The tumour suppressor p27kip1 interacts with NF-κB activator IKK and plays a role in inflammation

by

Charlene Antony

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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2009

Abstract

The tumour suppressor p27kip1 (p27) is a potent inhibitor of cell growth and proliferation. We identified NF-κB-activator, IKKα, as a novel interacting partner of p27 in a protein microarray screen. Both the IKKα and IKKβ components of the IKK complex were mapped to the C-terminal domain of p27. To investigate the physiological function of the p27-IKK interaction, we employed a well-established model of LPS-induced sepsis which is known to activate the IKK/NF-κB pathway. Lentivirally-mediated overexpression of p27 blocked LPS activation of NF-κB. Furthermore, in LPS-injected animals transduced with TAT-p27, a significant improvement in the left ventricular function of the heart was observed. TAT-p27 treatment was also shown to attenuate the endotoxin effect and significantly improve survival compared to both saline and TAT-LacZ controls. Our results indicate that p27 attenuates inflammation, possibly through inhibiting the IKK-dependent activation of NF-κB, thus supporting a novel link between both cell cycle regulation and inflammation.
Acknowledgments

First and foremost, I would like to sincerely thank my supervisor Dr. Rüdiger von Harsdorf for accepting me as his first graduate student and for providing me with guidance and support throughout my entire academic training. His constant encouragement and advice greatly contributed to a pleasant graduate experience.

I would also like to thank Dr. Ludger Hauck, the Senior Research Associate in our lab. Without his guidance this work would not have been possible. His mentorship was invaluable and was pivotal in every aspect of my research from experimental planning to thesis writing. His sense of humor and blunt remarks never ceased to make me laugh and added a lively flavor to our lab atmosphere. I remain indebted and attribute my success in my graduate studies to him.

A tremendous thank you extends to Dr. Phyllis Billia, our former postdoctoral fellow, whose friendship, advice and support was incredible. She is truly a selfless individual who really went the extra mile to help me in every aspect of my graduate experience as well as other challenges I encountered such as medical school applications and interview preps. She has been a mediator, confidante and true advocate. For all of that and more, I thank you.

I would also like to thank the members of my program advisory committee, Dr. Derek van der Kooy and Dr. Gordon Keller, for their thoughtful advice and support. I am sure I speak for Dr. von Harsdorf as well when I thank the both of you for all your help in the past two years.

I would also like to thank Dr. Karen Davis, Associate director of IMS, for lending a friendly ear and providing me with guidance and support during my graduate studies.

I am also very grateful for Daniela Grothe, the Head Technician in our lab. I am indebted to her constant support, troubleshooting advice, and for all of the experimental work she has performed during my entire research experience. Her love for playing music and cheery personality made the long days seem more enjoyable. I would not have made it this far without her support.
I would also like to mention our fellow lab technician, Jeremy Johns. He was a constant pillar of strength from day one. Supporting through all the difficult times and making me laugh in the rest. I will never forget our random explorations through the hospitals, Starbucks trips, restaurant outings, and watching Daily show and Family Guy clips. He is a true friend and will be greatly missed!

I would also like to thank our animal technician, Filip Konecny. His contribution to my Masters project is undeniable and I am very grateful for all of his help in the collection of the animal data.

A warm thank you extends to Natalie Stickle, Project Manager of the UHN Microarray Centre, for all of her help with operating the microarray scanner and image analysis software.

I would also like to thank all the members of our lab, past and present, for making each day more enjoyable, for all the laughs we shared, and for all the help you have shown me.

Last but not least, a sincere thank you goes to my family and friends for all of their unconditional love, prayers, and constant support. I dedicate this work to you.

**Data Attribution**

This work represents a collaborative effort on behalf of a number of individuals. FPLC purification of the p27 probe and the synthesis of TAT-p27 and TAT-LacZ were performed by Jeremy Johns. Daniela Grothe was involved with lentivirus production and plasmid transfections in the cell lines required for the immunoprecipitation and immunocytochemistry experiments. Ludger Hauck performed the neonatal cardiomyocyte isolations required for immunoprecipitation and immunocytochemistry. Finally, the animal handling, injections, and echocardiography were performed with the help of Filip Konecny and Sania Hasan.
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<td>c-abl oncogene 1, receptor tyrosine kinase (ABL1)</td>
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<tr>
<td>AKT</td>
<td>v-akt murine thymoma viral oncogene homolog 1 (PBK)</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia-telangiectasia mutated kinase</td>
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<tr>
<td>ATR</td>
<td>ATM-RAD3 related</td>
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<td>AURKA</td>
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<td>BAFF-R</td>
<td>B cell-activating factor receptor (TNFSF13B)</td>
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<td>BCA</td>
<td>bicinchoninic acid</td>
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<td>BCR</td>
<td>breakpoint cluster region</td>
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<td>BJ</td>
<td>Human fibroblast cell line</td>
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<tr>
<td>BlyS</td>
<td>B lymphocyte stimulator</td>
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<td>BMX</td>
<td>BMX non-receptor tyrosine kinase</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>BTK</td>
<td>Bruton agammaglobulinemia tyrosine kinase</td>
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<td>BW</td>
<td>body weight</td>
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<td>CAMK2D</td>
<td>Calcium/calmodulin dependent protein kinase II delta</td>
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<td>CC</td>
<td>coiled coil</td>
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<td>Cdk</td>
<td>Cyclin-dependent kinase</td>
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<td>CDKI</td>
<td>Cyclin-dependent kinase inhibitor</td>
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<td>CHUK</td>
<td>Conserved helix-loop-helix ubiquitin ligase (IKKα, IKKA, IKK1)</td>
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<td>Casein kinase 2 alpha prime</td>
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<td>CKI</td>
<td>Cdk inhibitor</td>
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<td>Term</td>
<td>Description</td>
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<td>Co-IP</td>
<td>co-immunoprecipitation</td>
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<td>Casein kinase 1, gamma 2</td>
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<td>C-terminus</td>
<td>carboxy terminus</td>
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<td>DIC</td>
<td>disseminated intravascular coagulation</td>
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<td>DNA</td>
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<td>DYRK1B</td>
<td>dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B (Mirk)</td>
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<td>EDD</td>
<td>end-diastolic diameter</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>EPHA3</td>
<td>EPH receptor A3</td>
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<td>extracellular signal related kinase</td>
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<td>ESD</td>
<td>end-systolic diameter</td>
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<td>Exportin 1</td>
<td>CRM1 Homolog (CRM1, XPO1)</td>
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<td>feline sarcoma oncogene</td>
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<td>fibroblast growth factor receptor 2</td>
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<td>FOXO3a</td>
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<td>human immunodeficiency virus 1</td>
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<td>hKIS</td>
<td>human interacting kinase stathmin</td>
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<tr>
<td>HLH</td>
<td>helix-loop-helix</td>
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<tr>
<td>HR</td>
<td>heart rate</td>
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<td>HT1080</td>
<td>human fibrosarcoma cell line</td>
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</table>
HW  heart weight
IP  intraperitoneal
IPTG  isopropyl β-D-1-thiogalactopyranoside
IKK  IκB kinase
IKKα  IκB kinase, alpha (CHUK, IKKA, IKK1)
IKKβ  IκB kinase, beta (IKKB, IKK2)
IKKγ  IκB kinase, gamma (NEMO)
IκB  inhibitor of kappa light polypeptide gene enhance in B-cells
IκBα  IκB inhibitor, alpha (NFKBIA)
IL-1β  interleukin 1, beta (IL-1)
IL-6  interleukin 6
IL-8  interleukin 8
KO  knockout
KPC1/2  Kip1 ubiquitylation-promoting complex
LacZ  beta-galactosidase
LPS  lipopolysaccharide
LT  lymphotoxin
LTβ-R  lymphotoxin beta receptor
LTR  long terminal repeat
LV  left ventricle
LYN  v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
LZ  leucine zipper
MAPK  mitogen-activated protein kinase
MAP2K2  mitogen-activated protein kinase (K) kinase 2 (MEK2)
MI  myocardial infarction
NBD  NEMO binding domain
NEK6  NIMA (Never in mitosis gene a)- related kinase
NEMO  NF-kappa B essential modifier (IKKγ)
NES  nuclear export signal
NF-κB  nuclear factor of kappa light polypeptide gene enhancer in B-cells
NIH 3T3  mouse fibroblast cell line
NIK  NF-κB-inducing kinase
<table>
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<tr>
<th>Acronym</th>
<th>Definition</th>
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<td>open reading frame</td>
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<td>p27kip1</td>
<td>cyclin-dependent kinase inhibitor 1B (CDKN1B, p27)</td>
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<td>platelet activating factor</td>
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<td>TAT</td>
<td>transactivator of transcription</td>
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<td>TL4</td>
<td>toll-like receptor 4</td>
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<td>TMB</td>
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<td>tumour necrosis factor receptor</td>
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<td>Tyrosine 88</td>
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<td>ZADH2</td>
<td>Zinc alcohol dehydrogenase, domain containing 2</td>
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<td>ZF</td>
<td>zinc finger</td>
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</table>
Heart failure (HF) is the leading cause of morbidity and mortality in developed nations. It is a condition in which the heart is unable to maintain a cardiac output sufficient to meet the body’s metabolic demands. Cardiac output becomes compromised following an initial insult such as a myocardial infarction (MI), resulting in impaired cardiac structure or function. Unfortunately, injury to the heart results in an irreversibly damaged myocardium due to a lack of significant cardiac regeneration. As a consequence, to improve cardiac performance, the remaining healthy myocardium undergoes an adaptive response of remodeling [1]. This compensatory mechanism, although initially beneficial, can become maladaptive and lead to progressive HF, the worsening of symptoms, and death.

