2 in cell cycle progression has thus not been elucidated.

To better understand the functions of Poldip2, we used a mouse deficient in Poldip2. Unexpectedly, homozygous deletion of Poldip2 induced perinatal lethality. Based on this observation, as well as the close relationship of Poldip2 to cell cycle proteins, we hypothesized that Poldip2 has multiple roles in cell division. We report here impaired growth in Poldip2 depleted cells, as well as altered expression of key cell cycle proteins such as Cdk1, CyclinA2 and p21, suggesting that Poldip2 targets a common regulator such as E2F1 or p53.

Methods

Animals.

Poldip2 gene trap mice in C57BL/6 background were produced by the Texas A&M Institute for Genomic Medicine (College Station, TX). Mice were genotyped using a standard 3-primer PCR method. All animal protocols were approved by Institutional Animal Care and Use Committee of the Emory University School of Medicine.

Preparation of mouse embryonic fibroblasts (MEFs).

MEFs were prepared from E13.5 embryos. Briefly, embryos were removed from the pregnant females with their yolk sacs intact. The yolk sac was removed and retained for genotyping. The head and internal organs of each embryo were removed and discarded. The dissected embryo was passed through an 18G needle to disperse the cells. The cells were plated on gelatin-coated 100-mm cell culture dishes in 15% FBS DMEM and passaged as described below.

Cell culture.

MEFs were grown in Dulbecco's Modified Eagles Medium containing 15% fetal bovine serum (FBS). The cells were cultured using a 3T3 method; they were passed every 3 days and seeded at a density of $3x10^5$ cells per 20 cm^2 dish. The cells were used for experiments between passages 2 and 7, at which point they became senescent.

Growth curve/Doubling curve.

Poldip2+/+, *Poldip2+/-*, and *Poldip2-/-* MEFs at passages 0-6 were seeded at 10^4 cells per 35mm dish (Corning). Cells were trypsinized and counted every 24 h for 5 days using a Scepter 2.0 cell counter (Millipore). For the doubling curve, cells were counted at each passage and seeded at $3x10^5$ cells per 20 cm^2. Population doublings after each passage were calculated as $\frac{\log(\textit{number of cells counted} / \textit{number of cells seeded})}{\log 2}$. This value was added to that of previous passages to produce a cumulative doubling curve.

Cell cycle analysis.

MEFs were trypsinized 24 h after passage and fixed in 60% ethanol overnight. The cells were pelleted and washed with PBS. Cells were then resuspended in staining solution

(1X PBS, 0.1% Triton-X, 0.2 mg/ml RNase A, 20 μg/ml propidium iodide (Sigma). Fluorescence signal was assessed using an LSRII (Becton, Dickinson) flow cytometer. Cell cycle analysis was performed using the Dean-Jett-Fox method in Flowjo (Treestar, Inc.).

Apoptosis.

MEFs were trypsinized 24h after passage and fixed in paraformaldehyde. Cells were stained with the apoptosis marker Annexin V, using the Annexin V:FITC Apoptosis Detection Kit I (BD). Fluorescence signal was assessed using a LSRII (Becton, Dickinson) flow cytometer. Data was analyzed using Flowjo (Treestar, Inc.).

MEF immortalization.

Poldip2+/+ or *Poldip2-/-* primary MEFs at passage 2 were seeded in 6-well plates (Corning). The cells were transfected with SV40 large T-antigen (Addgene plasmid 13970) using fugene HD (Promega). Cells were grown to confluence and transferred to 10-cm plates. Cells were then passaged at a ratio of 1:10 for 9 additional passages upon reaching confluence.

Western blot.

Whole cell lysate was extracted from MEFs using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholate, 1% NP-40) with fresh protease and phosphatase inhibitors (PMSF, aprotinin, leupeptin, NaF, activated sodium orthovanadate). Protein concentrations were measured by Bradford assay, and protein was diluted into Laemmli buffer for separation by SDS-PAGE. Following separation, proteins were transferred to a nitrocellulose membrane and assessed by western blotting with primary antibodies against β-actin (A5441;

Sigma), Cdk1 (sc-54; Santa Cruz), Cdk2 (sc-163; Santa Cruz), Cdk4 (559693, BD), CyclinA2 (sc-751; Santa Cruz), CyclinB (#4138S; Cell Signaling), CyclinD1 (sc-718; Santa Cruz), CyclinE (sc-481; Santa Cruz), CyclinF (sc-953; Santa Cruz), E2F1 (sc-193; Santa Cruz), p21 (ab7960; Abcam), p27 (#25525; Cell Signaling), p53 (sc-99;Santa Cruz), p-p53 (S20)(sc-18078; Santa Cruz), PCNA (ab2426; Abcam), Poldip2 goat antibody (5), Rb (#9313S; Cell Signaling), pRb(S780) (#9307S; Cell Signaling), pRb(S807/811) (#9308S; Cell Signaling) and pRb(T821) (44-582G; Invitrogen). Blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies depending on the species of the primary antibody [anti-mouse (NA931; GE), anti-rabbit (170-6515; Bio-rad), anti-goat (205-295-108; Jackson)], and assessed using enhanced chemiluminescence (ECL, GE). HRP-induced luminescence was detected with Amersham Hyperfilm ECL (GE). Detected bands were scanned and densitometry was performed using ImageJ.

RNA extraction/qRT-PCR.

Total RNA was extracted with the RNeasy Plus kit (Qiagen). Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen) using random primers. cDNA from Poldip2, p21, p19 and Cdk1 was amplified in a buffer containing SYBR green by polymerase chain reaction using the LightCycler 1.1 (Roche) glass capillary real time thermocycler.

Statistics.

MEFs were prepared from unique embryos for each experiment. Data were presented as mean±SEM from a minimum of 3 independent experiments. Significance was determined using 2-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. A threshold of $P<0.05$ was considered significant.

Results

Poldip2 knockout results in reduced fetal weight and perinatal lethality

To clarify the role of Poldip2 *in vivo*, we used mice that have a gene trap construct inserted into the first intron of Poldip2, disrupting gene expression (12). Expected Mendelian ratios would dictate that a cross of two heterozygotes should produce 25% wild type, 50% heterozygous and 25% homozygous animals; however, we observed 33% wild type, 65% heterozygous and 3% *Poldip2-/-* animals at birth (Figure 4.1A). To confirm the genotypes, mRNA (Figure 4.1B) and protein (Figure 4.1C) expression were measured in primary MEFs. As expected, Poldip2 mRNA and protein levels were about half that of wild type animals in heterozygotes, while *Poldip2-/-* animals had nearly undetectable levels of Poldip2.

We genotyped embryos at E13.5, E16.5, E18.5, and newborn stages to determine when *Poldip2-/-* embryos are lost. Surprisingly, *Poldip2-/-* embryos survive until birth (19.5 dpc) (Figure 4.1A), albeit at lower weight than wild type or heterozygous embryos (Figure 4.1D). There was no detectable weight difference between wild type and heterozygous embryos.

Poldip2 knockdown causes reduced cell growth and doubling time in MEFs

Given the reduced weight of *Poldip2-/-* embryos, we sought to investigate the contribution of Poldip2 to cell growth. We prepared MEFs from E13.5 *Poldip2* +/+, +/-, and -/- embryos. Since the rate of growth of cells in culture can depend on inoculation density, we chose to maintain a common seeding density for the duration of the experiment. Cells were plated and passed according to the 3T3 method (60). We compared the population doublings of cells of different genotypes (Figure 4.2A). The doubling of the wild type cells follows a predictable pattern of high growth in early passages, and slower growth at passage 5 or 6, which precedes the replicative senescence

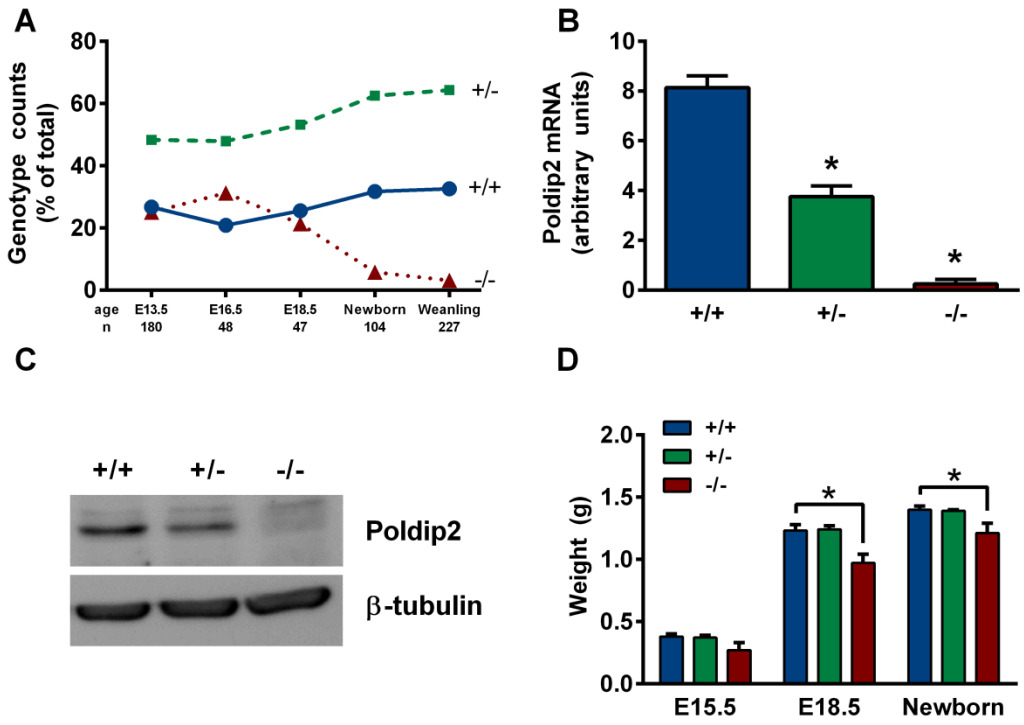

Figure 4.1. *Poldip2-/-* embryos exhibit perinatal lethality and reduced weight. (A) Progeny from heterozygote x heterozygote crosses were genotyped at different days post-conception and after birth. Mouse embryonic fibroblast Poldip2 (B) mRNA and (C) protein were measured to verify successful knockout. (D) Progeny at various stages of development were weighed and genotyped. Bars represent mean ± SEM of 3-4 independent mRNA experiments or 6-62 embryos or pups. * $P<0.05$ comparing *Poldip2+/+* with *Poldip2-/-*

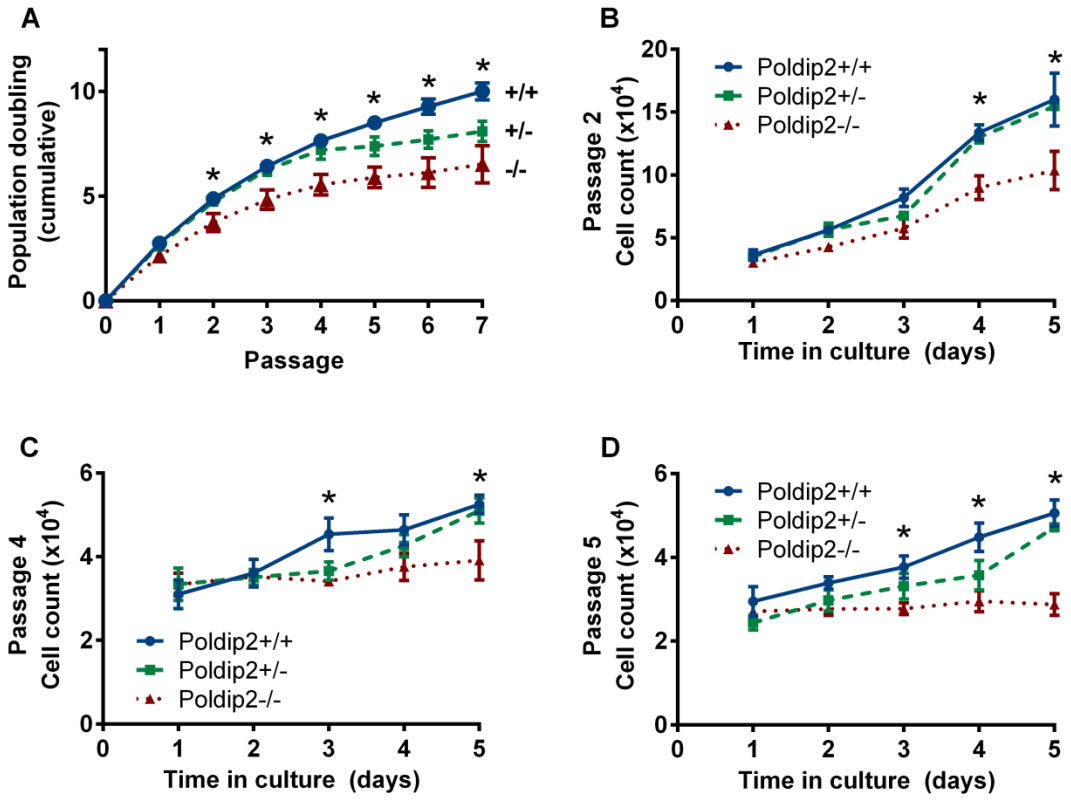

Figure 4.2. Reduced growth in Poldip2 null cells. Mouse embryonic fibroblasts were derived from *Poldip2*+/+, +/- and -/- E13.5 embryos. (A) Growth was assessed by counting cells at each passage and recorded as a cumulative population doubling. Additionally, growth was assessed by performing a growth curve at (B) passage 2, (C) passage 4, and (D) passage 5. Error bars represent mean ± SEM of 3-4 independent experiments. * P<0.05 comparing *Poldip2*+/+ with *Poldip2*-/-

that naturally occurs in MEFs (34). The *Poldip2*+/- cells show a pattern similar to the wild type, exhibiting slightly reduced growth that amounts to a difference of less than one doubling over seven passages. Strikingly, the *Poldip2-/-* MEFs have markedly reduced growth, which becomes obvious as early as passage 2. Over seven passages, the WT cells undergo three more doublings than the *Poldip2-/-* cells.

The growth of MEFs has been reported to be passage dependent, due to their propensity to senesce after 5-6 passages (141). We chose to measure the difference in growth rate between *Poldip2*+/+ and *Poldip2-/-* cells during several passages to investigate possible passage-dependent growth differences. *Poldip2-/-* cells grew significantly slower, which is most obvious in passage 2 (Figure 4.2B). By passages 4 (Figure 4.2C) and 5 (Figure 4.2D), cell growth has markedly slowed in all genetic groups. Wild type and heterozygous cells in later passages show reduced growth compared to early passage cells; however, the *Poldip2-/-* cells exhibit almost no growth in passage 4 or 5. To maximize growth differences, we chose to concentrate on *Poldip2*+/+ and *Poldip2-/-* cells for the remainder of the study. The early growth impairment in *Poldip2-/-* cells compared to *Poldip2*+/+ led us to hypothesize that the lack of Poldip2 was leading to premature senescence, apoptosis or a block/delay at a cell cycle checkpoint.