Cardiac remodeling is a physiologic and pathologic process that may occur after an MI, pressure overload (aortic stenosis, hypertension), volume overload (valvular regurgitation), idiopathic dilated cardiomyopathy, or inflammatory heart muscle disease (myocarditis) [1]. Despite differences in the etiologies of these diseases, they share commonalities in the pathophysiology of myocardial disorder.

Remodeling events are associated with an alteration in genome expression resulting in molecular and cellular changes. As surviving myocytes stretch under pressure overload, there is a reactivation of growth factors that are present in the embryonic heart but dormant in the adult heart [2, 3]. Consequently, there is an acceleration of protein synthesis and an increase in myocyte size, leading to hypertrophy of the left ventricle (LV) [3]. Initially, hypertrophic growth serves as a means of augmenting cardiac pump function and decreasing ventricular wall tension. However, long term hypertrophy can lead to increased oxygen consumption and diastolic dysfunction [1]. Once the limits on hypertrophic enlargement have been reached, continued stress results in myocyte dilation. Dilation of the heart eventually compromises ventricular systole and results in myocardial dysfunction. If these cellular changes continue, apoptosis (or programmed cell death) occurs, resulting in further permanent functional damage [4]. Apoptosis is also indirectly increased through the pro-inflammatory cytokines, tumour necrosis factor alpha (TNF-α, TNF), interleukin-1, beta (IL1B, IL-1) and interleukin 6 (IL-6), which are additional...
factors released during cardiac remodeling [1]. Elevated cytokine levels are associated with inflammatory processes as seen in myocarditis or can be triggered due to LV wall stress as a consequence of pressure or volume overload. Myocardial expression of cytokines contributes to a depression of contractile performance and adverse LV remodeling [5]. Thus, the end result of these remodeling events is an increased deterioration in cardiac performance.

As cardiac injury or overload ultimately leads to changes in gene expression, a central mediator of these remodeling processes is the transcription factor, nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB). HF patients typically show significant myocardial NF-κB activation and elevated levels of NF-κB activator and gene target, TNF-α, in the bloodstream [6, 7]. An increased NF-κB activation level is detected not only in end-stage HF but was shown in spontaneously hypertensive rats to commence at an early phase and increase as the disease progressed [8, 9]. Due to its involvement in the development and manifestation of cardiac pathologies, inhibition of the NF-κB pathway as a therapeutic means for HF holds substantial promise. However, knowledge about the intracellular mechanisms that govern NF-κB signaling in myocardial cells remains minimal.

1.1 Transcription factor NF-κB: activation and regulation

NF-κB represents a collection of 15 dimeric transcription factors composed of members of the v-rel reticuloendotheliosis viral oncogene homolog (REL, C-Rel) family of DNA-binding proteins that recognize a common deoxyoligonucleotide sequence motif. These dimers consist of either homo or hetero combinations of NF-κB1 (p105/p50), NF-κB2 (p100/p52), p65 (RelA), RelB, and c-Rel [10]. NF-κB is ubiquitously expressed in all adult tissues and regulates the expression of a large number of genes that play a key regulatory role in the immune system, inflammation, cell survival and cancer [11]. In nonstimulated cells, NF-κB is sequestered in the cytoplasm by an inhibitory complex IκB, which masks the nuclear localization signal of NF-κB thereby inhibiting nuclear uptake and the transcriptional transactivation capacity of NF-κB [11].

Inducers of NF-κB activation include proinflammatory cytokines, endotoxin, growth factors, microbial infections, and oxidant stress. Inflammatory stimuli are reported to activate a biphasic
pattern of NF-κB activation [12]. Lipopolysaccharide (LPS), a constituent of the outer membrane of gram-negative bacteria, stimulates the release of platelet activating factor (PAF), which in turn regulates the early phase of NF-κB activity by triggering the production of proinflammatory cytokines such as TNF-α and IL-1 [11, 12]. These cytokines are in turn potent activators of NF-κB and regulate the late phase of NF-κB activation through transcription of a number of inflammatory genes [13]. Both phases activate NF-κB by targeting its inhibitory molecule IκB. Rapid phosphorylation, ubiquitination and ultimate proteolytic degradation of IκB, frees NF-κB to translocate to the nucleus and induce the transcriptional transactivation of NF-κB-dependent genes [11]. NF-κB transcriptional activity is transient however, as it is coupled to an autoinhibitory feedback loop where NF-κB induces the expression of IκBα, an inhibitory component of the IκB complex. Once expressed, IκBα binds to and displaces NF-κB dimers from their DNA recognition sequences. The nuclear-export signal (NES) that is located at the amino terminus of IκBα expels the complex IκBα/NF-κB via an exportin 1 (CRM1 homolog, Xpo1, CRM1)-mediated export mechanism back to the cytoplasm as a transcriptionally inactive complex [14-16].

IκB kinase (IKK) is the main cytoplasmic complex which activates NF-κB upon extracellular cues. IKK-mediated phosphorylation of its main substrate IκB serves as a recognition site for IκB ubiquitination, which leads to IκB degradation and NF-κB activation [17]. IKK is composed of two catalytic subunits, namely IκB kinase alpha (IKKα, IKKA, IKK1, CHUK) and IκB kinase beta (IKKβ, IKKB, IKK2) and a regulatory subunit, NF-κB essential modifier (NEMO, IKKγ). Despite sharing high sequence similarity, IKKα and IKKβ are not functionally redundant and are involved in two separate pathways of NF-κB activation, namely the canonical and the alternative pathway [18]. Activation of NF-κB in either pathway is a result of differential targeting of various stimuli on IKKα and IKKβ (Figure 1.1).

The canonical/classical pathway is important for the initiation and propagation of the inflammatory response in various tissues including the heart [14, 22]. It is activated by a number of signals including the proinflammatory cytokines TNF-α and IL-1, viruses and the bacterial LPS, which transduce signals through the tumour necrosis factor receptor (TNFR) superfamily. These proinflammatory signals lead to the activation of the IKK complex, predominantly acting through IKKβ, to phosphorylate and target IκB for eventual proteasomal degradation [19, 20].
Figure 1.1 The classical and alternative pathways of NF-κB activation. In response to inflammatory stimuli, such as B lymphocyte stimulator (BlyS) and the CD40 ligand (CD40L), the classical pathway is activated and allows for the release of NF-κB dimer RelA: p50 through IKKβ-dependent phosphorylation of IκB, thus targeting IκB for proteasomal degradation. The alternative pathway is activated in response to developmental signals such as B-cell activating factor (BAFF) which acts through IKKα to process NF-κB component p100 to p52, thus allowing NF-κB components, RelB: p52, to translocate to the nucleus [22]. RANK = receptor activator of NF-κB, LTαβ = lymphotoxin-alpha or beta.
IKKβ-facilitated relief of IκB inhibition releases the NF-κB dimer, RelA: p50, the primary mediator of NF-κB function, to translocate to the nucleus. Once inside the nucleus, NF-κB mediates the transcriptional activation of several inflammatory genes in response to the original inflammatory signals [19, 20].

Despite IKKα existing in a complex with IKKβ, numerous studies have shown that IKKα is not required for activation of NF-κB in response to inflammatory signals [61]. In the heart, overexpression of dominant-negative IKKα had no effect on LPS-mediated NF-κB activation or resultant TNF-α production, revealing that IKKβ plays a central role in NF-κB regulation [14]. The regulatory IKKγ component however, was essential for IKK activation in the classical NF-κB pathway, but was not required for activation of the IKKα-dependent alternative pathway [21].

The non-canonical/alternative NF-κB pathway is most important for secondary lymphoid organ development and adaptive immunity [22]. It is activated by extracellular developmental stimuli acting through various TNFR receptors including the B cell-activating factor receptor (BAFF-R, TNFSF13B), B cell surface antigen CD40, receptor activator of NF-κB (RANK, TNFRSF11A) on osteoclasts, and lymphotoxin beta receptor (LTβ-R) on stromal cells (Figure 1.1). These signals activate NF-κB-inducing kinase (NIK), which was demonstrated in NIK knockout (KO) mouse embryonic fibroblasts when NIK failed to activate IKKβ in the presence of proinflammatory cytokines, TNF-α and IL-1 [13]. NIK in turn activates the IKK catalytic component, IKKα. IKKα triggers the processing of p100 to p52, leading to the nuclear accumulation of RelB: p52 NF-κB dimers to drive gene transcription [23-25] (Figure 1.1). As such, the alternative pathway regulates the transcription of genes that differ from the classical pathway by activating a distinct set of downstream mediators.

1.1.1 Role of IKK/NF-κB in cardiac dysfunction

NF-κB functions as a key regulator of cardiac gene expression programs downstream of multiple signal transduction cascades in a variety of both physiological and pathophysiological states. The regulation of IKKβ, a key activator of NF-κB, remains central to NF-κB transcriptional activity in myocardial associated processes of inflammation, apoptosis and hypertrophy [8, 14].
It is well known that endotoxin-induced cardiac dysfunction involves a complex inflammatory network and that NF-κB plays a central role in controlling the activation of this network [154, 158, 159]. NF-κB regulates a number of genes associated with the inflammatory pathways including the pro-inflammatory cytokines IL-1, IL-6, IL-8 and TNF [13]. The contribution of NF-κB activation in cardiac dysfunction associated with myocarditis or sepsis was observed when endotoxin- or burn trauma-induced NF-κB activation in mice was blocked through selective overexpression of its inhibitor IκBα. By inhibiting NF-κB, cardiac TNF-α production was prevented, which subsequently attenuated cardiac dysfunction [26, 27]. Furthermore, pharmacological inhibition of IKKβ or the overexpression of dominant-negative NF-κB component p65, significantly reduced the production of LPS-induced TNF-α production in cultured neonatal ventricular myocytes [14]. Similarly, numerous studies served to establish NF-κB as a central mediator of cardiac inflammation.

In addition to its inflammatory role, NF-κB also acts as a transcription factor to numerous genes involved in apoptosis. With respect to the heart, apoptotic events have been detected in cardiac tissue after ischemia/reperfusion [31], oxidative stress injury [32], postinfarction [33], and in patients with end-stage heart failure [34, 35]. Apoptosis has a profound impact on cardiac structure and function due to the inability of cardiomyocytes to regenerate. KO of NF-κB p50 subunit rescued cardiac function and improved survival in a transgenic model of cardiac inflammation due to TNF-α overexpression [28]. Cardiac protection observed in these models was largely attributed to an anti-apoptosis effect, suggesting that NF-κB is pro-apoptotic and not protective. This interpretation is consistent with several studies such as the observance of decreased myocyte death and the reduction in ischemia/reperfusion injury after NF-κB activity was reduced using an in vivo transfection of an NF-κB “decoy” oligonucleotide into a rat or pig heart [29, 38, 39]. Thus, NF-κB can promote apoptosis by activating transcription of pro-apoptotic genes.

However, a pro-apoptotic role for NF-κB is in contradiction to the numerous NF-κB target genes that suppress apoptosis. Expression of these anti-apoptotic genes counteracts immune responses that aim to eliminate premalignant or malignant cells. As such, inhibition of apoptosis is regarded as the most common mechanism through which NF-κB signaling contributes to the development of cancer [30]. In postnatal ventricular myocytes stimulated with TNF-α, NF-κB
activation was shown to prevent apoptosis [36]. As such, this anti-apoptotic function points to a cardioprotective nature of NF-κB. Thus, the role of NF-κB in the heart as either anti- or pro-apoptotic is still elusive.

In addition to its apoptotic role, the importance of NF-κB as a hypertrophic mediator in the heart was also recently demonstrated. *In vitro* studies have shown that activation of NF-κB is required for hypertrophic growth of cardiomyocytes in response to G protein-coupled receptor agonists including phenylephrine, endothelin-1, and angiotensin II (Ang II) [40, 43]. Recent *in vivo* studies have demonstrated that blocking NF-κB ameliorates myocardial hypertrophy in response to hypertension [41] and neurohumoral stimulation [42]. Furthermore, KO of the NF-κB p50 subunit in mice resulted in significantly reduced early ventricular rupture after MI as well as served to significantly suppress cardiomyocyte hypertrophy compared to wildtype mice [44]. Thus, NF-κB is an important regulator of cardiac hypertrophy, although the downstream transcriptional targets underlying this growth effect remains to be elucidated.

In summary, under the right circumstances, the inhibition of NF-κB activity may provide a new therapeutic strategy for heart failure by attenuating myocardial hypertrophy, remodeling, and inflammatory processes which can exacerbate a failing heart. Pharmacological means to inhibit various parts of the canonical pathway are currently investigated including pharmacologic inhibition of IKK with pyrrolidine dithiocarbamate (PDTC), acetyl salicylate, or the inhibition of the proteasome proteolytic pathway with lactacystin or MG-132, all of which lead to inhibition of NF-κB activation in cardiomyocytes [45-48]. Issues arise when developing useful IKKβ inhibitors due to the unforeseen off-target effects of some of these agents. For example, competitive ATP analogue SC-514 was reported to actually potentiate IKK activity by inhibiting the auto-inactivation of the kinase [49]. Furthermore, salicylamide compound IMD-0354 was found to not inhibit IKK-mediated phosphorylation of NF-κB component p65 in the heart despite an accompanying significant reduction in LPS-induced TNF-α expression [47]. This finding suggests that unknown downstream targets are also at play [14]. Considering these results, it is clear that developing effective therapies will require more precise knowledge of the regulation of IKK activity.
1.2 Cardiac regeneration

Addressing heart failure from a remodeling perspective fails to account for the underlying reason for why these cellular changes initially take place. The adaptive response of cardiac remodeling occurs due to the inability of the heart to regenerate itself. To compensate for the loss of cardiomyocytes and the resulting hemodynamic stress, the left ventricle (LV) undergoes a remodeling of the healthy myocardium. This structural change is ultimately detrimental, leading to progressive LV dysfunction and eventual heart failure. Conventional medical therapy aimed largely at preserving LV function, fails to correct the primary cause, which is loss of cardiomyocytes. In response to the limited treatment strategies available, repopulating the heart with “new” cardiomyocytes has gained considerable interest.

The concept of promoting regenerative growth of cardiomyocytes through cell cycle activation has recently shown substantial promise [50-52], yet very little has translated into the clinical arena. In general, the expressions of positive cell cycle regulators, such as cyclins and cyclin-dependent kinases (Cdks), are downregulated in the adult heart. In contrast, negative cell cycle regulatory genes, such as Cdk inhibitors (CKI), are present at high levels [50]. This expression pattern results in an overall absence of cardiomyocyte cell cycle activity and a corresponding lack of proliferative capacity. Thus, an understanding of negative cell cycle regulators necessary for cellular growth and proliferation is of value.

One particular cell cycle inhibitor that is highly expressed in terminally-differentiated cardiomyocytes is the cyclin-dependent kinase inhibitor 1B (CDKN1B, p27kip1, p27) [50]. p27 protein levels have been shown to be drastically reduced in both acute and end-stage human heart failure [53]. In addition, p27 KO mice demonstrate an increase in heart size, cardiomyocyte number and DNA synthesis in adult cardiomyocytes [54]. As such, further investigation into the physiological significance of p27 is beneficial due to its potential as a promising target for heart failure therapy.
1.3 p27kip1: tumour suppressor

The mammalian cell cycle is a highly coordinated process that is driven by several classes of cyclin/Cdk complexes. Cyclin D dependent kinases initiate the phosphorylation of the retinoblastoma (Rb) protein in mid-G1, which is completed by active cyclinE-Cdk2 and maintained by Cyclin A and B dependent Cdns in later stages of the cell cycle [55] (Figure 1.2). Rb hyperphosphorylation disrupts its association from various E2F family members, thus allowing transcription of genes necessary for DNA synthesis. These cyclin/Cdk complexes are tightly regulated by inhibitory proteins called CKIs to ensure proper cell cycle progression [55]. There exists two distinct CKI families, namely INK4 (e.g. p16ink4a) and CIP/KIP (e.g. p21cip1, p27kip1, p57kip2), which are classified based on their sequence homology and Cdk targets. Contrary to p16ink4a, which specifically inhibits Cdk4/6 complexes, CIP/KIP family members including p27, generally target all of the G1,Cdns (Figure 1.2). Thus, p27 regulates the transition from G0,G1 into S phase and is a key protein involved in regulating several physiological processes including proliferation, differentiation and malignant transformation [58-60]. p27 acts as a potent inhibitor of cellular growth and division by negatively regulating G1 progression through the inhibition of cyclinE-Cdk2 activity [59,61]. Accordingly, p27 protein levels are elevated in quiescent (G0) cells but decrease following mitogen stimulation to allow for G1 entry [56, 62].