Poldip2 knockdown does not alter expression of senescence markers or increase apoptosis

In order to determine whether *Poldip2*-/- cells enter senescence early, we measured the expression of senescence markers $p16^{INK4a}$ (Figure 4.3A) and $p19^{ARF}$ (Figure 4.3B) in passages 2-5. As expected, we observed an increase in the expression of $p16^{INK4a}$ and $p19^{ARF}$ as the passage number increased; however, there was no difference in the expression of senescence markers between genotypes. Because the gene trap construct in these mice includes a *lacZ* reporter, we were unable to use the β-

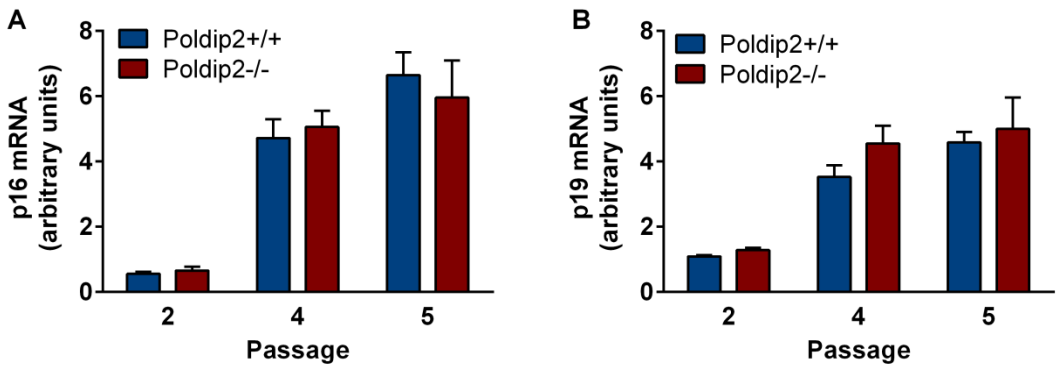

Figure 4.3. Poldip2 deletion does not affect expression of senescence markers. (A) p16 and (B) p19 mRNA expression was compared between *Poldip2+/+* (blue) and *Poldip2-/-* (red) cells by qRT-PCR and corrected for PPIA. Error bars represent mean ± SEM of 3 independent experiments.

galactosidase assay to confirm these findings. Nonetheless, it appears that lack of Poldip2 does not cause early senescence in MEFs.

Previous studies revealed interactions between Poldip2 and the p50 subunit of polymerase δ (1), polymerase η (6), and PCNA (1), which are involved in DNA replication and damage repair. We therefore tested whether reduced growth in *Poldip2* knockout cells could be caused by increased apoptosis due to DNA damage. In passage 5 MEFs, there was a 9.1%±1.3 basal level of Annexin V staining in wild type cells and 8.7%±0.9 *Poldip2-/-* cells were Annexin V positive (n=3, P=NS). The difference in growth between *Poldip2+/+* and *Poldip2-/-* cells thus does not appear to be due to increased apoptosis.

Poldip2 knockout delays or arrests growth in G1 and G2/M

After ruling out early senescence and apoptosis, we investigated the cell cycle distribution of *Poldip2+/+* and *Poldip2-/-* MEFs. We found that passage 2 *Poldip2-/-* MEFs are arrested or delayed in both G1 (Figure 4.4B) and G2/M (Figure 4.4D) phases of the cell cycle, resulting in a reduction of the number of cells in S phase (Figure 4.4C). Though there is a trend for more *Poldip2-/-* cells in G1 in passages 4 and 5 compared to wild type cells, the difference is not significant. The genotype-related difference in the number of cells in G2/M, however, becomes greater in passages 4 and 5 compared to passage 2 cells.

To begin to investigate the mechanism underlying these changes in the cell cycle, we assessed protein levels of key cell cycle regulators (Figure 4.4E). Of the Cyclin proteins, only the expression of Cyclins A2 and D1 was significantly altered in Poldip2 null cells. CyclinA2 expression in passage 5 was significantly lower in *Poldip2-/-* cells than in *Poldip2+/+* cells (Figure 4.4F). CyclinD1 was higher in passage 2 *Poldip2-/-* cells than in *Poldip2+/+* cells, but was unchanged in later passages (Figure 4.4G). Taken

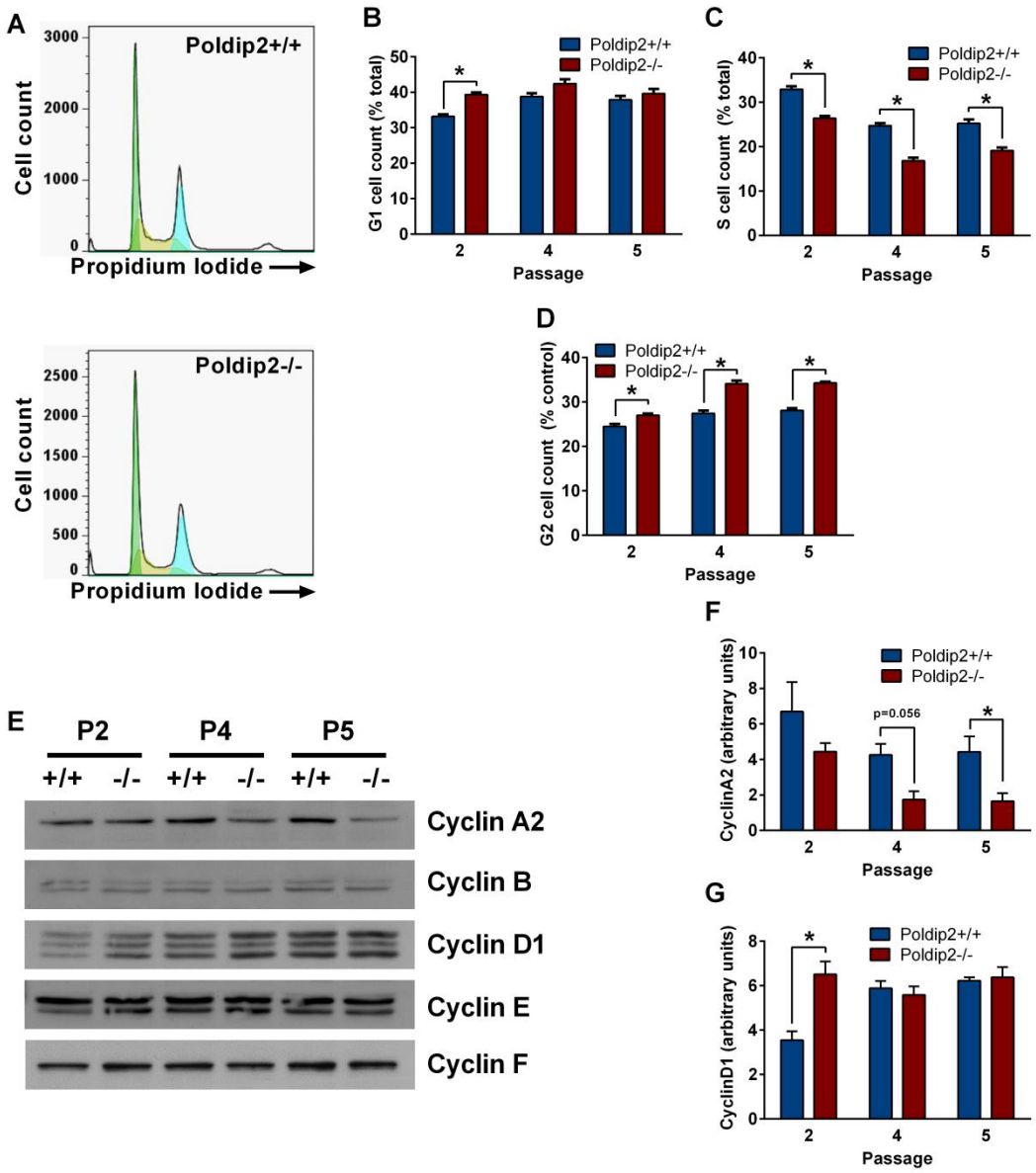

Figure 4.4. *Poldip2-/-* MEFs exhibit altered cell cycle characteristics. Asynchronous MEFs were collected at passage 2, 4 and 5 and stained with propidium iodide for FACS analysis of the cell cycle. (A) DNA content was measured by flow cytometry and fit to the Dean-Jett-Fox model to calculate the percentage of cells in (B) G1, (C) S and (D) G2/M. (E) Key cell cycle protein expression was measured by immunoblotting. Protein levels were quantified by densitometry and corrected to β-actin expression for (F) CyclinA2 and (G) CyclinD1. All three bands were used in the quantification of CyclinD1. Error bars represent mean ± SEM of 3-4 independent experiments. *p<0.05.

together, these data suggest that there is differential regulation of the cell cycle in different passages.

p53 phosphorylation and downstream targets are altered in *Poldip2-/-* MEFs

Cell cycle analysis indicates a delay in G1 and G2/M. Previous studies indicate that one cause of such a delay occurs via the p53 pathway (142). We measured expression of p53 and $p21^{CIP1}$, a p53 transcription target, by western blot. Total p53 was unchanged (Figure 4.5A), but phospho-p53 (S20), an indicator of p53 activity, was markedly increased in *Poldip2-/-* cells compared to wild type cells at passage 2, but not at later passages (Figure 4.5B). $p21^{CIP1}$, a cell cycle inhibitor that is positively regulated by p53, is significantly increased at both the protein (Figure 4.5C) and mRNA levels (Figure 4.5D) at later passages. These data suggest that Poldip2 may negatively regulate p53 or its loss elicits activation of p53 that results in increases in $p21^{CIP1}$ (Figure 4.5F); however, we cannot rule out the possibility that another transcription factor is responsible for the observed changes in $p21^{CIP1}$.

E2F target genes exhibit reduced expression in *Poldip2-/-* cells

The data in Figure 4.4 indicate a significant decrease in CyclinA2 levels after *Poldip2* deletion. CyclinA2 is not downstream of p53, suggesting additional targets of Poldip2. CyclinA2 expression is positively regulated in part through the E2F transcriptional activators and their binding partner, Rb, which prevents E2F-dependent transcriptional activity. Total protein levels of Rb, the main regulator of E2F, were unchanged (Figure 4.6A). Rb binding with E2F is regulated by its phosphorylation state. Two of the kinases responsible for phosphorylating Rb in G1 are Cdk2 and Cdk4. Whereas Cdk2 was unchanged (Figure 4.6B), Cdk4 was significantly increased in p2

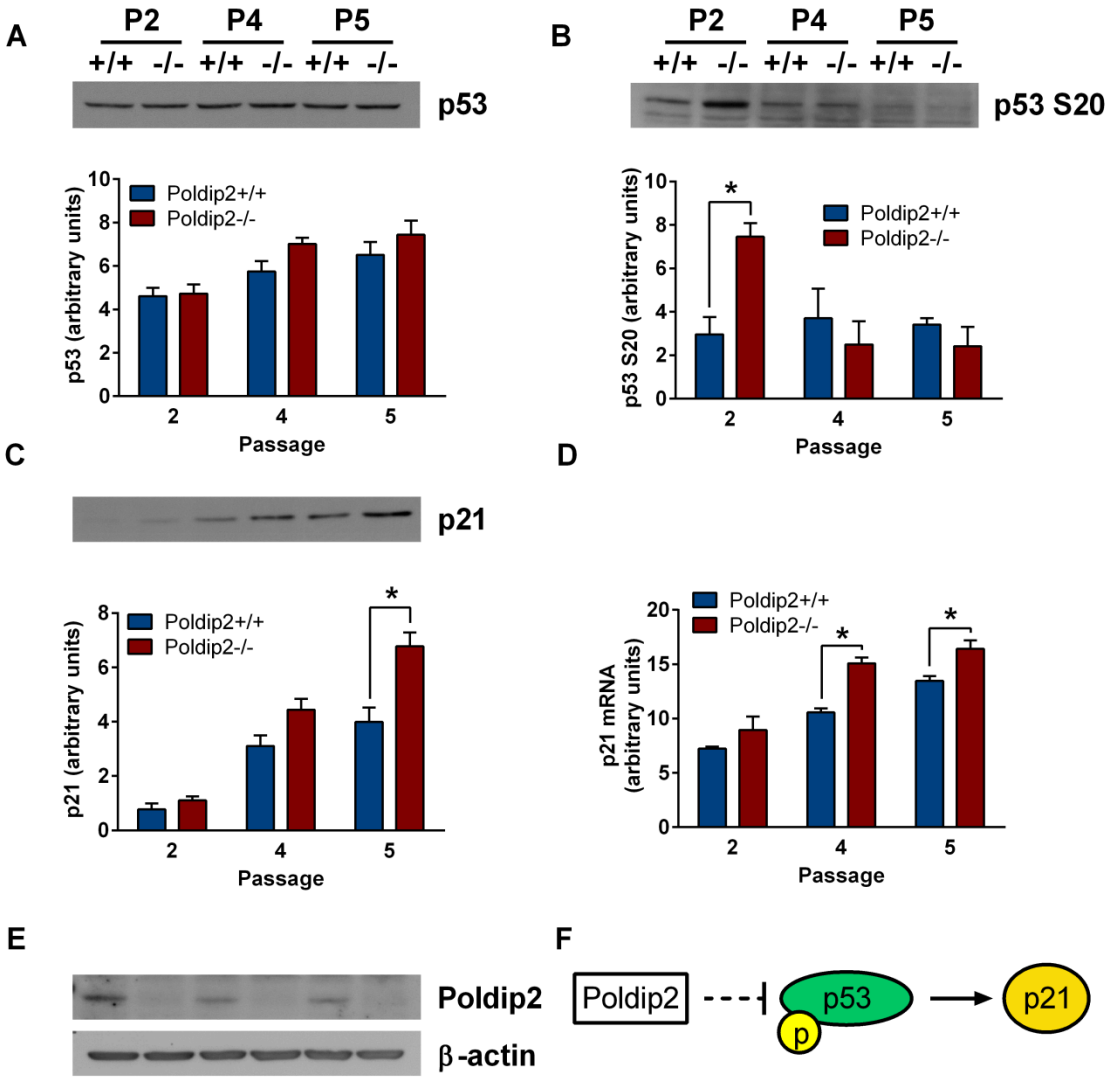

Figure 4.5. Poldip2 inhibits the p53 pathway. Immunoblotting was performed using lysates from *Poldip2+/+* (blue) and *Poldip2-/-* (red) MEFs in passages 2, 4 and 5. The blots were probed with antibodies against (A) p53, (B) phospho-p53(S20), (C) p21, (D) β-actin and Poldip2. (A-D) Densitometry was performed and corrected to β-actin. (D) p21 mRNA levels were assessed by qRT-PCR and corrected for PPIA. Error bars represent mean ± SEM of 3-4 independent experiments. *p<0.05. (F) Poldip2 is hypothesized to inhibit p53 activity, reducing the expression of p21.

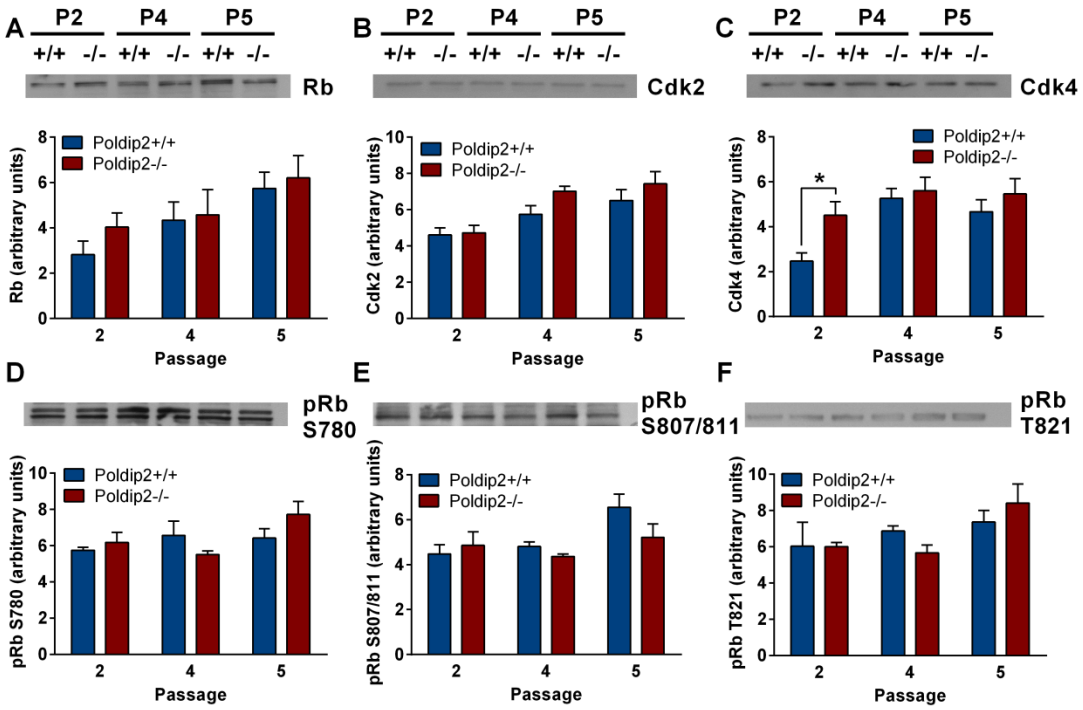

Figure 4.6. Poldip2 deletion does not affect Rb expression/phosphorylation. Immunoblotting was performed using lysates from *Poldip2+/+* (blue) and *Poldip2-/-* (red) cells in passages 2, 4 and 5. The blots were probed with antibodies against (A) Rb, (B) pRb S780, (C) pRb S807/811, (D) pRb T821, (E) Cdk2, and (F) Cdk4. Densitometry was performed and corrected to β-actin (A-F). Error bars represent mean ± SEM of 3-4 independent experiments. *$p<0.05$.