Until recently, p27 had been solely viewed as having a nuclear role of regulating cyclin-cdk activity and hence cell cycle progression; however, emerging studies reveal additional roles of p27 outside of the nucleus. With respect to its nuclear role, the N-terminus of p27 distinctly interacts with cyclin-Cdk complexes (Figure 1.3). In contrast, the C-terminal domain has been reported to bind with several proteins involved in processes not directly related to cell cycle control. When localized to the cytoplasmic compartment of the cell, p27 interacts with various proteins including the ras homolog gene family, member A (RhoA), ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1), growth factor receptor-bound protein 2 (Grb2) and Stathmin, which are proteins involved in cell contractility and motility [63-66] (Figure 1.3). p27 was also shown to interact with the cytoplasmic casein kinase 2, alpha prime (CK2-α') which is implicated in cardiac hypertrophy [67] (Figure 1.3). Thus, cellular localization of p27 is clearly important for p27 function.
Figure 1.2 Regulation of the mammalian cell cycle by cyclin dependent kinases, their cyclins and Cdk-inhibitors. One of the key events for entry into S phase is the phosphorylation of the retinoblastoma (Rb) protein by cyclin D and E associated kinases and its maintenance by cyclin A and B dependent Cdks. These Cdk kinases are in turn regulated by either the INK4 or CIP/KIP family of Cdk-inhibitors [56].

Figure 1.3 Major interacting proteins of p27kip1 and their functions. Binding sites for various protein partners correspond to individual p27 amino acid residues as indicated by arrows or a stretch of amino acids as depicted by a line. The precise region of interaction for RhoA remains unknown. See text for details regarding each protein [57].
Accordingly, compartmental-specific mechanisms of protein regulation are critical for the control of p27 function, localization and metabolism.

1.3.1 Regulation of p27: localization and degradation

The tumour suppressor p27 has several physiological roles including its well established function as a Cdk-inhibitor, governor of cell motility and a regulator of hypertrophy. These activities of p27 are regulated by multiple phosphorylation sites from differing signal transduction pathways. However, the precise functional roles of these phosphorylation sites and the kinases responsible are still of much debate. In addition to its phosphoprotein nature, the phase of the cell cycle and the specific subcellular compartment where p27 resides dictates its ubiquitin-dependent degradation and thus controls p27 cellular content [68, 69].

In the G₀ phase, p27 is present at high levels within the nucleus and contributes to the maintenance of the quiescent state of the cell (Figure 1.4). This high p27 protein expression is a result of phosphorylation at serine 10 (S10), accounting for ~70% of total p27 phosphorylation, and at threonine 198 (T198), which both serve to stabilize the protein. Several kinases have been implicated in S10 phosphorylation including v-akt murine thymoma viral oncogene homolog 1 (AKT, PBK) and the dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B (DYRK1B, Mirk) [reviewed in 72] (Figure 1.5). To allow for cell cycle progression in the G₀-G₁ transition, S10 phosphorylation is modified by a different nuclear kinase called human kinase interacting stathmin (hKIS) (Figure 1.5). This phosphorylation targets p27 for nuclear export via a CRM1-dependent mechanism. Once in the cytoplasm, ubiquination by the E3-ubiquitin ligase, Kip1 ubiquitylation-promoting complex (KPC1/2) leads to eventual proteasomal degradation of p27 [20-22] (Figure 1.4). Thus, phosphorylation of S10 in G₁ targets p27 for degradation and has a different functional consequence compared to the G₀ phase where S10 phosphorylation serves to stabilize p27 protein levels.

In the presence of antiproliferative signals, there is a stabilization and increase in p27 levels, blocking the G₁-S transition phase of the cell cycle. This cell cycle arrest is a result of p27 association with and inhibition of cyclin E-cdk2 complexes [59, 61]. To alleviate p27 inhibition, cyclinE-Cdk2 phosphorylate p27 on threonine 187 (T187) (Figure 1.5).
Figure 1.4  p27 protein level regulation during G₀/G₁ to S phase. During G₁, translation of p27 declines, while the nuclear export and KPC-mediated degradation of p27 increase. Thr187 phosphorylation increases in late G₁ and targets p27 for SCF^{SKP2}-mediated proteolysis [70].

Figure 1.5 Major phosphorylation sites of p27 along with the responsible kinases and their associated functions. See text for details regarding each phosphorylation site [71].
This phosphorylation is recognized by a nuclear E3-ubiquitin ligase complex called SCF^{Skp2} (S-phase kinase associated protein 1(Skp1)/Cullin/F-Box protein, SCF; S-phase kinase associated protein 2, Skp2), which targets p27 for proteasomal degradation [73-76] (Figure 1.4).

Interestingly, only free and active cyclinE-cdk2 is able to phosphorylate p27 at T187. CyclinE/Cdk2-p27 complexes are catalytically inactive due to p27-mediated remodelling of the Cdk2 catalytic cleft, thus rendering Cdk2 unable to phosphorylate T187. Recent studies have shown that phosphorylation of p27 on tyrosine 88 (Y88) by the Src-family kinase Lyn and its oncogene BCR-ABL, leads to partial activation of Cdk2 (Figure 1.5). This enables Cdk2 to subsequently phosphorylate p27 at T187, which soon renders Cdk2 fully active [77, 78]. This mechanism may at least partially explain how, despite the absence of free cyclinE-Cdk2, p27 phosphorylation and eventual proteasomal degradation mediated by the SCF^{Skp2} complex, can still occur. Collectively, these mechanisms help to amplify Cdk2 activity through inactivation of inhibitory p27.

A separate mechanism of non-proteasomal p27 inactivation was observed in breast cancer cells, whereby p27-mediated growth arrest is alleviated through an Akt-dependent phosphorylation of threonine 157 (T157) within the nuclear localization motif of p27. This resulted in the nuclear export and accumulation of p27 in the cytoplasm [79]. Phosphorylation of T157 or T198 by Akt also facilitates the binding of p27 to the cytoplasmic anchoring proteins, 14-3-3, which tethers p27 in the cytosol and prevents its nuclear translocation [80] (Figure 1.5). This phosphorylation site however, is not evolutionarily conserved and is absent from murine p27 [81].

Separate from its nuclear activity, p27 also has a distinct cytoplasmic role of regulating the actin cytoskeleton. p27 interaction with Rho GTPases are critical in governing actin dynamics and cell motility [63]. Interestingly, this role is cell type-specific, as p27 stimulates migration of hepatocellular carcinoma cells [82] while inhibits cell mobility of vascular smooth muscle cells, umbilical vein endothelial cells, neurons and oral cancer cells [83-85].

In addition, our group has recently established p27 as an intrinsic inhibitor of cardiac hypertrophy through binding and inhibition of the cytoplasmic kinase, CK2-α'. Regulation of p27
by this kinase occurs on serine 83 (S83) and T187, and thus introduces a new site of regulation for p27 activity [67] (Figure 1.5).

In summary, p27 activity is regulated by its protein stability, subcellular localization and post-translational modifications through phosphorylation. Ultimately, the necessity for several levels of regulation is dictated by the crucial role of p27 in controlling cell growth and division.

1.3.2 Significance of p27 in growth control

The tumour suppressor p27, plays a critical role in regulating the growth of proliferating cells [86, 87]. p27 KO mice displayed a phenotype of gigantism, multi-organ hyperplasia and tumour development [88]. The tumour suppressor function of p27 is considered haploinsufficient since the loss of only one p27 allele was necessary to increase the overall number of tumours and shorten survival of the affected heterozygous mice. In cancer patients however, p27 is rarely mutated or deleted. Rather they display an increased p27 degradation, which often correlates with aggressive tumours and poor prognosis [89]. In human breast cancer cells, sustained cell proliferation is achieved through an Akt-dependent mechanism of p27 phosphorylation on T157 leading to impaired nuclear translocation and cytoplasmic p27 accumulation [79]. These findings highlight the role of p27 as a tumour suppressor, whose activity is often disrupted either through compromising its stability or cellular location to allow for cancer progression. In conclusion, p27 is dispensable for proper embryogenesis and postnatal development and differentiation; however, it does play a significant role in controlling body and organ size through cell cycle regulation.

In concert with p27 downregulation in most cancer models, highly proliferating cells express low levels of p27 to allow for cell cycle progression. In contrast, differentiated postmitotic cells display an absence of proliferative potential, which correlates with high levels of p27 expression. The lack of a regenerative capacity of cardiomyocytes is thought to be as a consequence of the unavailability of G1-cyclin/Cdns, key positive cell cycle regulators, and the abundance of inhibitory p27 [50]. As a result, the heart fails to activate cell division in response to mitogens and undergoes a maladaptive response of hypertrophy where individual cardiomyocytes increase in size. Our laboratory has recently established a novel role for p27 as an intrinsic inhibitor of cardiac growth and thus having an anti-hypertrophic function in cardiomyocytes [67]. Evidently,
this new role for p27 is associated with different regulatory mechanisms and downstream targets compared to the cell cycle activity of p27.

1.3.3 p27: an anti-hypertrophic agent in cardiomyocytes

During fetal and early neonatal development, mammalian cardiac myocytes proliferate actively and grow through both hyperplastic and hypertrophic mechanisms. This ability to divide however ceases shortly after birth, with all subsequent growth of cardiomyocytes occurring by an increase in myocyte size [50]. As a consequence, the heart lacks a regenerative capacity and will undergo a compensatory process of hypertrophy in response to increased biomechanical stress such as hypertension or deregulated neurohumoral signals. Hypertrophy is a physiological multifarious process that includes cell enlargement, myofibril disarray and re-expression of fetal genes, ultimately leading to heart failure and death in human patients [90].

Our laboratory has shown that the ectopic expression of p27 abrogates the development of cardiac hypertrophy in experimental mouse models. In the presence of Ang II, a well-described inducer of cardiomyocyte hypertrophy, the ubiquitously-expressed serine-threonine kinase CK2-\(\alpha'\) phosphorylates p27 on both S83 and T187 residues, thus targeting p27 for ubiquitination and eventual proteasomal degradation (Figure 1.6). This removal of inhibitory p27 allows for cardiomyocyte hypertrophy to progress in the presence of active CK2-\(\alpha'\). Conversely, p27 that is resistant to CK2-\(\alpha'\)-dependent phosphorylation, blocks Ang II-triggered CK2-\(\alpha'\) activation and prevents cardiac hypertrophy (Figure 1.6). Silencing of CK2-\(\alpha'\) abolished cardiac hypertrophy. In contrast, the overexpression of siRNA resistant CK2-\(\alpha'\) or the silencing of p27 induced cardiomyocyte hypertrophic growth in the absence of Ang II stimulation. Furthermore, p27 KO mice developed an age-dependent cardiac hypertrophy characterized by an increase in the length and width of the cardiomyocytes and an enhanced contractile ability [67]. Taken together, these findings served to establish a role for cytoplasmic p27 as an anti-hypertrophic agent that is distinct from its nuclear role of regulating cellular proliferation through Cdk2.
Figure 1.6 Regulation of cardiomyocyte hypertrophy by p27 and CK2-α'. In the presence of hypertrophic stimulus Ang II, CK2-α' phosphorylates p27 at S83 and T187. This leads to p27 ubiquitination and degradation, thus disrupting the p27-CK2-α' interaction. This amplifies the activation of CK2-α' and allows for hypertrophy to occur possibly through a downstream unidentified factor, Y. Furthermore, it possible that p27 degradation may also release another p27 binding partner, X. Dashed lines denotes hypothetical factors and pathways [91].
1.3.4 Investigating unknown p27 targets

The Cdk-inhibitor p27 belongs to a family of “natively unfolded” or “intrinsically unstructured” proteins. This allows p27 to adopt a large number of possible conformations, which upon protein binding converges to a well-defined structure with high target-affinity [92, 93]. The absence of a pre-folded protein facilitates p27 binding to a number of targets and allows for the biological advantage of “speed” in molecular identification [reviewed in 57].

Until recently, p27 was almost solely viewed as a nuclear protein with the main function of modulating cyclin-CDK activity and hence, cell cycle progression. Emerging studies now clearly indicate that p27 plays additional roles outside of the nucleus such as controlling cell motility and hypertrophy. Based on recent findings, it is clear that aspects of p27 metabolism still remain uncertain. As such, there is accumulating evidence pointing to the possibility that unknown p27-regulated proteins have yet to be identified.

Interestingly, within adult myocardium, high expression of p27 is found despite the lack of its main known target, cyclinE1-Cdk2 [50]. This indicates a possibility that p27 interacts with non-Cdk partners in cardiomyocytes. Based on our findings, the elimination of p27 gene function in p27 KO mice, completely diminished the ability of kinase dead CK2-α’ to block hypertrophy. In contrast, transduction of degradation-resistant p27, refractory to CK2-α’-mediated phosphorylation, prevented the development of hypertrophy in the presence of Ang II (Figure 1.6) [67]. These results imply that p27 may exert its growth restraining effects through non-CK2-α’ interacting partner(s) (Figure 1.6).

Based on our hypothesis that p27 targets unknown proteins in addition to Cdk's and CK2-α’, we screened a protein microarray with a recombinant p27 probe in an effort to identify novel protein interactors of p27.

1.4 Protein microarray technology

Understanding complex cellular systems requires identifying and analyzing each of its components and how they functionally interrelate. As proteins represent the final player in
cellular events, determining their biochemical activity and how they are regulated by other proteins is critical. A powerful means to aid in assessing the functionality of a protein is to build a protein interaction network. Traditionally, this was achieved through the laborious studying of a single protein at a time, but recent technological advances have developed high-throughput screening methods to optimize the study of large numbers of proteins at once. Mass spectroscopy and yeast two-hybrid assays have aided in mapping protein-protein interactions but are associated with several limitations [94]. The requirement of a lengthy time investment, expensive and specialized equipment including the expertise to run the equipment, and large amounts of sample make these techniques laborious. Recently, protein arrays have emerged as a powerful tool to allow for simultaneous interaction profiling screens of thousands of proteins, which greatly accelerates the rate in which novel interactions can be found [95-97].

Structurally, protein microarrays are grids composed of purified full-length protein or functionally crucial protein domains. Advantages of these arrays include low reagent consumption, rapid interpretation of results, and the ability to easily control experimental conditions [97]. Protein chips are a promising approach for a wide variety of applications including the identification of protein-protein interactions, small molecule targets, and substrates of protein kinases. Their usefulness can also be extended to the monitoring of disease states and for clinical diagnostics [98, 99].

Constructing a protein microarray is associated with several challenges however. It requires the efficient production of thousands of pure proteins that can be immobilized onto a solid surface without loss of functionality. Furthermore, the functional mammalian proteins are required to have proper protein folding and post-translational modifications. To address this issue, a switch from bacterial protein expression to homologous systems such as baculovirus driven expression in insect cells have been utilized [97]. However, changing the expression system does not necessarily ensure that all the proteins produced on the array will be functional. Despite these limitations, protein microarrays have been shown to hold great promise for studying protein interactions. Recently, Schnack et al. identified protein phosphatase 1, regulatory subunit 14A (PPP1R14A), as a novel substrate of Cdk5/p25 through the use of a protein microarray [101]. In addition, Snyder et al. reportedly used an array containing the majority of the yeast proteome to identify a new binding motif for calmodulin [99].
In our study, the ProtoArray® Human Protein Microarray v4.0 from Invitrogen was employed. This chip was based on the yeast protein microarray technology developed by Zhu et al. to detect molecular interactions between proteins [99]. The array consisted of 8,222 unique, full-length, and partially full-length (20%), human proteins derived from defined open reading frames (ORFs) alongside 1326 built-in controls (Table 1.1). These recombinant proteins were expressed in insect cells as amino-terminal glutathione S-transferase (GST) or histidine (His) fusion proteins, purified under native conditions, and spotted in duplicate in picomolar amounts on nitrocellulose-coated glass slides. The microarrays were incubated with a V5 epitope-tagged p27 protein probe, washed to remove excess and nonspecifically bound p27 molecules then scanned to visualize bound p27 (Figure 1.7). The V5 epitope tag, used as method for detection of the p27 probe, is a 14 amino acid (GKPIPNPLLGLDST) epitope derived from the P and V proteins of the paramyxovirus, SV5 [102]. By identifying where p27 bound, a list of candidate p27 interactions is obtained which can be compared and validated with a second microarray run.

1.4.1 Determining significant p27 targets

The objective of our protein-protein interaction experiment was to identify proteins on the microarray which display significant binding to the p27 protein probe. Invitrogen’s ProtoArray® Prospector software is designed to analyze the results from experiments using a fluorescently conjugated antibody against V5-tagged proteins. The relative amount of bound p27 protein to the human proteins printed on the microarray is quantified by measuring the fluorescence intensity arising from each protein spot on the slide. These fluorescent signals were quantified then used by the ProtoArray® Prospector software to compute a value called a Z-Score ($Z_k$) for each of the human protein features present on the chip. A Z-Score indicates how far and in what direction the value of an individual data point in a population falls from the mean in units of standard deviations.

\[ Z_k = \frac{X_k - \mu_s}{\sigma_s} \]

$X_k$ = the signal value from the $k^{th}$ protein feature

$\mu_s$ = mean signal for all the protein features

$\sigma_s$ = signal sample standard deviation for all the protein features
Table 1.1

A) List of (A) human proteins and (B) control spots present on ProtoArray® Human Protein Microarray v4.0 (Source of control protein chart: Invitrogen).