Poldip2-/- cells compared to control cells (Figure 4.6C). Interestingly, the cyclin binding partner of Cdk4, CyclinD1 was similarly increased in p2 *Poldip2-/-* cells (Figure 4.4E). However, we did not see increased phosphorylation of Rb sites that would indicate elevated Cdk2 (T821) and Cdk4 (S780, S807/811) activity (143). In fact, none of the sites measured, pRb S780 (Figure 4.6C), pRb S807/811 (Figure 4.6D), or pRb T821 (Figure 4.6E) showed a significant difference in phosphorylation between *Poldip2+/+* and *Poldip2-/-* cells.

Total E2F1 levels were also unchanged (Figure 4.7A). However, we did observe a decrease in expression of the E2F target gene, CyclinA2 (Figure 4.4F). Another E2F target, Cdk1 (Figure 4.7B), was similarly downregulated in later passages in *Poldip2-/-* compared to wild type cells. Moreover, the E2F target protein and Poldip2 binding partner PCNA was significantly reduced in passage 4 (Figure 4.7D), with a trend towards reduction in passage 5. mRNA of Cdk1 was measured to verify that the change in protein was a result of changes in mRNA expression (Figure 4.7C), and indeed Cdk1 mRNA was reduced at passage 5 in *Poldip2-/-* cells compared to controls. These data suggest that Poldip2 positively regulates the E2F pathway in a direct or indirect fashion (Figure 4.7F).

SV40 immortalization restores Poldip2 growth to wild type levels

Due to the effects on p53 and E2F1 downstream targets, we hypothesized that inactivating Rb and p53 by expressing SV40 large T-antigen in *Poldip2-/-* cells would rescue the deficiency in proliferation (Figure 4.8F). Wild type and *Poldip2-/-* MEFs were transfected with SV40 large T-antigen at passage 2. Cell cycle distribution analysis of the cells by flow cytometry (Figure 4.8A) showed that expression of SV40 large T-antigen in *Poldip2-/-* cells restored cell cycle distribution to the wild type pattern (Figure 4.8B). Immortalization with SV40 also prevented the impairment of growth induced by loss of Poldip2 (Figure 4.8C). Protein levels of the E2F target genes CyclinA2, Cdk1 and

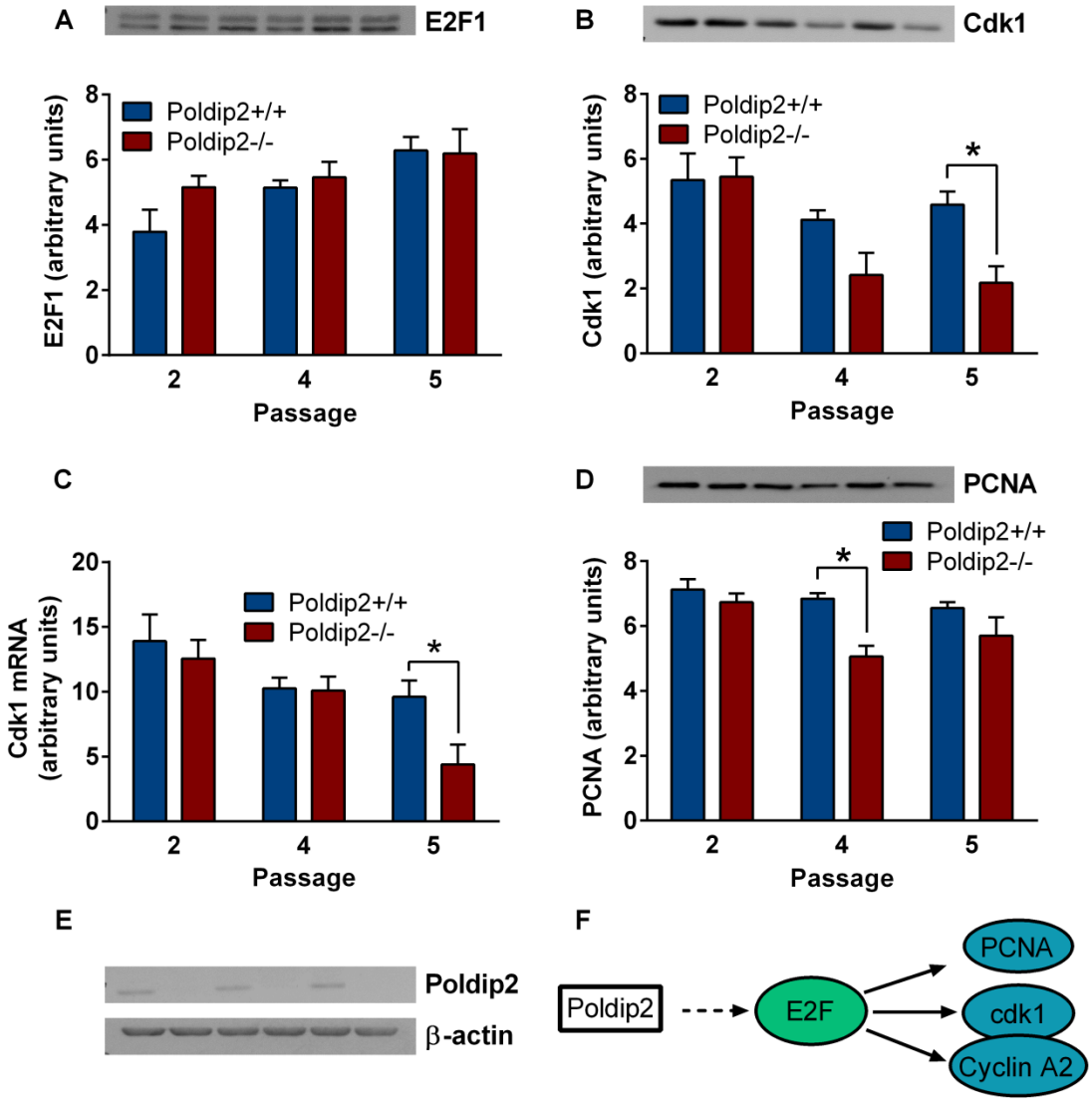

Figure 4.7. Poldip2 activates the E2F1 pathway. Protein levels of E2F1 target genes were measured by immunoblot. Lysates from *Poldip2+/+* (blue) and *Poldip2-/-* (red) cells in passages 2, 4 and 5 were probed with antibodies against (A) E2F1, (B) Cdk1, (D) Cdk1, (E) β-actin and Poldip2. (C) Cdk1 mRNA levels were measured by qRT-PCR and corrected with PPIA. Error bars represent mean ± SEM of 3-4 independent experiments. *p<0.05. (E) Poldip2 is hypothesized to activate E2F transcriptional activity, resulting in the expression of Cdk1, CyclinA2 and PCNA.

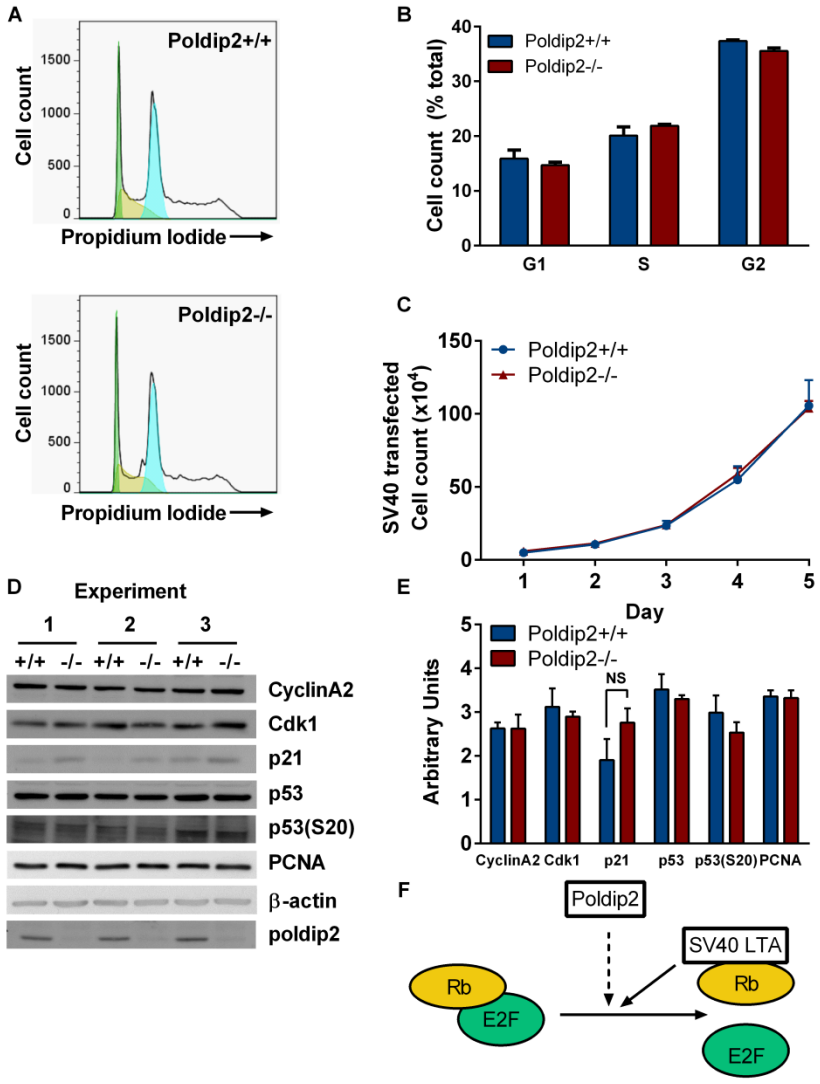

Figure 4.8. SV40 immortalization of *Poldip2-/-* MEFs restores growth and cell cycle distribution. *Poldip2* +/+ and -/- MEFs were immortalized with SV40 large T-antigen. (A, B) Cell cycle analysis was performed as in Figure 4.4 using flow cytometry (G1- green, S-yellow, G2/M-blue). (C) A growth curve was performed to compare *Poldip2*+/+ (blue, solid line) to *Poldip2-/-* (red, dashed line) MEFs. (D) Expression of the indicated proteins was assessed by western blot in 3 independent batches of immortalized cells. (E) Densitometry was performed and corrected to β-actin. Error bars represent mean ± SEM of 3 experiments. (F) SV40 large T-antigen binds and sequesters Rb, promoting E2F activity, and inhibits p53.

PCNA were restored to wild type levels (Figure 4.8D and 4.8E). Phosphorylation of p53 S20 was not readily detectable, but $p21^{Cip1}$ levels showed a trend towards being elevated in SV40 immortalized *Poldip2-/-* MEFs, similar to untransformed *Poldip2-/-* MEFs (Figure 4.8E and 4.5C). These results indicate that SV40 immortalization is sufficient to overcome the effect of Poldip2 loss on E2F-dependent cell cycle regulators and proliferation.

Discussion

In this study, we uncovered a novel role of Poldip2 in cell cycle regulation (Figure 4.9). We demonstrated that mice lacking Poldip2 are smaller during embryonic development and suffer perinatal lethality. The loss of Poldip2 reduces growth of MEFs and changes the cell cycle distribution of asynchronous cells. Poldip2 loss appears to increase p53 activity in early passages, resulting in increased expression of p21, a cell cycle inhibitor. Additionally, E2F/Rb-dependent gene expression is repressed in *Poldip2-/-* cells as evidenced by the loss of CyclinA2 and Cdk1. Finally, we showed that the cell cycle delays and expression of cell cycle regulators resulting from the loss of Poldip2 can be rescued by inhibiting p53 and Rb with SV40 LTA expression. This is the first time that Poldip2 has been reported to be implicated in cell cycle checkpoint regulation.

These results at first seem to contradict FACS analysis performed in a previous study of Poldip2, which found no difference in the cell cycle after treatment with siRNA against Poldip2 (6). However, that study was performed in SV40-transformed human fibroblasts. Our data clearly show that SV40 transformation eliminates cell cycle alterations that are readily apparent in primary mouse embryonic fibroblasts. In fact, many experiments in earlier publications describing Poldip2 function and localization were performed in immortal or cancer cell lines such as HEK293 (1; 2), HeLa (1-4), and

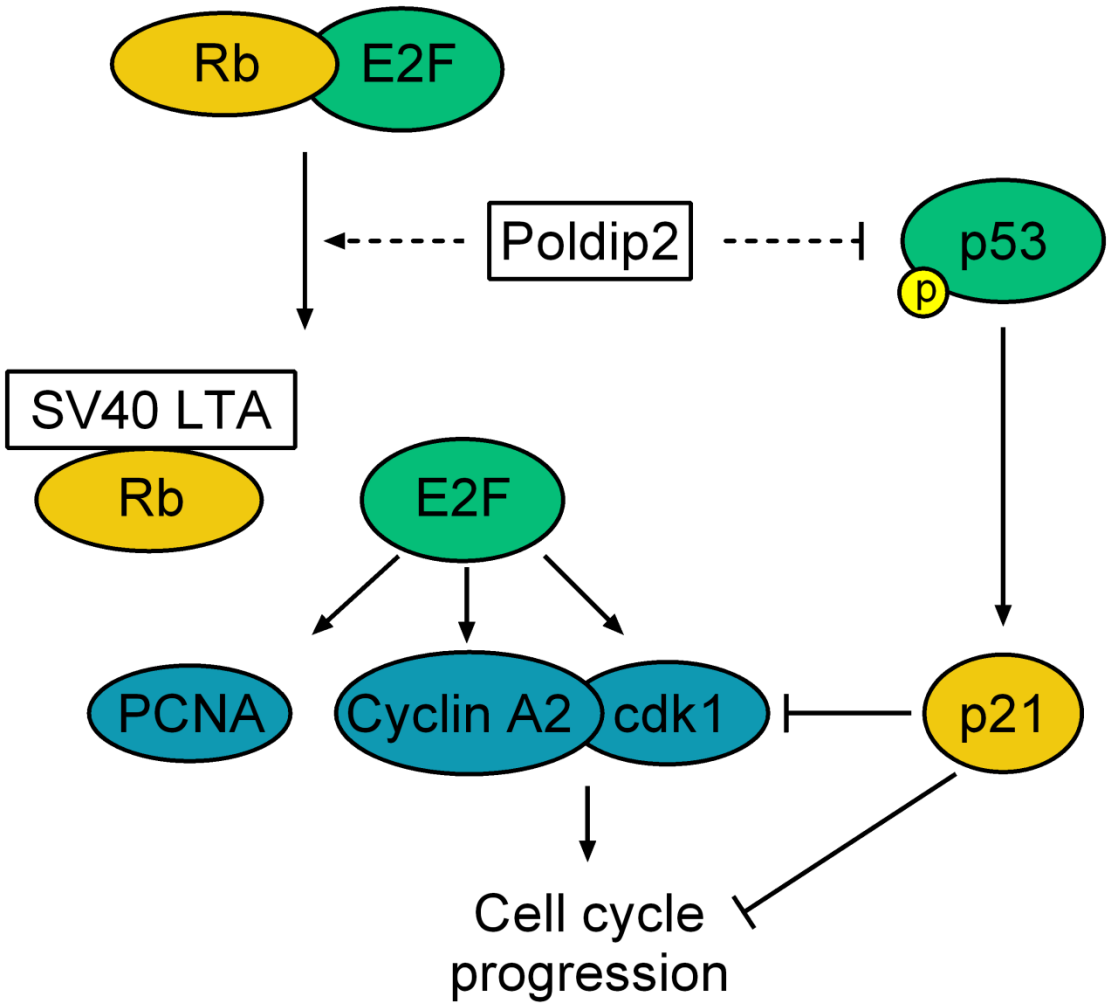

Figure 4.9. Proposed mechanism by which Poldip2 promotes cell cycle progression. Poldip2 activates the transcription of E2F target genes such as Cyclin A, Cdk1 and PCNA. These act to promote cell cycle progression. Poldip2 has been previously shown to bind PCNA. Poldip2 also limits the activation of p53 by phosphorylation and reduces expression of the cell cycle inhibitor p21. Reduced p21 promotes cell cycle progression by relieving inhibition of the activity of Cyclin/Cdk complexes. SV40 immortalization inhibits p53 activity and sequesters Rb away from E2F, promoting cell cycle progression.