<table>
<thead>
<tr>
<th>Human Protein ProtoArray® Content</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Kinases</td>
<td>447</td>
</tr>
<tr>
<td>Transcription Factors</td>
<td>239</td>
</tr>
<tr>
<td>Membrane Proteins</td>
<td>1552</td>
</tr>
<tr>
<td>Nuclear Proteins</td>
<td>1152</td>
</tr>
<tr>
<td>Signal Transduction</td>
<td>970</td>
</tr>
<tr>
<td>Secreted Proteins</td>
<td>137</td>
</tr>
<tr>
<td>Cell Communication</td>
<td>1166</td>
</tr>
<tr>
<td>Metabolism</td>
<td>2748</td>
</tr>
<tr>
<td>Cell Death</td>
<td>248</td>
</tr>
<tr>
<td>Protease/peptidase activity</td>
<td>185</td>
</tr>
</tbody>
</table>

B) Control Spots required for PPI Data Analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® Antibody (Rabbit anti-mouse IgG Antibody</td>
<td>Serves as a positive control for fluorescence scanning and for</td>
</tr>
<tr>
<td>labeled with Alexa Fluor® 647, Alexa Fluor® 555, and</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 488)</td>
<td>orientation of the microarray image.</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>A negative control for non-specific protein interactions.</td>
</tr>
<tr>
<td>Biotinylated Anti-mouse Antibody</td>
<td>A positive control for interaction with streptavidin-labeled detection</td>
</tr>
<tr>
<td></td>
<td>reagent.</td>
</tr>
<tr>
<td>V5 Control Protein (biotinylated, V5-tagged control</td>
<td>A positive control for detection with the Anti-V5-Alexa Fluor® 647</td>
</tr>
<tr>
<td>protein)</td>
<td>Antibody and the streptavidin-labeled detection reagent.</td>
</tr>
<tr>
<td>Human IgG Protein Gradient</td>
<td>A positive control for the immune response serum profiling application.</td>
</tr>
<tr>
<td></td>
<td>Interacts with Alexa Fluor® 647 goat anti-human IgG.</td>
</tr>
<tr>
<td>Anti-Human IgG Antibody Gradient (goat anti-human IgG)</td>
<td>A positive control for the immune response serum profiling application.</td>
</tr>
<tr>
<td></td>
<td>Interacts with serum IgG antibodies which are then bound by Alexa Fluor®</td>
</tr>
<tr>
<td></td>
<td>647 goat anti-human IgG.</td>
</tr>
<tr>
<td>Yeast calmodulin (Cnd1p)</td>
<td>A positive control for protein-protein interaction application and</td>
</tr>
<tr>
<td></td>
<td>interacts with the Array Control Protein.</td>
</tr>
<tr>
<td>GST Protein Gradient</td>
<td>Serves as a negative control and signals are used by ProtoArray® Prospect</td>
</tr>
<tr>
<td></td>
<td>or software for background and statistical significance calculations.</td>
</tr>
</tbody>
</table>

Table 1.1 List of (A) human proteins and (B) control spots present on ProtoArray® Human Protein Microarray v4.0 (Source of control protein chart: Invitrogen).
Features that have a Z-score greater than the user-defined cut-off value are considered positive for binding to the p27 probe.

The default Z-score as set by Invitrogen is three which corresponds to a confidence interval of 99%. Our own results showed that the known p27 targets, CK2-α' and cdc2, were below three. Thus, we defined our cut off value to be above two which corresponds to a 97% confidence interval. Since Z-score values are based on a measure of signal intensity, it corresponds to how much antibody is bound to a spot on the array. As such, these values do not correlate with the strength of the interaction between the p27 probe and the target. Therefore, all targets obtained above a Z-score of two were considered equally significant.

In addition to the Z-score value, a protein feature is only considered a “hit” when the coefficient of variation for the signals from the two replicate spots on the array is less than 0.5. Once proteins that score as positive on a single microarray are identified, ProtoArray® Prospector also allows for the comparison of results across several microarrays. As such, we determined the reproducibility of the candidate interactions by probing multiple arrays with our recombinant p27 probe.
1.5 Project Rationale

The increased prevalence of heart failure alongside limited treatment options has advanced interest in alternative therapies for heart failure patients. As the lack of a substantial regenerative capacity of the heart is primarily responsible for progressive myocardial dysfunction, a thorough understanding of the regulation of cardiomyocyte differentiation is of value. We have previously shown that the cell cycle inhibitor p27 is an intrinsic inhibitor of cardiac growth by blocking protein kinase CK2-α'. Catalytically inactive CK2-α', however, was unable to block hypertrophy in p27 KO mice, while re-introduction of p27 in these mice prevented hypertrophy [36]. Based on these findings, we hypothesized that the growth restraining activity of p27 may be mediated through negative regulation of CK2-α' unrelated kinases.

Therefore, the objectives of this thesis were:

1) To identify novel interacting partners of p27.
   Several questions of p27-dependent regulation of cardiomyocyte hypertrophy remain unanswered. Furthermore, emerging studies reveal additional roles for p27 outside of the nucleus including regulation of cell motility. This evidence suggests the existence of unidentified targets for p27. In an effort to address this, we screened a human protein microarray using a recombinant p27 probe. This experiment served as a means to quickly and efficiently obtain a list of possible novel p27 interacting partners which can then be further investigated.

2) To characterize the interaction between the novel target(s) and p27.
   a) To investigate whether p27 binds to the novel target(s) as recombinant proteins in vitro and in vivo and to determine the target-interaction domain of p27. To identify the region that the novel target(s) interacts with p27, we will generate amino- and carboxy-terminally truncated mutant versions of p27 and perform co-immunoprecipitation analysis of these recombinant proteins when overexpressed in living cells.
   b) To determine the physiological significance of the interaction between the novel target(s) and p27.
Chapter 2 Materials and Methods

2.1 Construction of the recombinant p27 microarray probe

For generation of the recombinant p27 protein necessary as a probe for the protein microarray, an N-terminal histidine (His) tag [103] was cloned in frame with a human p27 cDNA (Genbank D86924) to allow for purification and isolation of the p27 protein from the bacterial lysate. A V5 epitope was also cloned in frame with p27 on the C-terminus to facilitate its detection on the microarray. Exponentially growing *Escherichia coli* BL21(DE3)pLysS cells (L1191; Promega), transformed with pRSET-C (for His6-tagged proteins) encoding a C-terminal V5-conjugated p27, were induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 4 hours at 225 rpm at 37°C. After centrifugation (4000 rpm, 20 min at 4°C), the cell pellet obtained from 1L of culture volume was lysed in 25 ml of non-denaturing NETN lysis buffer (20 mM Tris pH 8.0, 100 mM NaCl, 20mM Imidazole, 1.0 mg/ml lysozyme (Sigma-Aldrich), and protease inhibitor cocktail (Roche)). Following sonification, the lysate was centrifuged (13,000 rpm, 30 min at 4°C) and the supernatant was separated on immobilized Ni$^{2+}$ metal ion affinity chromatography (Chelating Sepharose Fast Flow, GE Healthcare) using fast protein liquid chromatography (FPLC) (ӒKTA, GE Healthcare) and eluted in Probing buffer (phosphate buffered saline (PBS), 5mM MgCl$_2$, 0.5mM DTT, 0.05% Triton X-100). The identity of p27 and the presence of both the His and V5 epitope tags were verified through immunoblot analysis and visualized with an enhanced chemiluminescence system employing horseradish-peroxidase conjugated secondary antibodies. Purity of the p27 preparation was evaluated through Coomassie staining of a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and p27 concentration was determined with a bicinchoninic acid (BCA) protein assay using bovine serum albumin (BSA) as a protein standard (Pierce).

2.2 Antibodies

Antibodies directed against the following proteins were used: mouse monoclonal antibody to His6Gly (R940-25; Invitrogen), mouse monoclonal antibody to V5 (R960-25; Invitrogen), mouse monoclonal antibody to FLAG (F3165; Sigma-Aldrich), Alexa Fluor® 647-conjugated Anti-V5 (451098; Invitrogen), rabbit polyclonal antibody to p27 (2552; Cell Signaling), rabbit polyclonal antibody to p27 (AB3003; Upstate), mouse monoclonal antibody to p27 (610241; BD
Transduction), rabbit polyclonal antibody to IKK-alpha (2682; Cell Signaling), rabbit polyclonal antibody to IKK-beta-L570 (2678; Cell Signalling), mouse monoclonal antibody to IκBα (4184; Cell Signaling), rabbit monoclonal antibody to NF-κB p65 (4764; Cell Signaling), highly cross-adsorbed Alexa Fluor® 488–conjugated goat antibody to rabbit IgG (A-11034; Molecular Probes), Alexa Fluor® 594–conjugated goat antibody to mouse IgG (A-11029; Molecular Probes).

2.3 Assessing the recombinant p27 probe by ELISA

The integrity of both the p27 protein and the V5 epitope was assessed by an enzyme-linked immunosorbent assay (ELISA). An indirect ELISA was performed by initially binding varying concentrations of p27 protein (0µg-100µg) diluted in microarray probing buffer (1X PBS, 5 mM MgCl₂, 0.5 mM DTT, 0.05% Triton X-100, 5% Glycerol, 1% BSA) to the bottom of a 96-well plate followed by incubation of the anti-V5 antibody (1:1000) for 2 hrs at room temperature (RT). Wells were then incubated with anti-mouse horse radish peroxidise conjugated secondary antibody (1:1000) for 1 hour at RT. Tetramethylbenzidine (TMB) and sodium acetate substrate was incubated for 30 min at RT then 1M H₂SO₄ was added to stop the color reaction. Wells were washed four times with Probing buffer between each step. The absorbance was measured using a CytoFluor® series 4000 Fluorescence Multi-Well Plate Reader (Applied Biosystems). A sandwich ELISA was performed in a similar manner to an indirect ELISA; however, the sandwich ELISA involved initially binding 100ng/well of anti-p27 antibody to the bottom of a 96-well plate prior to p27 protein incubation and detection with the anti-V5 antibody.

2.4 Probing the protein microarray with recombinant p27

ProtoArray® Human Protein Microarrays nc v.4.0 (PAH0524013, Invitrogen) were used according to the manufacture’s protocol (PPI Protoarray® Manual). Each array (dimensions: 1 inch x 3 inch) was equilibrated at 4°C for 15 min prior to blocking with 30 mL of PBST Blocking buffer (1% BSA in PBST) for 1 h at 4°C. Recombinant p27 protein probe diluted in Probing Buffer (1x PBS, 5mM MgCl₂, 0.5mM DTT, 0.05% Triton X-100, 5% glycerol, and 1% BSA) at a concentration of 50µg/ml was sequentially incubated for 1.5 h at 4°C on two arrays. Slides were washed three times with 25 mL of Probing buffer before incubation with Alexa Fluor® 647 anti-V5 antibody diluted in Probing buffer for 30 minutes on ice in the dark. Washes
were repeated prior to centrifugation at 800xg for 5 min at RT followed by 1 hr of air drying in the dark at RT. Control arrays (2112 control spots) using a calmodulin kinase control probe were run to verify acceptable experimental conditions of low signal to noise ratio, appropriate signal intensity for positive controls and to test the efficiency of the Alexa Fluor® 647 conjugated antibody to V5. Data acquisition was performed using LS Series Laser scanner (Tecan AG group; University Health Network Microarray centre, 9th floor TMDT-MaRS) using Invitrogen-specified settings (wavelength: 635 nm; PMT gain: 120; laser power: 100%; pixel size: 10 µm; lines to average: 1.0; focus position: 0 µm).

2.5 Data analysis of protein microarray

The scanned image was analyzed by GenePix Pro 3 image analysis software using lot specific information from Invitrogen’s Protoarray® Central Portal (www.invitrogen.com/protoarray, Protoarray® Lot Specific Information) associated with the array barcode. Data analysis was performed using ProtoArray® Prospector v4.0 software (www.invitrogen.com/protoarray, online tools) that provides rapid interpretation of experimental results generated from the ProtoArray® microarrays. Statistically significant protein hits were determined based on Z-score values to determine which protein signals were significantly above both spotted control proteins and background signals. Z-scores were defined as the value that indicates how far and in what direction a signal from a specific protein feature deviates from the mean of the signals from all the protein features in terms of standard deviations where n= 2/array. Z-score values above the value of two were considered statistically significant which pertains to a 97% confidence level.

2.6 Lentiviral constructs and ectopic protein expression

cDNAs (Origene and Open Biosystems) were cloned into slightly modified pSIH1-H1-copGFP (SI501-A1; System Biosciences) lentiviral vectors that were devoid of the copGFP sequence (pS1, pS2, and pS5; each vector contained a unique cloning site). A C-terminal V5 epitope was cloned in frame with each protein target selected from the microarray screen. A p27 lentiviral plasmid (pS1-p27) containing only an N-terminal His tag and a pS2-p27 construct containing a C-terminal V5 tag were also produced.
Viral-driven protein expression consisted of two sequential steps: 1) Producing the lentiviruses from lentiviral plasmids using HEK293FT producer cells and 2) Incubating these lentiviruses on various cell lines for protein production.

For production of the lentiviruses, lentiviral plasmids were transfected into 70-80% confluent HEK293FT producer cells cultured in DMEM growth medium (DMEM; 11995 Invitrogen, 10% fetal bovine serum, 50 U/mL Pen/Strep, 1x MEM). The lentiviral plasmid transfection required two separate mixtures: a) 130 µl of DMEM (11995; Invitrogen), 1.3 µl of Vira Power Packaging Mix (K4975-00; Invitrogen), and 0.4 µg of lentiviral expression plasmid and b) 130 µl of DMEM and 7.2 µl of Lipofectamine 2000 (11668; Invitrogen), which were incubated at RT for 5 min each before combining both mixtures for a further incubation of 30 min at RT. This final DNA-lipofectamine mixture was added dropwise to the cells and was incubated for approximately 72 hrs. Lentiviral particles suspended in the supernatant were then harvested and concentrated 100-fold by polyethylene glycol precipitation using 0.5x PEG-it Virus Precipitation Solution (LV810A-1; System Biosciences) at 4°C for 2 days. Viral pellets were resuspended in 100 µl of DMEM and both target and p27 viruses were co-transduced along with 6mg/ml of Polybrene (H9268; Sigma-Aldrich) into 70-80% confluent NIH 3T3, BJ Fibroblast, GP2 293 and HT1080 cell lines incubated in DMEM growth medium. Cells were harvested after 60 hrs, washed in cold PBS (without Ca/Mg), lysed in cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin; 9803, Cell Signalling), and then immunoblotted with an antibody against the tags to check for protein expression.

To increase the reliability of protein production, lentiviral plasmids were directly transfected into the cell line rather than using the viruses to drive protein production. Lentiviral plasmids were transduced directly into 70-80% confluent GP2 293 and HT1080 cells cultured in DMEM growth medium with a mixture of 130 µl of cold DMEM, 2.5µg of purified target plasmid, and 1 mg/ml of Lipofectamine 2000 that was incubated for 30 min at RT prior to incubation on the cells. Cells were harvested after 48 hrs and washed with cold PBS (without Calcium/Magnesium).
2.7 Co-immunoprecipitation of recombinant proteins

For the co-immunoprecipitation (Co-IP) experiment between the target proteins and p27, proteins expressed directly from the lentiviral plasmids were used, which were each cloned with C-terminal V5 epitope tags. The only exception were the plasmids pCR-FLAG-IKKα (15467, P.I. Hiroyasu Nakano; Addgene) and pCR-FLAG-IKKβ (15465, P.I. Hiroyasu Nakano; Addgene) which were purchased with N-terminal FLAG tags.

Each target plasmid was co-transduced after 12 hours with a mixture of 130 µl of cold DMEM, 2.5µg of pCMV5-p27kip1 plasmid (pCMV5-p27; 14049, P.I. Joan Massague; Addgene), and 1 mg/ml of Lipofectamine 2000 that was incubated for 30 min at RT prior to incubation on the cells. The pCMV5-p27 plasmid was a p27 construct that was absent of any tags.

Cells were harvested after 48 hrs and washed with cold PBS (without Ca/Mg). Cell pellets were resuspended and lysed in 100µl of RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin; 9806, Cell Signaling) and incubated on ice for 30 min followed by mechanical disruption 5-7x with a 1cc 27G syringe. Supernatant isolated after centrifugation (13,000 rpm, 1hr at 4°C) was incubated in BSA blocked 1.5mL microcentrifuge tubes with an antibody against the V5 epitope covalently linked to protein G-agarose beads (20398, Thermo Scientific) overnight at 4°C. Beads were washed in RIPA buffer then heated for 8 minutes at 100°C in 60µl of SDS sample buffer. Samples were centrifuged at RT for 3 min at 13,000 rpm and the supernatant was run on SDS-PAGE for analysis.

Co-IP of IKKα and IKKβ with full-length p27 and its fragments involved the use of the plasmids pCR-FLAG-IKKα and pCR-FLAG-IKKβ which were each co-transduced with either pS2-p27, pS2-p27fragA consisting of amino acids 1-86 of p27 or pS2-p27fragB consisting of amino acids 87-198 of p27. All of these p27 constructs were cloned with C-terminal V5 epitope tags.
2.8 Primary rat ventricular cardiomyocyte culture

Spontaneously beating cardiomyocytes from the left ventricle of neonatal rats were cultured for immunocytochemistry and lysed for immunoprecipitation analysis. Briefly, hearts from 3-day postnatal Wistar rats were dissected, minced, and enzymatically isolated with collagenase II (0.5 mg/ml, Invitrogen) and pancreatin (1 mg/ml, Sigma). To selectively enrich for cardiomyocytes, the resultant cell suspension was preplated onto 10 cm-collagen I (Gibco) coated dishes in culture medium DMEM/F12 containing 3 mM Na-pyruvate, 2 mM glutamine, Penstrep antibiotics (5000 units/ml Penicillin, 5000 µg/ml Streptomyocin, Gibco), 0.2% BSA (Sigma-Aldrich), 0.1 mM ascorbic acid and 0.5% insulin-transferrin-selenium (Sigma-Aldrich) for 60 min. After preplating, cardiomyocytes were exposed to 25 µM arabinosylcytosine (AraC; Sigma-Aldrich) and 5% horse serum (Sigma-Aldrich) for 36 hrs to inhibit non-cardiomyocyte proliferation. Less than 10% of non-cardiomyocytes remained in the cultures after cardiomyocyte isolation.