C2C12 (9). These results may need to be reexamined in primary cells to verify that localization and function of Poldip2 were not altered by the immortalization.

Interestingly, one of the few Poldip2 studies performed mainly in primary cells (rat brain endothelium) finds alterations in mitosis related to chromosome segregation defects (8). Consistent with our results, the authors of this study suggest that Poldip2 likely has multiple interacting partners and might be involved in the control of a cell cycle checkpoint, which could explain the observed defect. The present work provides direct evidence that Poldip2 does in fact regulate cell cycle progression.

In this study, we focused on two key cell cycle regulatory pathways, E2F and p53. Although we do not observe a change in overall p53 expression, it has been established that expression alone is not the determinant of transcription activity (144). We observed an increase in p53 phosphorylation at serine 20 at an early passage, which has been reported to increase p53 activity(145), and increased expression of $p21^{cip1}$, a p53 transcriptional target and cell cycle inhibitor. These data support the hypothesis that p53 activity increases in the absence of Poldip2. Though the p53 and Rb/E2F pathways are often described as independent pathways, there is a large degree of crosstalk seen between the two. Downregulated E2F1 for instance, can stimulate p53 by increasing oncogenic stress (146).

The influence of Poldip2 on CyclinA2 and Cdk1 was not observed until passage 4. There is a precedent for Cdk1 reduction in later passages of knockout MEFs: in a Cdk2/Cdk4 double knockout study, Berthet et al. (147) observed a similar late decrease in Cdk1 protein and Cyclin A. This was found to be due to a hypophosphorylation of Rb. Hypophosphorylation of Rb results in increased binding to E2F, reducing the transcription of cell cycle regulators, including Cdk1 and Cyclin A. Although we did not observe a change in Rb phosphorylation in *Poldip2-/-* MEFs, there were a large number of Rb phosphorylation sites that we could not assess due to insufficient cross-species

reactivity of antibodies. However, Rb binding to E2Fs could be altered in other ways. If Poldip2 directly binds Rb, it could prevent Rb mediated inhibition of E2Fs independent of Rb phosphorylation, acting as a redundant mechanism to Rb phosphorylation. Both HPV E7 and SV40 large T-antigen sequester Rb in this manner, resulting in immortalization of many cell types (148). Another possibility is that Poldip2 binds directly to E2Fs, preventing Rb from binding even in a hypophosphorylated state. Poldip2 could also aid E2Fs in binding DNA; indeed, one of Poldip2's domains (YccV) is a DNA binding domain in bacteria (88). Further study is necessary to elucidate the effect of Poldip2 on E2F target gene expression

A number of knockout and transgenic models of cell cycle regulators display phenotypes similar to our Poldip2 model. A study of an Rb transgenic model containing extra copies of the Rb gene reported smaller embryos in transgenic mice compared to littermate controls (149). The same study found that high Rb levels correlated with embryonic lethality in some transgenic embryos. Since Poldip2 may decrease Rb activity in normal cells, the *Poldip2-/-* cells would effectively have more active Rb, similar to the transgenic model of Rb. An E2F1 knockout mouse model was viable, but exhibited tissue specific effects, including apoptosis and increased tumorigenesis (150). Although E2F1 is best known for its role in the G1/S transition, there is evidence to suggest that it plays a tumor suppressor role, inducing apoptosis and cycle arrest in other contexts (146).

The p53 and E2F/Rb transcriptional pathways have been of much interest in cancer biology, due to their complex regulation of the cell cycle and apoptosis. One of the desirable methods of targeting cancer has been to overcome tumor cell resistance to senescence and apoptosis, while leaving normal cells untouched. The p53 tumor suppressor and cyclin dependent kinases have been the targets of drug and genetic therapies because p53 is lost in many human cancers, and Cdks play such a key role in cell cycle regulation (151). The role of Poldip2 in tumor formation is not well known yet,

though a study of the sense-antisense gene pair of TNFAIP1/POLDIP2 found poor prognosis in breast cancer patients with upregulated Poldip2 expression (13). A second key area where antiproliferative research can make an impact on is the prevention of post-angioplasty restenosis. A recent trial found that bare stents have a 21% restenosis rate within 24 months (152). Antiproliferative drugs in a drug eluting stent could lower recurrence rates by limiting vascular smooth muscle cell growth after angioplasty.

Our current understanding of Poldip2 is far from complete. Future studies are necessary to uncover exactly how Poldip2 influences the cell cycle and E2F/p53 target proteins. However, this study and others highlight the importance of Poldip2 in growth, including the cell cycle and DNA duplication/repair (1; 2; 6; 8). Perinatal lethality in the absence of Poldip2, as well as the reduced growth in *Poldip2-/-* primary cells, indicate that Poldip2 is essential for normal cell growth and proliferation. A further understanding of Poldip2 signaling may uncover novel targets for antiproliferative drugs, and provide a better understanding of the mechanism of current therapies.

Chapter 5

Conclusion

Overview

When I began this work, our lab had discovered and characterized the interaction between Poldip2 and the NADPH oxidase subunit p22phox in VSMCs. Little has been previously published on Poldip2 function; much of the current knowledge comes from speculation based on interacting proteins. Poldip2 knockdown has shown a variety of consequences including mitochondrial morphology defects (3; 9), impaired DNA replication and chromosome segregation (8), altered cytoskeleton dynamics (5), and reduced ROS production (5). However, these studies have been primarily descriptive and have not explored the mechanism of how Poldip2 might be affecting these processes. The work presented in this dissertation furthers our knowledge of Poldip2 by examining Poldip2 structure, exploring the gene expression consequences of Poldip2 downregulation, and identifying a potential function in the cell cycle. The primary findings of this work are:

1) The two identified functional domains YccV and ApaG are homologous to bacterial proteins of the same name. Both domains are well conserved, but ApaG in particular contains hydrophobic surface regions including a highly conserved surface region that resembles a solvent accessible binding pocket.
2) Poldip2 knockdown disrupts gene expression in key signaling pathways regulating the cell cycle, circadian rhythms, mitochondria function and metabolism.
3) Poldip2 knockout mice are not viable. Cells generated from knockout animals exhibit reduced proliferation and altered checkpoint protein expression, which can be restored by inhibiting p53 and Rb via SV40 virus expression.

In this chapter, I will summarize my findings and discuss their significance with respect to understanding the context of Poldip2 function. I will consider the implications of these results, potential caveats and future research directions.

Summary and Significance

In Chapter 2, I investigate the structure of the Poldip2 protein by *in silico* analysis. By performing a sequence alignment comparing the human Poldip2 gene to 50 other species including mammals, amphibians, insects and birds, I discovered a high degree of homology between all species. The degree of conservation was especially pronounced within the YccV and ApaG domains. Although no crystal structure of Poldip2 has been resolved, I used the known structures of bacterial YccV and ApaG domains to estimate N-terminal, C-terminal and full length structures of the protein. By performing a second sequence alignment comparing the human ApaG domain to bacterial homologues, I identified key residues that were conserved across all species. Many of these residues were adjacent on one surface of the ApaG model, which resembles a binding pocket of some kind. Additionally, by performing a solvent accessibly analysis, I found this pocket is indeed accessible. This analysis identifies key regions that are likely important for Poldip2 function. The highly conserved binding pocket could indicate that Poldip2 has enzymatic activity or closely associates with another protein or an ion.

The work in Chapter 3 furthers our understanding of the pathways in which Poldip2 takes part. A pathway analysis of genes altered by Poldip2 downregulation was performed using gene arrays with RNA from Poldip2+/- aortas and Poldip2-/- mouse embryonic fibroblasts. Notably the cell cycle, circadian rhythms, mitochondrial function and metabolism all had multiple genes altered in the arrays. These pathways provide evidence of Poldip2 functionality.

Chapter 4 uncovers more mechanistic evidence of cell cycle regulation in a mouse knockout model of Poldip2. We discovered that Poldip2 knockout mice die at birth. Additionally, proliferation of cells derived from Poldip2 knockout embryos was markedly

reduced. Further investigation of the MEFs uncovered a disrupted cell cycle and key changes in cell cycle checkpoint proteins.

What can we learn from the structure of Poldip2?

When Poldip2 was originally identified in 2002 as a polymerase delta and PCNA interacting protein, the only information about the protein available in the literature was the primary sequence (1). The authors discuss the conservation of the C-terminal tail of Poldip2, and the C-terminal homology with both ApaG and the F box A proteins.

Both ApaG and F-box A have been proposed to be involved in protein binding (86; 153). Interestingly, one of the best known functions for F-box proteins is as members of ubiquitin ligase (E3) complexes. The F-box protein connects the enzymatic complex to the substrate, as is the case in the Cdc4 SCF complex. The cell cycle is a tightly regulated process requiring the ubiquitin-mediated degradation of proteins to properly pass through specific phases. In a yeast study, the absence of Cdc4 caused cells to arrest at G1/S and G2/M (154). These results are similar to what happens in MEFs that lack Poldip2 (Figure 4.4).

There are however, key sequence differences between Poldip2 and the ubiquitin ligase protein Cdc4, namely the lack of WD40 repeats in Poldip2. WD40 is a 40-60 amino acid repeating sequence that typically terminates with tryptophan and aspartic acid to form a series of parallel β-folds (155; 156). These can form a propeller structure that is involved in protein binding, and potentially aid in ubiquitin transfer (157). The lack of the WD40 domain does not eliminate the possibility that Poldip2 may be involved in an E3 ligase or other enzymatic activity complex, but it does mean that Poldip2 does not closely resemble other proteins with that function (with the exception of the F box A domain).

Another key piece of evidence from the primary amino acid structure of Poldip2 is the presence of the GXGXXG G-loop motif in the ApaG domain. The importance of this motif is clear considering that it is 100% conserved in all 51 Poldip2 sequences and bacterial ApaG sequences (Figures 2.2 and 2.5). Additionally, the domain is located on the surface of the Poldip2 ApaG domain with solvent accessibility (Figure 2.6). Due to the high level of conservation with even bacterial proteins, one can speculate the interaction target with this domain is a specific small molecule or amino acid. Any alteration of this sequence would therefore disrupt the interaction and perhaps protein activity associated with this binding. In future work, the creation of a Poldip2 construct with hydrophobic surface residues in the ApaG binding pocket mutated to alanines could be used to disrupt protein-protein interactions without altering other potential activities that Poldip2 may have, such as those associated with G-loop binding.

Previous work has implicated the G-loop in binding adenosine diphosphate, which could indicate Poldip2 binds ADP/GMP, NAD or FAD due to the common adenosine diphosphates in these molecules. In a crystallography study of the bacterial ApaG, the authors do not find an interaction with ATP or GTP (86). The authors suppose that the sequence might instead be involved in a protein interaction due to the lack of a nearby positively charged surface to interact with the nucleotide phosphate group. However, in the Poldip2 structure, there is an adjacent solvent accessible binding pocket, which could accommodate Mg^{2+} or some other charged ion to enhance phosphate binding. Post-translational modification to Poldip2 or protein interaction could also act to shift the conformation to further expose the binding region to its partner. Binding with NAD^{+} and FAD was not tested, but remains a real possibility. In order to test Poldip2 binding to ADP containing molecules, in vitro binding assays should be performed in the presence and absence of other Poldip2 associated proteins like Polδ, PCNA and p22phox.

Evidence from our lab demonstrated binding between the NADPH oxidase subunit p22phox and Poldip2, which enhances Nox4 activity (5). Based on my structural analysis, one possible scenario is that the interaction with p22phox causes Poldip2 to pass NADPH from the G-loop to the NAD binding region on the cytoplasmic tail of Nox4 complex to enhance enzymatic activity. Alternatively, Poldip2 could remove the spent $NADP^+$ from the Nox4 NAD binding site to make room for the active NADPH. This would support a role for Poldip2 as a protein that enhances Nox activity, but is not required for activity. By creating a point mutation of Poldip2 within the G-loop GXGXXG, I hypothesize that certain functions of Poldip2 would be severely impaired. Compared with overexpressing wild type Poldip2, overexpressing the mutant should not increase Nox4 activity due to disrupted p22phox or NAD binding.

Although the ApaG domain provides high identity conservation with eukaryotic and prokaryotic homologues, the full length Poldip2 protein also contains a well conserved N-terminal YccV domain (Figure 2.1). The YccV domain in part is responsible for the localization of Poldip2 due to the presence of a 40AA mitochondrial targeting sequence (2). The bacterial YccV protein has been demonstrated to be involved in bacterial DNA replication by regulating the DnaA protein. In two separate reports about the mechanism of DnaA regulation, there is controversy about the mechanism. One report proposes direct binding to oriC to regulate gene expression (88). A second paper proposes that the YccV protein acts as a heat shock protein (named HspQ) to degrade DnaA (90). Due to the bacterial origin of the mitochondria, there are many similarities between mitochondrial DNA replication and bacterial replication. Because Poldip2 is localized to the mitochondria in some cell types (2) and siRNA against Poldip2 causes mitochondria morphology defects (3; 9), it seems likely that the YccV domain contributes to regulation of DNA replication in the mitochondria. Interestingly, F-box proteins have also been implicated in mitochondrial replication. The F-box protein Mus-

10 was demonstrated to alter morphology and mtDNA stability in knockout cells (91). If the ApaG domain contributes in a similar fashion, both domains could be involved in mitochondrial replication and function. To investigate the binding the DNA binding ability of Poldip2, EMSA or DNase footprinting assays should be performed, followed by a more detailed analysis of the binding sequence. Comparing the sequences with the overall genome would allow predictions of whether Poldip2 might bind to the promoters of certain proteins.

In addition to the mitochondria, in eukaryotic cells there is evidence of Poldip2 in the nucleus. Though nuclear DNA replication is fundamentally different from that of mitochondria, certain similarities exist, particularly in error checking mechanisms (158; 159). Poldip2 could act in a similar fashion in both cases. In the nucleus, Poldip2 binds the p50 subunit of polymerase δ and PCNA, which are both involved in error checking and DNA damage response (1). In the mitochondria, Poldip2 associates with proteins in the mitochondrial DNA nucleoid including mtSSB (9), TFAM, Hsp90β, and LonM (3). Furthermore, mitochondrial proteins LonRF1, Pdk1 and Mgarp were decreased in the Poldip2 knockdown gene arrays (Tables 4.1 and 4.2). Perhaps Poldip2 acts as a stress or DNA damage sensor in mitochondria and the nucleus. Nox4 has been proposed to be a protective NADPH oxidase (160); during periods of stress sensed in the mitochondria or nucleus, Poldip2 could in part rapidly activate Nox4 protectively. Interestingly, Nox4 localization in cardiomyocytes is in part mitochondrial (161), though it is unclear whether mitochondrial Nox4 is cardioprotective. Poldip2 must also play additional roles in cell growth apart from its regulation of Nox4, because in knockout of Nox4 is not embryonic lethal (14), whereas Poldip2 knockout mice die at birth (12).

Making comparisons between primary structures to identify conserved domains and motifs can be valuable in identifying proteins with similar functions. However, due to the nature of protein folding, amino acids that are not within the same region of the

primary sequence can end up adjacent in the folded structure. By comparing Poldip2 primary sequences and overlaying the degree of similarity on the *in silico* tertiary structure of Poldip2, I have gleaned additional information about its structure that highlights the importance of the conserved G-loop and binding pocket regions of the protein.

What do Poldip2 knockout gene arrays uncover about Poldip2 signaling?

Although several studies have described potential functional roles for Poldip2, there has been no previous publication on the effect of Poldip2 on gene expression. I have identified several key cellular pathways with multiple gene transcripts affected by Poldip2 knockdown: cell cycle, circadian rhythms, mitochondria function and metabolism. Further verification of these Poldip2 downstream targets by other means such as qRT-PCR or measuring protein levels by immunoblot is necessary to eliminate false positives that sometimes occur in high throughput assays such as the gene array used to identify these targets.

Although each of these pathways exhibits multiple affected genes, the mechanism of Poldip2 regulation of gene transcription remains to be discovered. There is some evidence that the YccV domain that makes up the N-terminal segment of Poldip2 may be involved in DNA binding in bacteria, but there is no experimental evidence to suggest that Poldip2 is a transcription factor or repressor. DNA binding by Poldip2 could be tested using a gel mobility shift assay or with more modern methodologies such as fluorescence anisotropy (162). Though we cannot rule out the possibility that Poldip2 can directly bind DNA, it is possible that Poldip2 regulates the activity of transcriptional proteins by post-translational modification, or by influencing transcription factor nuclear localization or activity by binding. Poldip2 could also be more distantly related to transcription factor activity by affecting upstream signaling pathways that ultimately

alter transcription. With these identified pathways, we can begin to narrow down the signaling mechanisms regulated by Poldip2.

In order to narrow down the mechanism of Poldip2 regulation of these pathways, several areas of investigation should be undertaken. Beginning with an identified endpoint that Poldip2 knockdown alters, one could work backwards through known signaling pathways. If increasing the activity of a protein upstream of the chosen endpoint overcomes the effect of Poldip2 knockdown, this would suggest that Poldip2 is upstream of that protein. For example, in the case of the circadian rhythm pathway one obvious target is the transcription factor Tef, which was reduced in Poldip2 knockdown. Tef is known to be regulated by the activity of Bmal1. If Tef expression is recovered by increasing Bmal1, this would suggest that Poldip2 is upstream of Bmal1. If Tef expression is not recovered, then Poldip2 could be a limiting step in Bmal1 regulation of Tef. In the case of clock genes, this strategy can be challenging because the signaling pathway is not linear and circadian proteins exhibit a great deal of crosstalk with other circadian and cell cycle proteins.

It would be illuminating to perform an analysis of circadian periodicity by comparing wild type and knockout cells to identify overall changes in circadian dynamics, similar to the cell cycle analysis performed in Chapter 4. This would require synchronization of the circadian rhythms of cultured cells. The lack of synchronization of circadian rhythms in cultured cells could explain why there were changes in circadian proteins in the aorta samples, but not the culture MEFs (Tables 4.1, 4.2). One strategy to synchronize circadian rhythms in culture is to pulse heat shock the cells, which synchronizes Per2 rhythms in cultured fibroblasts (163). However, heat shock may also cause the activation of other proteins that associate with Poldip2, namely the heat shock proteins Hsp70 and Hsp90, which associate with Poldip2 in the mitochondria (3).

Overall, much remains to be done to better understand the role of Poldip2 in gene regulation. The relatively small fold changes (<2) of many of the gene array identified proteins in Poldip2 knockout cells could indicate that Poldip2 is well upstream of gene regulation, but they give a starting place for signaling studies of Poldip2 function.

How does Poldip2 regulate the cell cycle?

Although Poldip2 has been previously proposed to be involved in the DNA damage response and replication due to interaction with PCNA and the p50 subunit of polymerase δ (Pold2), it is now clear that Poldip2 has additional roles in the cell. A study on the functional link between Poldip2 and Polδ proves that recombinant Poldip2 expression has an inhibitory effect on Polδ activity (2). If Poldip2 negatively regulates Polδ or PCNA, one would expect that reduced Poldip2 would conversely increase the activity of these complexes.

Overexpression of PCNA has been associated with increased proliferation by overcoming growth arrest pathways (164). Reduced Poldip2 has been associated with mitotic spindle disorganization and problems with chromosome segregation. Additionally, my own work has demonstrated that the lack of Poldip2 results in reduced cell growth due to increased checkpoint activity (Figure 4.2). This contrasts with the expected consequence of reduced Poldip2 if it primarily reduces the activity of Polymerase δ. It should also be noted that the original experiment showing Poldip2 regulation of Polδ activity was performed with recombinant, not endogenous, protein, which could lead to different processing and perhaps protein activity. The consequence of prolonged overexpression of Poldip2 remains to be tested. This could be done with a lentivirus to stably overexpress Poldip2. The effect of Poldip2 overexpression on the cell cycle could then be investigated with the expectation that Poldip2 overexpression would not alter the cell cycle, or possibly increase cell proliferation.

It remains possible that Poldip2 could reduce Polδ activity in certain contexts. Poldip2 was shown to interact with the translesional DNA polymerase Polη (6). Loss of Poldip2 results in increased Polη DNA foci and reduced survival in response to UV irradiation. One possibility is that in the case of DNA damage, Poldip2 reduces the activity of the replicative polymerase, Polδ, and directs the action of repair polymerases such as Polη. Poldip2 knockout MEFs exhibit fewer cells in S-phase (Figure 4.4), which could be due to cells lacking Poldip2 failing to complete DNA repair by regulating translesional polymerases such as Polη, resulting in faster DNA replication. A recent publication challenges the role of Poldip2 in DNA damage repair by demonstrating that Poldip2 does not colocalize with PCNA and Polη in UV-induced DNA damage foci (10). Another possibility is that binding Poldip2 improves the fidelity of replicative polymerases in a manner that has the consequence of reduced replicative activity. In this case, reduced Poldip2 would result in faster replication, but also more errors, which could lead to detrimental mutations. To test this hypothesis, an in vitro polymerase assay followed by Sanger sequencing of individually cloned PCR products should be performed to measure the fidelity of DNA replication in the presence and absence of Poldip2. This would not only give a quantitative measure of fidelity, but would identify the specific residues that mutate, allowing for the identification of particular types of DNA errors.

Although there is much evidence to support a role for Poldip2 in DNA replication and repair, there is reason to believe it has additional activities. A high throughput yeast two-hybrid screen confirmed the interaction with PCNA and Pold2, but also identified binding with several other growth related proteins, including PDPK1, MAP3k71p1, and HDAC6 (7). The gene array data presented earlier in this report shows key cell cycle genes such as Gadd45γ, MAPKBP1, and Pde1a have altered expression as a consequence

of Poldip2 downregulation (Tables 3.1 and 3.2). It is possible that Poldip2 acts as a cell cycle checkpoint protein to link DNA duplication to cell cycle progression.

Although the lack of Poldip2 causes clearly reduced proliferation in MEFs, the degree of the effect and the cell cycle proteins involved changes depending on the passage of the cell (Figure 4.4-4.7). This phenotype is not limited to Poldip2; the Tert-/- mouse model exhibits no noticeable phenotypes until several generations of knockout have been bred (165). This is due to mice having long telomeres and a short lifespan. The reduction in telomere size takes more than one generation to achieve crisis levels. Furthermore, higher passage NIH3T3 cells with dominant negative telomerase exhibit increased G1 and reduced S phase (166), similar to passage 2 Poldip2-/- MEFs (Figure 4.4). Telomere crisis is unlikely to be the cause of reduced growth in Poldip2-/- MEFs due to the lack of increased senescence marker expression (Figure 4.3), but could be ruled out by performing a TRAP assay to assess telomerase activity (167). Although telomerase changes are unlikely to cause the observed phenotype, some other key cell process or component could require multiple divisions before reaching a critical level that reduces proliferation. This would explain why Poldip2-/- embryos are not significantly reduced in size until E18.5 (Figure 4.1).

Poldip2 has been established as a key protein in normal cell cycle progression. The mechanism is not yet well understood. The cause of E2F pathway disruption remains unclear. Though E2F1 expression was unchanged, expression and activity of E2F activators E2F2 and E2F3a should be assessed in the absence of Poldip2. E2F inhibitors could also play a role. Untested phosphorylation sites on Rb could be altered or expression of E2F repressors E2F3b-E2F8 could be elevated in Poldip2 knockout cells. The E2F repressors p130 and p107 should also be tested for altered regulation in Poldip2 knockout cells. Other pathways that can influence cell cycle checkpoints such as mitochondria function and circadian rhythms remain to be tested.

Concluding Statement

Many cellular functions have been attributed to Poldip2 since its discovery in 2003, although data on the molecular mechanisms by which Poldip2 exerts its effects are scarce. Our lab became interested in the protein primarily due to effects in the vasculature on cytoskeleton dynamics and ROS production via Nox4. Further study has uncovered its role as an essential protein in embryonic and cellular growth.

Cell cycle proteins, particularly those involved in DNA replication, have proven to be effective targets in antiproliferative therapies. Drugs such as the Cdk4/6 inhibitor palbociclib (Pfizer) have shown great promise in recent clinical trials (168). The drug acts by blocking phosphorylation of Rb, which prevents cell cycle progression by E2F. The action of Poldip2 on the cell cycle also works though E2F activation (Figure 4.7).

Novel cell cycle targets such as Poldip2 could be good targets for antiproliferative therapy. Proliferative diseases such as cancer, post-stent restenosis, atherosclerosis, and autoimmune disease are costly and potentially deadly diseases that are the result of the overproliferation of certain cell types. Current treatment regimens prolong patient lifespan, and can treat some types of cancer if started early in progression, but are still far from a cure. Current therapies cause adverse off target effects resulting in poor lifestyle during treatment, and significant improvements in efficacy must be made to treat late stage and aggressive cancers. All of these could be improved with novel cell cycle targets. Having a greater understanding of proliferation related signaling pathways could lead to improved efficacy and fewer side effects.

Based on my structural analysis of Poldip2, I propose that the highly conserved binding pocket could be a good target for small molecule therapy. Due to the fact that Poldip2 knockout causes embryonic lethality and reduced growth in a cell culture setting, inhibiting the activity by blocking binding to this site could have therapeutic benefits.

In order to further progress in the understanding of Poldip2 function, I have proposed a number of future experiments. By creating a point mutation of Poldip2 within the G-loop GXGXXG using site-directed mutagenesis, I hypothesize that certain functions of Poldip2 would be severely impaired. Compared with overexpressing wild type Poldip2, overexpressing mutant protein should not increase Nox4 activity due to disrupted p22phox or NAD binding. An additional Poldip2 construct with hydrophobic surface residues in the ApaG binding pocket mutated to alanines could disrupt protein-protein interactions without altering activity associated with G-loop binding. I would also perform in vitro assays to assess binding of Poldip2 to ADP containing molecules in the presence and absence of other Poldip2 associated proteins. I would verify the findings of the microarray by western blot and qRT-PCR. In order to investigate the target of Poldip2 regulation in the cell cycle, I would perform western blot and immunoprecipitations with Rb and E2F family members.

Overall, Poldip2 has been demonstrated to be an essential protein in eukaryotic physiology. In this thesis, I have explored the structure of Poldip2, identified key gene expression pathways influenced by Poldip2 depletion, and uncovered a novel role for Poldip2 as a cell cycle regulator. Much remains to be discovered about the structure and function of Poldip2, but this thesis provides groundwork for future research and highlights the importance of better understanding Poldip2.

Appendix A

Nox proteins in signal transduction

Text and figures reproduced from Brown et. al. (14) with permission.

Note: Although the effects of Poldip2 on proliferation are likely unrelated to Nox activity, Poldip2 may influence signaling pathways downstream of p22phox containing Nox proteins. In this review, I discuss in detail the Nox family members that interact with p22phox, and the downstream signaling pathways that may be influenced by Poldip2 regulation of Nox.

NADPH oxidases: a brief history

Early Nox research was carried out in neutrophils, studying the respiratory burst NADPH oxidase complex (169). The catalytic subunit of this protein is now known as Nox2, or gp91phox. Nox2 has been extensively studied and reviewed (170-172), so I will summarize here the role of Nox2 in signaling pathways only briefly. Although functional studies indicated the probable existence of Nox2 homologues, new family members have only been cloned and studied in the past decade.

The first homologue of Nox2 to be cloned was Nox1, originally described in 1999 as Mox1 (173), and almost simultaneously as NOH-1 (174). Although the dual oxidase (Duox) enzymes (longer homologues of Nox2) were not cloned until shortly after Nox1, earlier research had characterized a putative thyroid NADPH oxidase (175), so the Duoxes were believed to exist before they were finally cloned (176; 177). Almost immediately thereafter, in 2000, Nox3 was described as a gp91phox homologue expressed in fetal kidney and a cancer cell line (178). Nox3 was later determined to be primarily expressed in the inner ear in adults (179). Nox4, originally Renox, was discovered in the kidney (180; 181) and soon afterwards was described in osteoclasts (182). Nox5 was discovered in 2001 by two different groups (183; 184).

Structurally, all members of the Nox family contain at least six transmembrane domains and cytosolic FAD and NADPH binding domains (Figure A.1). Nox1-4 lack extra functional domains that Nox5 and Duox1/2 contain. Nox5 contains EF-hand Ca^{2+} binding domains (183; 185), while Duox1/2 have an extracellular peroxidase domain in addition to the EF-hand and gp91phox homology domains (Figure A.1), though it does not appear to be functional (186).

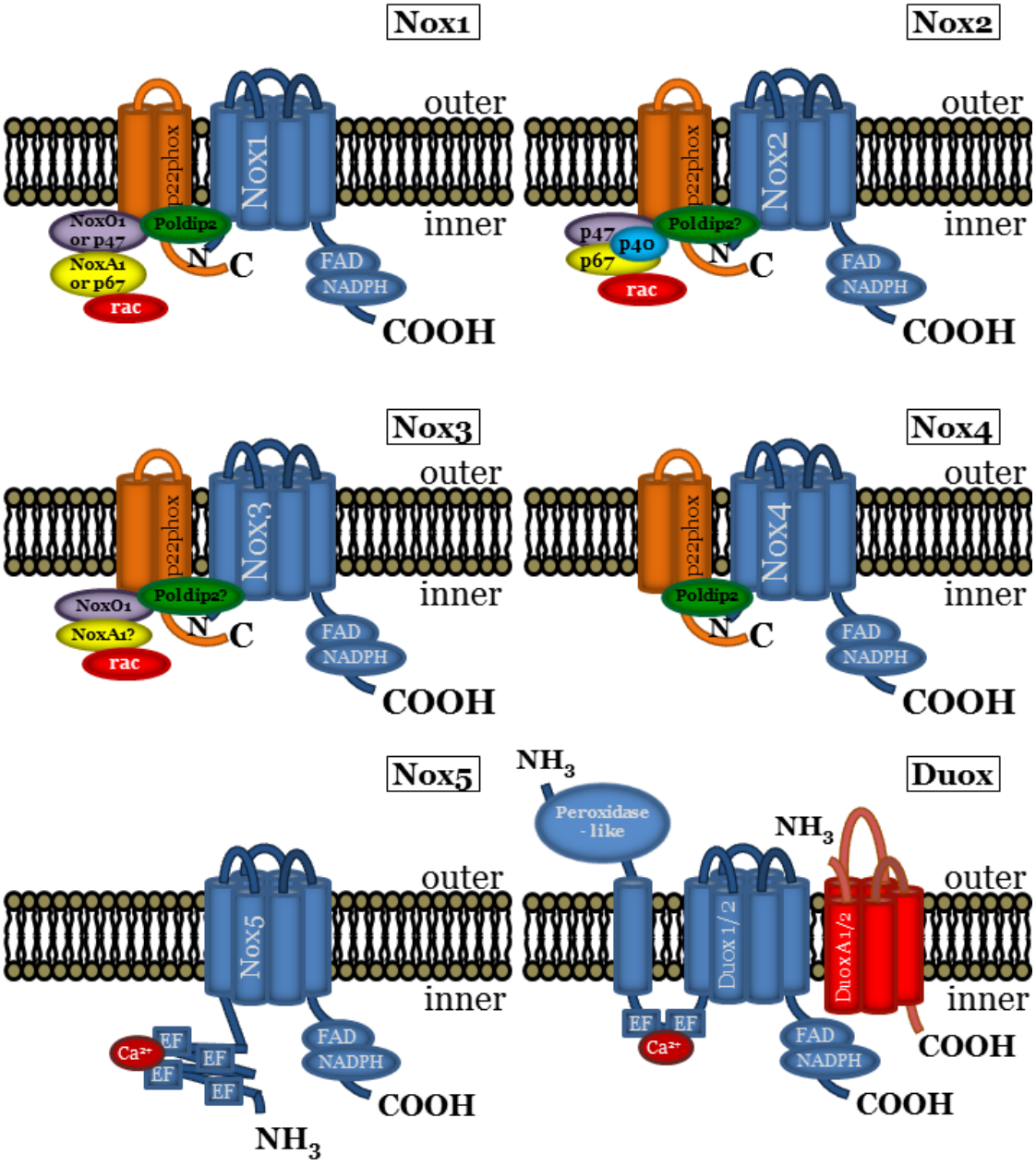

Figure A.1. Nox family members and their regulatory subunits. In Nox1-4, the transmembrane subunit p22phox associates with active and inactive Nox. Poldip2 associates with p22phox in the Nox1 and Nox4 complexes, and is predicted to be in Nox2 and Nox3. Nox1-3 are activated by a Rac-mediated mechanism, whereas Nox4 is constitutively active when associated with p22phox. Poldip2 enhances the activity of Nox4, but it is unknown how Poldip2 association with other Noxes influences their activity. Modified figure reproduced from Brown et al. (14) with permission.

NADPH oxidase activation

A number of regulatory subunits have been identified for the Noxes, and various stimuli such as angiotensin II (Ang II), thrombin, platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β) have been shown to alter the activity or expression of the Nox proteins and subunits, and ultimately the amount of ROS produced. Activation mechanisms for Nox1-3 are similar, and involve complex formation with regulatory cytosolic subunits. Regulation of Nox4 is poorly understood, but may be primarily at the expression level (187), although the identification of Poldip2 as a Nox4 regulator suggests some regulation of activity as well (188). In contrast, Nox5 and the Duoxes appear to be activated by Ca^{2+} (185; 189). Detailed mechanisms of activation for individual Nox enzymes are included below.

Physiological targets of Nox-derived ROS

The ROS produced by NADPH oxidases seem to have two general downstream physiological roles. Superoxide produced by Nox2 is required for the respiratory burst. A role in host defense has been proposed for other Nox enzymes as well, including Nox1 in the colon and Duox1 and 2 in the lung (190). This topic has been extensively reviewed elsewhere (172; 191-193). The second role of Nox is in signaling: $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2) that are derived from Nox enzymes can specifically and reversibly react with proteins, altering their activity, localization, and half-life. Many signaling processes are known to be affected by Nox-derived ROS. They will be described in greater detail below with respect to the Nox family member that initiates the ROS signal.

Compartmentalization

It should be noted that the roles of different Nox family members, though they all produce $O_2^{\bullet -}$, are physiologically distinct. This is due in part to compartmentalization

within the cell. Antioxidants and ROS metabolizing enzymes are in place to reduce non-specific reactivity. Nox1 has been identified in caveolae on the plasma membrane (194; 195). Nox2 is found in phagosomes and on the leading edge of lamellipodia (196). Both Nox1 and Nox2 have also been localized to "redoxisomes", endosomes responsible for early receptor-mediated signaling in non-phagocytic cells (171; 196; 197). The subcellular localization of Nox3 has only been studied in overexpression systems, where it was shown to target to the plasma membrane (198). Nox4 has been identified in focal adhesions (195), the nucleus (199), mitochondria (161), and the endoplasmic reticulum (200), where it interacts with kinases and phosphatases distinct from those found in caveolae and endosomes. Nox5 has been found to localize to internal membranes in the absence of stimulus; however, in response to added phosphatidylinositol 4,5-biphosphate (PIP_2), Nox5 localizes to the plasma membrane via an interaction between PIP_2 and the Nox5 N-terminal polybasic domain (201). Duox1/2 are found on the plasma membrane (202).

ROS produced by Nox proteins can act both intra- and extracellularly. Nox2 can produce ROS extracellularly via exocytosis that occurs after agonist activation of the enzyme. Nox1 on the other hand, has been demonstrated to induce endocytosis upon activation, which produces intracellular-acting ROS in endosomes. Other Nox members primarily produce ROS intracellularly, and are believed to reside within intracellular membrane structures or vesicles, from which ROS enter the cytosol. The mechanism by which ROS escape from these signaling endosomes is under active investigation, but has been studied most extensively for Nox1.

Physiology and pathology

Physiological roles

The Nox family of proteins has been demonstrated to be essential in normal physiology. Expression of NADPH oxidases is ubiquitous in mammals, though the individual Nox isoforms have different distributions between tissues and species. Nox proteins have been shown to regulate many fundamental physiological processes, including cell growth, differentiation, apoptosis, and cytoskeletal remodeling. In addition, they have more specialized functions, such as host defense (Nox2) (172), otoconium formation in the inner ear (Nox3) (187), iodination of thyroid hormone (Duox2) (203), and control of vascular tone (Nox2) (204). As research in this area expands, we are bound to gain a better understanding of the myriad functions of this enzyme family.

One controversial potential role of the Nox proteins is oxygen sensing. It is clear that ROS species play a role in the hypoxia response; however, the source or sources of ROS are a matter of dispute. Early in vitro studies showed that Nox enzymes were less active in hypoxia than normoxia (205). However, in vivo it was found that ROS production increases in low oxygen to activate the transcription factor hypoxia inducible factor-1 (HIF-1) (206) and redox sensitive K+ channels (207). There is evidence to support both mitochondrial (208) and NADPH oxidase-derived (209) ROS in oxygen sensing, but overall, the mechanisms are not well understood.

The presence of Poldip2 in the mitochondria and its interaction with Hsp proteins could indicate a role in hypoxia or oxygen sensing. The Poldip2 interacting protein Hsp70 in particular has been previously implicated in oxygen sensing (210). Poldip2 could be a link between mitochondrial oxygen sensing and the Noxes, but further study is necessary to understand the functional connection between Hsp proteins and Poldip2.

Nox proteins in disease

The dysregulation or absence of certain Nox isoforms has been linked to a variety of diseases in essentially every organ system. The earliest discovery was an immune disorder, chronic granulomatous disease (CGD), caused by the absence of active Nox2 or its subunits (211-213). Patients with CGD exhibit chronic infections and impaired wound healing (214). Nox derived ROS have been implicated in the pathogenesis of a number of neurological diseases, including Alzheimer's disease. Nox has even been proposed as a potential pharmacological target for slowing Alzheimer's disease progression (215). Overactivation of Nox1 and Nox2 has been shown to be involved in the development of H. pylori-induced gastrointestinal inflammation (216), hypertension (217; 218), and restenosis after angioplasty (219; 220), while excess ROS produced by Nox5 are related to atherosclerosis (221) and cancer (222; 223). Moreover, Duox1/2 dysregulation has been associated with thyroid dysfunction (224) and cystic fibrosis (225). Nox proteins have also been linked to rheumatoid arthritis and diabetes. A complete description of pathologies associated with the Nox proteins is beyond the scope of this dissertation, but can be found in several recent reviews (192; 204; 216; 226).

Nox-derived ROS

Superoxide

Superoxide, the primary product of Nox enzymes, is produced physiologically via a one-electron reduction of molecular oxygen. Superoxide is highly reactive and short lived, which makes determining a biological half-life difficult. Superoxide can dismutate to a second signaling intermediate, H_2O_2, spontaneously (rate constant = 8×10^4 $M^{-1}s^{-1}$) or enzymatically via superoxide dismutase (SOD) (rate constant = 2×10^9 $M^{-1}s^{-1}$) (227). As a consequence, $O_2^{\bullet -}$ must be produced in very close proximity to its target to be effective as a signaling molecule. Superoxide is also capable of reacting with nitric oxide

(NO), forming highly reactive and potentially damaging peroxynitrite ($OONO^-$) (rate constant = 4-16 x 10^9 $M^{-1}s^{-1}$) (228). This also inactivates NO, which can have pathological consequences, particularly in vascular endothelial cells.

Superoxide is known to react with $(FeS)_4$ clusters, which may release ferric ions (229). In the case of aconitase, $O_2^{\bullet-}$ inactivates the enzyme, leading to reduced mitochondrial function (230). There is *in vitro* evidence of $O_2^{\bullet-}$ reacting with heme groups such as cytochrome C; however, the physiological significance of this reaction remains to be determined. Finally, the formation of peroxynitrite from $O_2^{\bullet-}$ can then lead to reversible glutathionylation of proteins on reactive cysteines, as has been described for the Na+-K+ ATPase (231).

Superoxide is also known to react with protein thiols such as cysteine residues, but it has been pointed out that the reaction rate of SOD converting $O_2^{\bullet-}$ to H_2O_2 is much faster than that of $O_2^{\bullet-}$ with biothiols (232). H_2O_2 also reacts with protein thiols, and although the reaction rate of $O_2^{\bullet-}$ with protein thiols is chemically faster than that of H_2O_2, the greater stability and diffusibility of H_2O_2 increases its probability of reacting with the protein thiols involved in ROS signaling. This suggests that physiological protein thiol oxidation is most likely H_2O_2-dependent.

Although production of $O_2^{\bullet-}$ is the main biological function of Nox proteins and is important in the bactericidal activity of Nox2, much of the signaling that occurs is directly mediated by its dismutation product H_2O_2. Superoxide is not able to diffuse across biological membranes due to its negative charge. There is, however, evidence for channels that are capable of transporting $O_2^{\bullet-}$, which will be discussed in a later section.

Hydrogen peroxide

Hydrogen peroxide is more stable than $O_2^{\bullet-}$ and is capable of crossing biological membranes. Because of the presence of SOD in the cell, H_2O_2 is formed rapidly from

Nox-generated $O_2^{\bullet-}$, or in the case of Nox4, perhaps prior to the release of $O_2^{\bullet-}$ from the enzyme (233). H_2O_2 is also tightly regulated biologically by catalase, glutathione peroxidase, and peroxiredoxins, which convert H_2O_2 to water and other metabolites. H_2O_2 can reversibly react with low pKa cysteine residues (234) on proteins to initially form a disulfide bond (–SSR) and sulfenic acid (–SOH). Sulfinic acid ($–SO_2H$) and sulfonic acid ($–SO_3H$) can be formed by additional oxidation; however, these latter reactions are essentially irreversible, and not useful for signaling (235; 236). Oxidation of thiols by H_2O_2 has been demonstrated to have diverse physiological consequences, as indicated by the myriad signaling pathways described below.

Nox proteins in signal transduction

Nox1

Tissue distribution and physiological function

Nox1 was the first of the novel NADPH oxidase catalytic subunits to be cloned. Shortly after the discovery of Nox1, an alternatively spliced form of the gene was discovered (Nox1β), which lacks exon 11 (174) and is incapable of producing $O_2^{\bullet-}$ (237). A second splice variant (Nox1 γ) was also identified, but was later discovered to be an artifact of the technique used (237; 238), likely caused by stable loop formation of Nox1 mRNA (239).

Nox1 mRNA is most highly expressed in colon epithelia (216), but is also expressed at lower levels in VSMCs, endothelial cells, uterus, placenta (239), prostate, osteoclasts (240), retinal pericytes, neurons, astrocytes and microglia (193). There is evidence of species-specific distribution of Nox1 as well. Rodent stomach expresses Nox1, which has been shown to be upregulated by *Helicobacter pylori* lipopolysaccharide (LPS) (241; 242). However, the expression of Nox1 in human

stomach has been questioned (216), though another Nox isoform could play a similar role in humans.

The physiological role of Nox1 in colon remains somewhat controversial. Two proposed roles are immune defense and cell proliferation (or pathologically, inflammatory bowel disease and carcinogensis) (216; 243). Nox1 ROS production has been shown to be increased in response to LPS and flagellin (244). Several studies have correlated increased Nox1 activity to increased proliferative signaling processes such as mitogen-activated protein (MAP) kinase (245) and c-Src (246). Another recent study suggests that increased Nox1 activity promotes colon adenocarcinoma migration (247).

Though Nox1 has a low basal expression in VSMCs, it has been extensively studied because it is upregulated at the mRNA level and activated by vascular pathological stimuli such as Ang II and PDGF (248-250). Nox1 mRNA has been shown to be increased in rat arteries during restenosis after balloon injury (220), in the aortas of hypertensive rats (251), and in diabetic arteries (252). It has been shown to regulate smooth muscle cell growth, both hypertrophy and hyperplasia, and migration (204; 253). In addition, Nox1 may be important in regulating blood pressure (254).

Nox1 is also active in the central nervous system (CNS). A study of Nox1 knockout mice found that these mice exhibit a reduction in the augmented sensitivity to pain that accompanies inflammation (hyperalgesia), which is apparently mediated by a reduction in transient receptor potential vanilloid receptor 1 (TRPV1) channel activation via impaired calcium mobilization and impaired translocation of PKCε to the membrane (255). In microglia, like in the colon, LPS has been shown to activate Nox1, which suggests a role in host defense (256). Nox1 in neurons has also been implicated in neurite growth (257).

Mechanisms of activation

At the protein level, Nox1 associates with the membrane subunit p22phox, which is necessary for enzymatic activity (200; 258; 259). Nox1 is activated by forming a complex with cytosolic activators in a similar manner to Nox2, and can interact with p47phox (260), p67phox (260) and the small GTPase Rac (261), but is most highly activated by the p47phox and p67phox homologues, NoxO1 and NoxA1 (262). In contrast to the cytosolic localization of p47phox in resting cells, NoxO1 is constitutively associated with Nox1, and lacks the autoinhibitory region found on p47phox, which may be responsible for some constitutive activity (263). Analogous to Nox2 activation, NoxA1 and Rac membrane translocation are required for activation and initiation of $O_2^{\bullet-}$ production (264). The best-studied activation of Nox1 occurs via Ang II in vascular smooth muscle cells (VSMCs). Ang II stimulates the AT-1 receptor, which rapidly activates phospholipase C (PLC) though the heterotrimeric G-protein subunit Gα/11 (265). PLC cleaves PIP_2 into inositol trisphosphate (IP_3) and diacylglycerol (DAG). DAG and Ca^{2+} released by IP_3 activate protein kinase C (PKC), which phosphorylates p47phox (250; 266). Continued activation of Nox1 by Ang II requires ROS-sensitive, Src-mediated transactivation of the epidermal growth factor (EGF) receptor, leading to phosphatidylinositol 3-kinase (PI-3K)-dependent activation of Rac (250; 267). Poldip2 associates with Nox1 (5), however the influence of Poldip2 on Nox1 activity has not been established.

Subcellular localization

Various subcellular localizations of Nox1 have been reported. In keratinocytes, Nox1 was found to have a nuclear localization with some cytoplasmic distribution (268). Recent studies suggest a plasma membrane distribution, specifically in caveolae on the cell surface (194; 195). Poldip2 is also reported at the cell surface in association with the

cell adhesion receptor Ceacam1 in certain contexts (4) and could interact with Nox1 at the cell membrane. ROS production by Nox1 in vascular cells occurs in early endosomes and requires the expression of chloride channel 3 (CLC-3) (269). Although the mechanism of why the channel is required is not well defined, the authors suggest that CLC-3 may act to neutralize the electron flow into the endosome that occurs during Nox1-mediated $O_2^{\bullet-}$ generation. Another suggestion, based on the observation that CLC-3 can transport $O_2^{\bullet-}$ across endothelial cell membranes (270), is that CLC-3 may transport $O_2^{\bullet-}$ out of the endosome and into the cytosol (271). This model is attractive because Nox-derived ROS are detectable in the cytosol, although the orientation of Nox1 is similar to that of Nox2, which releases $O_2^{\bullet-}$ from phagosomes extracellularly. Since $O_2^{\bullet-}$ is a charged species, it cannot freely diffuse across membranes and would require dismutation to an uncharged species such as H_2O_2, or transport.

Signal transduction

The primary ROS produced by Nox1 is $O_2^{\bullet-}$, although H_2O_2 is thought to be the most important signaling molecule in Nox1 signal transduction. Due to the short-lived nature of ROS, the localization of Nox in the cell is believed to determine the downstream signaling effects (196). In the case of Nox1-derived ROS, there is evidence for a role in inactivating phosphatases, modifying kinase pathways, regulating cell cycle proteins and altering the activity of transcription factors (Figure A.2).

Before Nox1 had been formally cloned and identified, it was observed that the hypertrophic agent Ang II stimulates a NADPH oxidase in VSMCs (272) leading to activation of a variety of signaling cascades including the p38 MAP kinase/mitogen-activated protein kinase activated protein kinase-2 (MAPKAPK2)/Akt (265; 273; 274), Ras (via glutathionylation) (275), and EGF receptor transactivation (276) pathways. Ang II-induced hypertrophy can be inhibited by diphenylene iodonium (DPI), a non-specific

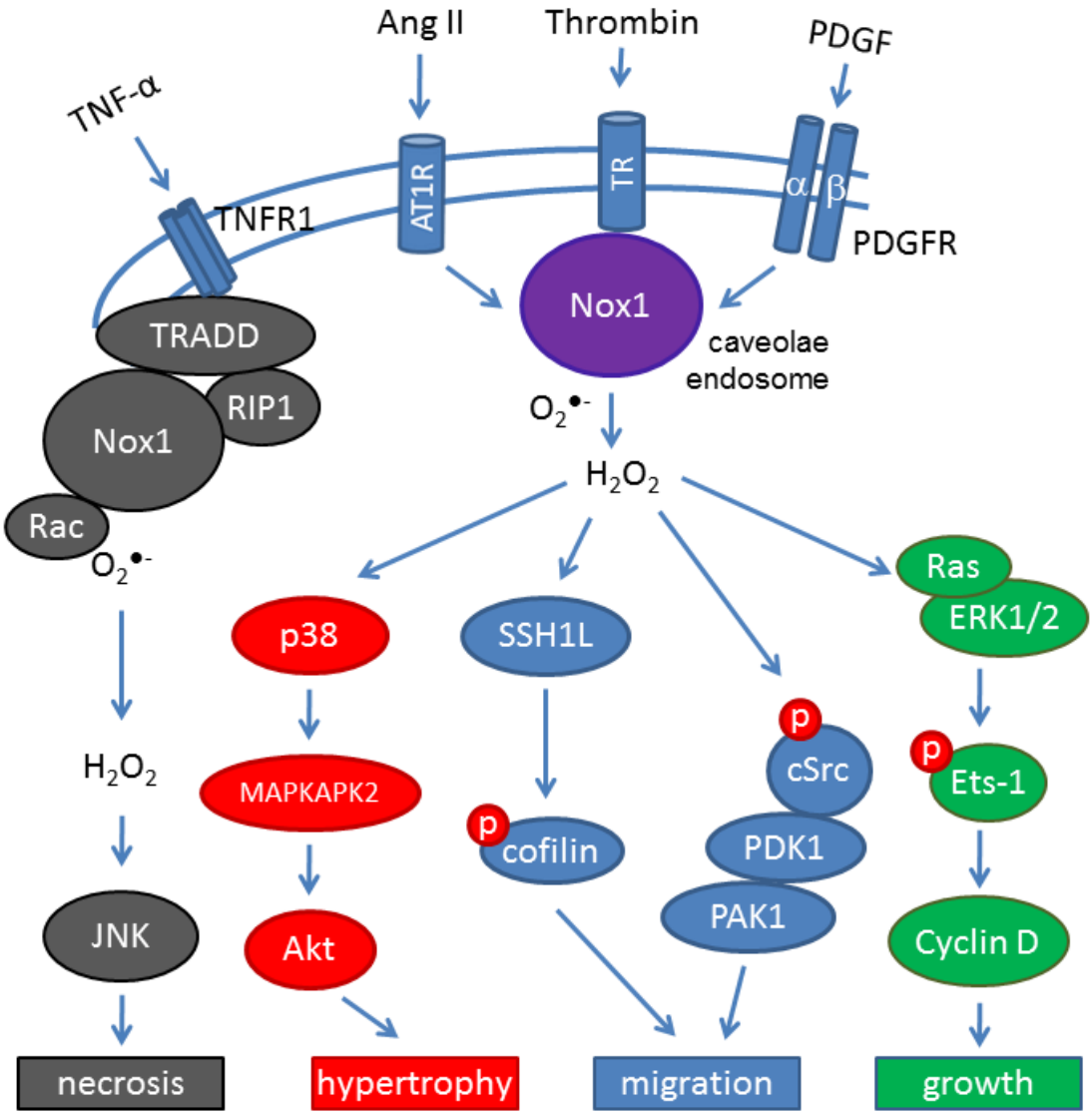

Figure A.2. Nox1 signaling pathways. TNF-α stimulates TNFR1, resulting in the recruitment of TRADD, RIP1, Rac, and Nox1 to the receptor. The complex produces ROS that activate JNK to initiate necrosis. Other activators of Nox1 include Ang II, thrombin, and PDGF. Nox1 activation initiates hypertrophy by activating p38 MAPK, which associates with MAPKAPK2 and Akt. Nox1 also activates SSH1L, which activates cofilin to promote cell migration. Nox1 increases cSrc phosphorylation, which activates PDK1, followed by PAK1. Nox1 also stimulates growth by activating Ras and ERK1/2, which activate Ets-1 causing upregulation of cyclin D, promoting cell cycle progression. Figure reproduced from Brown et al. (14) with permission.

inhibitor of Nox catalytic subunits and other flavin containing proteins, and catalase (277). Though multiple Nox members are present in the vasculature, Ang II selectively activates Nox1, so the ROS-dependent hypertrophic effects are likely mediated by Nox1 (248; 249). Additionally, protein tyrosine phosphatase (PTP) SHP-2 and Akt activation by Ang II have been demonstrated to be regulated in a Nox1-dependent manner in a study of spontaneously hypertensive rats (251).

Along with hypertrophy, Nox1 has also been implicated in cell migration. Migration in response to PDGF (278) or fibroblast growth factor (FGF) (279) is impaired in VSMCs from Nox1 knockout mice, while Nox1 siRNA attenuates arachidonate-induced migration of HT29-D4 adenocarcinoma cells (247). The downstream targets of Nox1 with respect to migration have been studied extensively for PDGF. PDGF induced H_2O_2 formation mediates smooth muscle cell migration via activation of c-Src, which subsequently activates phosphoinositide-dependent kinase-1 (PDK1) and p21-activated protein kinase (PAK1) (280). A parallel pathway in which PDGF stimulates Nox1-dependent ROS-mediated regulation of actin turnover has also been described. Nox1 activates Slingshot (SSH)1L phosphatase, through disruption of an inhibitory partnership with 14-3-3 proteins (281; 282). Once active, SSH1L dephosphorylates and activates cofilin (283). The PDGF-induced activation of SSH1L is required for cofilin activation and migration in VSMC (282). This Nox1-dependent pathway was demonstrated to be functionally important in a femoral artery wire-injury model using Nox1 knockout mice, in which neointima formation was decreased compared to wild type mice (278). There is also a significant amount of information about how FGF-mediated Nox1 activation affects migration. As is the case for PDGF, Nox1 appears to target cytoskeletal remodeling. FGF-induced activation of c-Jun N-terminal kinase (JNK) and subsequent phosphorylation of the cytoskeletal adapter protein paxillin were shown to

be mediated by Nox1 (279). Consistent with this theme, Nox1 mediates integrin turnover in carcinoma cells (247).

There is also substantial literature suggesting that Nox1 has role in cell proliferation. H_2O_2 generated via Nox1 was demonstrated to mediate cell growth and transformation when overexpressed in NIH 3T3 fibroblasts (284). More recently, Cyclin D1 has been identified as a target of Nox1 regulation of the cell cycle (285). Lung epithelial cells overexpressing Nox1 exhibit increased proliferation, higher protein expression of Cyclin D1, and increased extracellular signal regulated kinase (ERK) 1/2 activity. H_2O_2 is believed to mediate these effects, as they are blocked by catalase (286). Of interest, Nox1 is also probably important for thrombin-induced proliferation of VSMCs, given that its growth effects in the aorta, whose VSMCs express only Nox1 and Nox4, are blocked in p47phox knockout mice (287). Finally, the activated oncogene, Ras, has been proposed to constitutively activate and upregulate Nox1, which is necessary for its oncogenic properties (288). This activation results in upregulated vascular endothelial growth factor (VEGF) via activation of the transcription factor SP1 (289). The disruption of stress fibers and focal adhesions associated with Ras-activation are mediated by the oxidative inactivation of low molecular weight (LMW)-PTP, which reduces Rho activity (290). A study of human colon cancers demonstrates a correlation between activating Ras mutations and overexpressed Nox1 (291), supporting the in vitro data. Poldip2 has been demonstrated to activate Rho, and could contribute to the observed Nox effects on the cytoskeleton (5).

Nox1 has also been implicated in cell death and necrosis. Tumor necrosis factor-α (TNFα) promotes complex formation between Nox1, TRADD, RIP1, and Rac1, which in turn promotes necrotic cell death by prolonged JNK activation (292). Another group showed that TNFα also regulates the Nox1 complex transcriptionally (293).

Nox2

Tissue distribution and physiological function

Nox2 is known to be essential in innate host defense, both by producing ROS to attack invaders after phagocytosis and by acting as a signaling molecule to initiate a number of inflammatory and immunoprotective responses (172). Though Nox2 is most highly expressed in phagocytes, expression has also been detected in CNS, endothelium, VSMCs, fibroblasts, cardiomyocytes, skeletal muscle, hepatocytes, and hematopoietic stem cells (226). In vascular cells, Nox2 is activated by Ang II, endothelin-1, VEGF, TNFα, and mechanical forces (294; 295). Superoxide produced by Nox2 can react with NO in the cells to regulate bioavailability with the consequence of creating the reactive molecule $OONO^-$, which has been implicated in oxidative stress. NO is an important vasodilator and signaling molecule in endothelial cells. Dysregulation of Nox2 activity can lead to endothelial dysfunction and contribute to hypertension (296).

Mechanisms of activation

Nox2, or the neutrophil NADPH oxidase, is the first discovered and most extensively studied of the Nox members. The Nox2 complex is composed of the membrane subunits gp91phox (Nox2) and p22phox, and is stimulated by agonists such as F-Met-Leu-Phe (297-299). Upon exposure of neutrophils to this peptide, p47phox is phosphorylated on 8-9 serines by either proline-directed kinases or PKC (300). S359 and S370 are phosphorylated first, and then S379 acquires a phosphate, exposing an SH3 binding site that interacts with the proline-rich region of p22phox and facilitates translocation to the membrane. Finally, S303 and S304 are phosphorylated, leading to full catalytic activity (301). p67phox then binds to the translocated p47phox, providing a binding site for activated Rac and forming the functional enzyme. Nox2 has also been

found to complex with p40phox, but the functional consequences of this interaction are controversial (226).

Subcellular localization

Nox2 is localized in submembranous phagosomes in neutrophils, and in caveolae on the leading edge of lamellipodia in endothelial cells (196). Nox2 has also been identified in endosomes (302), including those responsible for early receptor-mediated signaling called redoxisomes in non-phagocytic cells (171; 196; 197). In transfected HEK293 cells, Nox2 is distributed to the plasma membrane (303).

Signal transduction

Nox2 signaling in neutrophils has been extensively studied. In host defense Nox2 localized in phagosomal membranes is activated by the presence of microbes and cytokines to generate $O_2^{\bullet -}$ (Figure A.3). Superoxide in the phagosome dismutates to H_2O_2, which, along with chloride ions, can be converted to hypochlorous acid (HOCl) by extracellular myeloperoxidase (MPO). HOCl is an effective antimicrobial oxidant. This pathway has clear physiological relevance in immune defense. Individuals lacking Nox2 or with mutations in other necessary components of the neutrophil NADPH oxidase are afflicted with CGD, and are highly susceptible to infection (213).

It has become clear that the production of ROS in phagosomes is not the only role of Nox2, even in the context of host defense. Numerous cytokines activate ROS production in neutrophils, which then inactivate PTPs (304) leading to cytoskeletal rearrangement (305) or other signaling consequences (306). Nox2-derived ROS in macrophages have also been implicated in apoptosis by activating the ASK1-p38 MAP kinase pathway (307).

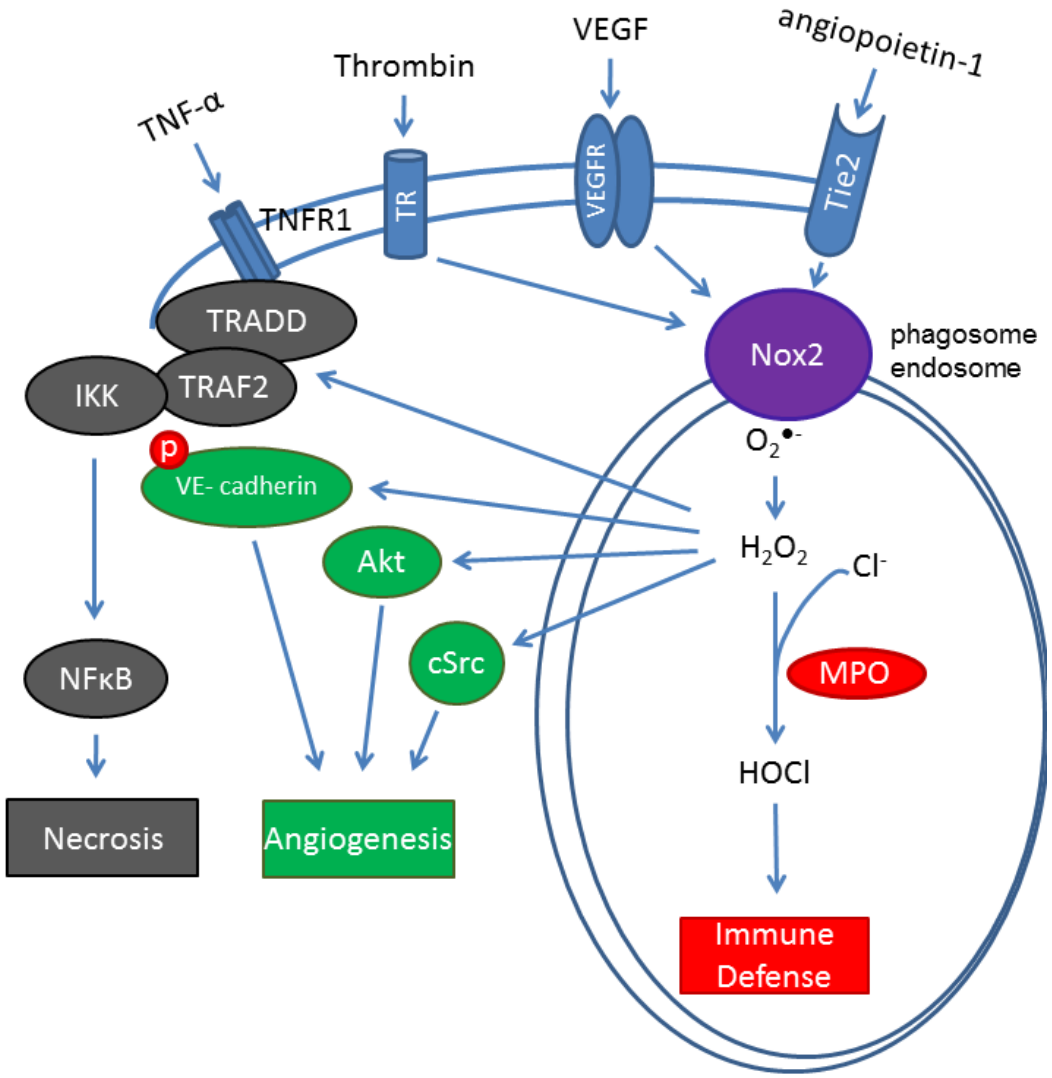

Figure A.3. Nox2 signaling pathways. Nox2 is localized to endosome and phagosome membranes. In necrosis, TNF-α activates TNFR1, which recruits TRADD to the receptor. TRAF2 then binds to TRADD in a Nox2-derived ROS-dependent manner and activates IKK, leading to NFκB activation and necrosis. Nox2 in endosomes is also activated by thrombin, VEGF, and angiopoietin-1. Nox2-derived ROS promote angiogenesis by activating VE-cadherin, Akt, and cSrc. Nox2 acts in host defense in phagosomes by producing $O_2^{\cdot-}$, which is dismutated to H_2O_2. The reaction of H_2O_2 with Cl^- is catalyzed by MPO to form HOCl, which is bacteriocidal. Figure reproduced from Brown et al. (14) with permission.

In other cell types, Nox2 signals to kinase/phosphatase cascades in a similar manner to Nox1. For example, a recent study in fibroblasts found Nox2 in endosomes to be involved in TNFα induction of the transcription factor NF-κB (308). The authors propose that once the TNFα receptor is activated and endocytosed, TRADD is recruited to the receptor. Nox2-derived ROS promotes TRAF2 binding to the TNFR1/TRADD complex, which then activates IkB Kinase (IKK) and promotes NF-κB activation. This pathway may contribute to cell death, as TRAF2-deficient MEFs are resistant to ROS-induced cell death (309; 310).

Nox2 signaling in endothelial cells has emerged as an important angiogenesis-regulating pathway. Nox2 is activated in endothelial cells by VEGF (311), angiopoietin-1 (312), hypoxia (171) and thrombin (313). ROS produced in this process have been implicated in endothelial cell proliferation and migration. It is clear that Nox2-derived ROS take part in VEGF-induced VEGF receptor 2 phosphorylation, activation of cSrc and Akt, and phosphorylation of VE-cadherin to promote angiogensis; however, the molecular mechanisms remain to be fully elucidated (314).

Nox3

Tissue distribution and physiological function

Nox3 was first discovered in 2000, along with Nox4 and Nox5, based on sequence homology to gp91phox (184). The finding that Nox3 is expressed in the inner ear led to an examination of balance in a Nox3 mutant mouse model (Nox3^{het}) (315). Indeed, these mice exhibit a head tilt, similar to that seen in *nmf333* mice, a mouse strain with a point mutation in p22phox (316), and in *hslt* mice, a mouse strain with a spontaneous mutation in the region of the NoxO1 gene (317). Both of these mice are unable to remain on the surface of the water during a swim test, and fail to respond to

linear acceleration of the head with vestibular-evoked potentials, indicating a severe balance disorder, which has been attributed to a lack of functional Nox3 (316).

Nox3 has also been shown to have functional significance in lung endothelial cells. A study of toll-like receptor (TLR) 4 knockout mice found increased expression and activity of Nox3, which resulted in increased elastolytic activity, an indicator of emphysema development. DPI and siRNA against Nox3 reversed the phenotype (318). This suggests that Nox3 may serve physiological roles distinct from the inner ear. Indeed, it has been detected in fetal spleen, kidney, lung and skull by PCR (179; 184), which may indicate that Nox3 plays an important role in tissue development, but is turned off in adult tissue, a concept that requires further investigation.

Mechanisms of activation

Nox3 three-dimensional structure is predicted to be similar to that of Nox1 and Nox2 (226). Nox3 is highly expressed in the inner ear, along with the Nox subunits p47phox, NoxO1 and NoxA1 (179; 319). Studies on the activation of Nox3 have shown contradictory results. Ueno et. al. (320) demonstrated that p22phox is a necessary subunit for Nox3 $O_2^{\bullet -}$-producing activity. This could mean that Poldip2 influences Nox3 activity as well, though it has not been tested. Recent studies suggest a weak constitutive activity when Nox3 is coexpressed with p22phox, but full activation requires Rac and various other combinations of cytosolic Nox subunits (321-324). Contradictory results were obtained when the Nox3 system was reconstituted in HEK293 cells with combinations of NoxO1, NoxA1, p47phox and p67phox, but most studies agree that NoxO1 and p67phox each universally activate Nox3. The fact that the head tilt phenotype is shared in Nox3 and NoxO1 deficient mice strongly suggests a functional interaction between the two (317). However, it is likely that the precise molecular composition of the Nox3 complex is tissue dependent.

Subcellular localization

Very little information is available about the targeting of endogenous Nox3, but tagged Nox3 coexpressed with p22phox in HEK293 cells is localized to the plasma membrane (198). Evidence of Poldip2 on the plasma membrane (4) potentially places Poldip2 in the same subcellular space as Nox3, though an interaction between the two proteins has not been investigated. In the absence of p22phox, Nox3 is detected in the cytoplasm.

Signal transduction

Based on the head tilt phenotype of Nox3 mutant mice (315), a recent observation may offer a clue to downstream effects of Nox3-derived ROS. The drug cisplatin is known to induce hearing loss and increase $O_2^{\bullet-}$ production via Nox3 (325). Mukherjea et. al. (326) reported that the TRPV1 channel in the cochlea is upregulated in response to cisplatin. The upregulation is prevented by DPI and the antioxidant lipoic acid. Finally, siRNA against TRPV1 reduces cisplatin-induced ototoxicity, which suggests that TRPV1 may be downstream of Nox3 and may mediate cisplatin toxicity effects. It is not known whether this pathway also mediates the head tilt phenotype seen in the Nox3^{het} mice.

Nox4

Tissue distribution and physiological function

Nox4 is highly expressed in the kidney (180), but has been found to be expressed and functionally important in many cell types including mesangial cells (327), smooth muscle cells (195), endothelial cells (328), fibroblasts (329), keratinocytes (330), osteoclasts (182), neurons (331), and hepatocytes (332). Nox4 tissue distribution is fairly

ubiquitous (187), and in general Nox4 is highly expressed compared to other Nox homologues.

Nox4-derived ROS have been implicated in a variety of physiological processes, including cellular senescence (180; 181; 333), apoptosis (334), survival (335), insulin signaling (336), migration (337; 338), the unfolded protein response (339), and differentiation (329; 340-342). In addition, Nox4 has been proposed to play a role in oxygen sensing by enhancing the O_2 sensitivity of TWIK-related acid sensitive K channel 1 (343). The best established functions of Nox4 revolve around cell growth, death and differentiation. Because these responses are often antagonistic, it is likely that Nox4 regulates a fundamental physiological process common to all of them, such as cytoskeletal reorganization or gene expression.

Mechanisms of activation

Nox4, originally Renox (180), is unique among the catalytic Nox subunits in that it only requires the membrane subunit p22phox for ROS producing activity, and appears to be constitutively active (344). This observation has led to the proposal that Nox4 is an inducible Nox, and its activity is proportional to Nox4 protein expression alone. In cardiac fibroblasts (345), lung (346) and pulmonary artery (329) smooth muscle cells, TGF-β induces increased expression of Nox4. Insulin stimulates Nox4 expression in adipocytes (347) and IGF-1 has been found to induce expression in VSMCs (337). Importantly, Peshavariya et al. (348) recently showed that the regulation of Nox4 also occurs at the translational level by a mechanism dependent on p38 MAP kinase.

The identification of the p22phox-interacting protein, polymerase delta-interacting protein (Poldip2) provides new evidence of regulation of Nox4 activity. Poldip2 overexpression also has downstream consequences on Nox4 pathways by

participating in its regulation of the cytoskeleton in VSMCs (188). The importance of Poldip2 in Nox4 regulation of other systems remains to be determined.

Subcellular localization

There have been conflicting reports on the localization of Nox4. In VSMCs, Nox4 has been identified in focal adhesions (195), the nucleus (199), and the endoplasmic reticulum (200). Nuclear and endoplasmic reticular localization have been confirmed in other cell types, including HEK293 cells and endothelial cells (194; 199; 349; 350). One study identified Nox4 splice variants with potentially distinct subcellular localizations (351). However, it is not known whether or not all these variants are translated physiologically. One splice variant, the 28kD Nox4D, has been found in the nucleus of VSMCs (352).

Signal transduction

Nox4 differs from other Nox enzymes because the $O_2^{\bullet-}$ produced by Nox4 is rapidly converted to H_2O_2, so $O_2^{\bullet-}$ release from this enzyme is almost undetectable (353). In rat VSMCs tested in basal conditions, siRNA against Nox4 does not reduce $O_2^{\bullet-}$ production as measured by DHE-HPLC, but does reduce production of H_2O_2 measured by Amplex Red assay (233). The E-loop on Nox4, which is absent on other Nox family members, contains a histidine residue that is essential for the conversion of $O_2^{\bullet-}$ to H_2O_2 by the enzyme (15). The molecular mechanism of the conversion is not well understood.

With such a variety of physiological processes proposed to be regulated by Nox4-derived ROS, it is not surprising that specificity of downstream signaling dictates the final response (Figure A.4). In adiposities, insulin-induced ROS production inactivates PTP1B, which enhances the phosphorylation of the insulin receptor (336). In VSMCs, IGF-I-induced migration is dependent upon Nox4-mediated activation of matrix

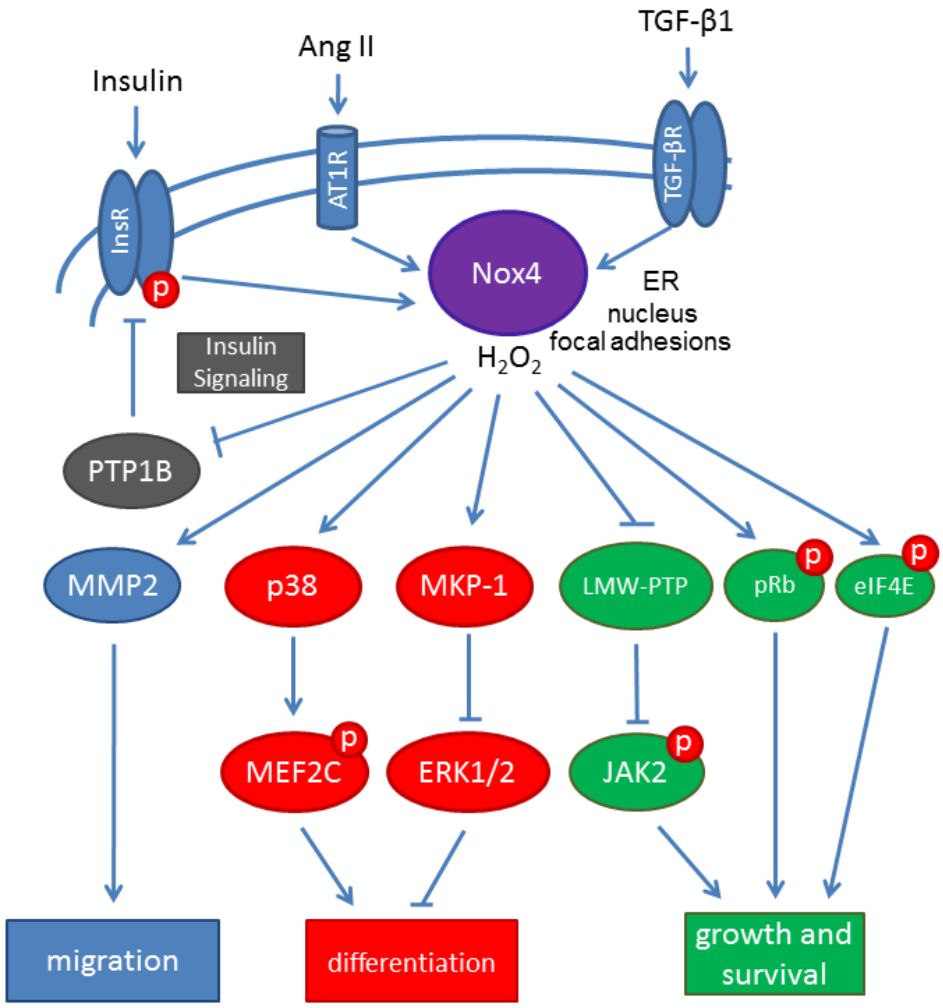

Figure A.4. Nox4 Signaling Pathways. Nox4 is constitutively active, but activity and/or expression can be increased by insulin, Ang II and TGF-β1. Nox4 inhibits insulin signaling by inhibiting the phosphatase PTP1B, which prolongs the phosphorylation of the insulin receptor. Nox4 promotes migration by activating MMP2. Nox4-derived ROS activate p38 MAP kinase, which phosphorylates and activates MEF2C to promote differentiation. In addition, H2O2 produced by Nox4 activates MKP-1, which inhibits the activation of ERK1/2. Nox4-derived ROS inhibit LMW-PTP, which prolongs the phosphorylation of JAK2 to promote growth. Nox4 also induces phosphorylation of pRb and elF4E to promote growth and hypertrophy. Figure reproduced from Brown et al. (14) with permission.

metalloproteinase-2 (MMP2) (337), while PDGF-induced migration requires Nox4-mediated focal adhesion turnover (188). In contrast, Nox4 overexpression inhibits angiotensin II-induced migration of adventitial myofibroblasts by an unknown mechanism (354).

Growth and survival effects of Nox4 activation have been reported to be mediated by Akt in mesangial cells stimulated with Ang II (327). In pancreatic cancer, LMW-PTP inactivation by Nox4 promotes prolonged phosphorylation of JAK2, a tyrosine kinase that phosphorylates signal transducers and activators of transcription (STAT) proteins and enhances the growth response (355). Nox4-associated ROS have also been implicated in progression through the G2/M checkpoint of the cell cycle via regulation of Cdc25 phosphorylation (356). Nox4-mediated growth and survival have also been observed in VSMCs treated with urokinase plasminogen activator (357) or TGF-β (346), and in hypoxia-mediated activation of pulmonary adventitial fibroblasts (358). In the latter case, hypoxia induces TGF-β, which increases IGFBP-3 expression via a phosphatidylinositol 3-kinase/Akt-dependent pathway. IGFBP-3, in turn, induces Nox4, leading to proliferation (358). Nox4 has also been shown to mediate TGF-β-induced phosphorylation of retinoblastoma protein (pRb) and the eukaryotic translation initiation factor 4E binding protein-1 (eIF4E), which regulate cell cycle progression and hypertrophy, respectively, in airway smooth muscle cells (346).

Studies performed with VSMCs (342), fibroblasts (329), adipocytes (347), and embryonic stem cells (341; 359) show that ROS production by Nox4 promotes differentiation. In adipocytes, Nox4 was shown to upregulate MAP kinase phosphatase-1 (MKP-1), which reduces activation of ERK1/2 (347). However, the detailed molecular mechanisms by which Nox4 regulates MKP-1 expression, and by which MKP-1 regulates differentiation, are not known. In mouse embryonic stem cells, Nox4-derived ROS activate p38 MAP kinase, resulting in the phosphorylation and translocation to the

nucleus of MEF2C, a transcription factor important in cardiomyocyte differentiation (341). Nox4-mediated differentiation of VSMCs appears to be related to regulation expression of the smooth muscle-specific transcription factor serum response factor (SRF) (342; 359).

Cell and tissue specificity of Nox proteins

One of the puzzling observations in the Nox field is that cells and tissue often express multiple Nox proteins in the same cell, but that these enzymes regulate different functions in different cell types. It is clear from the previous discussion that Noxes are involved in a plethora of signaling pathways and cellular responses. Yet, they all produce the same ROS. This suggests that the complement of Nox proteins within a cell, and more importantly, their subcellular localization and coupling to external stimuli, are critical determinants of the integrated response to Nox activation.

In many, if not most, cell types, different Nox homologues are coupled to different agonists and therefore different physiological responses. For example, in VSMCs, Ang II and PDGF activate Nox1 (248), while TGF-β and serum withdrawal activate Nox4 (329; 360). In this cell type, Nox1 is growth-promoting, while Nox4 is pro-differentiating. In contrast, Nox4 is activated by Ang II and mediates hypertrophy of mesangial cells (327). While Nox1 is acutely activated by agonists in VSMCs, it is regulated by transcriptional control of NoxA1 expression in the gut (216). In endothelial cells, Nox2 and Nox4 appear to play antagonistic roles. Nox2 is activated by Ang II and TGF-β, and Nox4 is upregulated by serum withdrawal and insulin (303). Finally, in cardiac fibroblasts, Nox4 and Nox5 are oppositely regulated by TGF-β and appear to mediate the transition to myofibroblasts and inflammatory pathways, respectively (329). In all of these cell types, activation of individual Noxes leads to activation of specific signaling pathways that are dictated by their subcellular localization. Given these non-

redundant functions and tissue-specific responses, it is imperative to study Nox enzymes in specific cellular contexts.

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