2.9 Endogenous protein interaction assays

For Co-IP analysis, cardiomyocyte extracts were lysed in 500 µl of cell lysis buffer for 1 hour on ice followed by mechanical disruption 5-7x with a 1cc syringe. Supernatant was isolated after centrifugation (13,000 rpm, 1hr at 4°C) and was transferred to BSA blocked 1.5 mL microcentrifuge tubes containing pre-incubated and PBS washed protein G beads (20398, Thermo Scientific) covalently bound to an antibody against either the protein(s) of interest or p27. The samples were incubated overnight at 4°C and the beads washed in cell lysis buffer and heated for 8 minutes at 100°C in 60µl of SDS sample buffer. Samples were then centrifuged at RT for 3 min at 13,000 rpm and the supernatant was run on SDS-page for analysis.

For immunocytochemistry, cardiomyocytes were cultured in 24-well plates on collagen-coated coverslips and sequentially fixed with 500 µl/well of cold paraformaldehyde (PFA) followed by 0.3M glycine pH 7.1 for 10 minutes with agitation at RT. Wells were washed three times with 500 µl/well of 1x TBS then blocked overnight at 4°C with 500 µl/well of antibody dilution buffer with 5% Triton X-100. Both primary antibody (1:25) and secondary antibodies (1:150) were diluted in antibody dilution buffer with 5% Triton X-100. The primary antibody was incubated for 2 hours at RT followed by three consecutive washes with 1x TBS/NP40 (0.1%) before
incubation of the secondary antibody for one hour at RT in the dark. Washes were repeated with 1x TBS/NP-40 followed by incubation of the cells with DAPI stain for one minute at RT. After one wash with 1x TBS, cells were fixed with 4% formalin in 1x TBS for three minutes at RT. The cardiomyocyte coverslips were then incubated with DABCO (D-2522; Sigma) and sealed with Enkitt (03989; Sigma) on glass slides and stored at -20 °C. Images were captured using the PALM MicroBeam Laser Scanning microscope (Carl Zeiss MicroImaging).

2.10 In vitro LPS studies
Hela cells cultured on coverslips in 24-well plates were transduced with 20 µl/well of concentrated pS2-p27 lentivirus for 60 hours using the protocols as described. Transduction efficiency was assessed using green fluorescent protein CopGFP. Cells were then incubated with 500 µl of fresh media containing 1µg/ml of lipopolysaccharide (LPS from *Escherichia coli* 0111:B4, L2630, Sigma) for 0, 10, 30, and 60 minutes. At each time point, LPS-containing media was removed and cells were fixed and stained using the protocol described.

2.11 Synthesis of TAT-p27 and TAT-LacZ proteins
For generation of the TAT-conjugated p27 and LacZ proteins necessary for the in vivo experiments, cDNAs for p27 (Genbank D86924) and LacZ were cloned in the pRSET based pTAT vector (kind gift from S. Dowdy, Howard Hughes Medical Institute, La Jolla, USA) providing the N-terminal HIV-1 TAT protein transduction domain and a His-tag for purification and isolation of the proteins from the bacterial lysate [67]. Protocol for isolation and purification of the proteins were similar to the p27 probe with a few minor modifications. Bacterial cells containing the TAT-p27 protein were lysed in HEPES buffer (20 mM Heps, 100 mM NaCl, 25 mM Imidazole, 50 mM L-arginine, 50 mM L-glutamic acid) while the cells containing TAT-LacZ were lysed in NETN. Furthermore, both proteins were eluted into sterile water after the desalting step during FPLC purification at a concentration of 1 mg/ml.

Transactivator of transcription (TAT) is a highly cationic 9 amino acid residue (RKKRRQRRR) PTD from the human immunodeficiency virus (HIV-1) that facilitates diffusion of proteins across physiological membrane barriers [67, 104].
2.12 LPS-challenged mice studies

We treated 10-12 wk old adult male C57BL6/J mice (22-25g, n=10 per group) from Jackson Laboratories with LPS (10mg/kg body weight) injected intraperitoneally (IP). After 24 hours, the LPS-treated mice were IP injected with either recombinant TAT-conjugated p27 protein (8.5mg/kg) or 200 µl of 0.9% NaCl, pH 5.5 (JB1323, Baxter corp.) as a control. Left ventricular contractility was assessed with transthoracic echocardiography using the General Electric Vivid 7 Dimension imaging system equipped with a 14-MHz linear probe (General Electric, Milwaukee, Wisconsin). Animals were induced with a mixture of 2% Isoflurane in oxygen, received continuous inhaled anaesthetic for the duration of the imaging session using a small animal ventilator (Benson), and were imaged in the left lateral decubitus position. M-mode short axis views of the LV were obtained and archived. Analysis of the M-mode images was performed using GE built-in analysis software. LV contractility of the mice was assessed before LPS injections at baseline, then at days 1, 3, and 5 post-LPS injection. LV end diastolic diameter (EDD) and end systolic diameter (ESD) were measured and used to calculate fractional shortening (FS) by the following formula: \( FS = \frac{EDD - ESD}{EDD} \).

For the survival studies, the LPS-treated mice (30mg/kg body weight, n=5-10 per group) were IP injected after 6 hrs with either recombinant TAT-conjugated p27 protein (8.5mg/kg), TAT-beta-galactosidase (TAT-LacZ, 8.5mg/kg), or 200 µl of 0.9% saline. The concentration to be administered was experimentally determined in our laboratory based on our previous publication [67].

2.13 Statistical analysis

Analysis of variance (ANOVA) was used to analyze the data. Significant ANOVA values were subsequently subjected to post hoc comparisons of individual means using the Bonferroni method. Survival analysis was performed using the logrank test for trend. P values less than 0.05 were considered significant.
Chapter 3  Results

3.1 Construction of the recombinant p27 probe

To identify novel targets of the cell cycle inhibitor p27kip1, we probed a human protein microarray using a recombinant p27 probe. To construct the probe, we cloned an N-terminal His tag [155] in frame with a human p27 cDNA to allow for purification and isolation of the p27 protein from the bacterial lysate. The bacterial expression p27 construct also carried a carboxy-terminal V5 epitope tag [53] to facilitate the detection of the p27 probe with a fluorescently labelled anti-V5 antibody on the microarray. Immunoblot analysis confirmed the identity of the p27 protein and the presence of both the His and the V5 tags (Figure 3.1). Interestingly, a different p27 expression construct cloned in parallel containing both the V5 epitope and His-tag on its amino-terminus was not recognized by the V5 antibody on the immunoblot despite its reactivity with anti-His (data not shown). As such, further experiments were carried out solely using the recombinant p27 probe with the V5 tag on the C-terminus. The p27 probe was then purified using FPLC with a Ni$^{2+}$ chelated resin, which captures histidine-tagged proteins. After subjecting the lysate to FPLC, the purity of the p27 protein was evaluated using a Coomassie stained SDS-PAGE gel (Figure 3.2).

To ensure that the C-terminal V5 epitope tag was functional and accessible within the native confirmation of p27, both an indirect and sandwich ELISA were performed. The indirect ELISA involved the use of the anti-V5 antibody for detecting varying concentrations of p27 antigen diluted in microarray probe buffer and bound to the surface of a 96-well plate. The sandwich ELISA, on the other hand, involved a 96-well plate pre-bound with an antibody against p27, followed by incubation with the p27 probe and exposure to an antibody against the V5 tag. Both ELISAs displayed similar sensitivity to antigen concentrations. The lowest detectable concentration of p27 antigen was determined at 250 ng/ml. For the concentration of 100 µg/ml, a maximal absorbance value of 3.8 for the indirect ELISA and 3.5 for the sandwich ELISA was obtained (Figure 3.3A & B). However, the probe concentration to be used on the array of 50 µg/ml, displayed absorbance values of 3.5 for the indirect ELISA and 3.2 for the sandwich ELISA. Thus, increasing antigen concentration correlated with a linear increase in absorbance until 50 µg/ml, with only slight changes in absorbance for concentrations above 50 µg/ml.
Figure 3.1

**A**  N  
\[\text{His} \rightarrow \text{P27 sequence} \rightarrow \text{V5 Epitope} \rightarrow \]

**B** Anti-p27  
**C** Anti-His  
**D** Anti-V5

**Figure 3.1** Reactivity of bacterially produced recombinant p27 protein with anti-p27 or anti-tag specific antibodies. (A) Schematic of the recombinant p27 probe cloned with an N-terminal His tag and a C-terminal V5 epitope. (B-D) Immunoblot analysis of recombinant p27 (30 kDa, relative molecular weight) employing (B) anti-p27, (C) anti-His and (D) anti-V5 antibodies.

Figure 3.2

**Crude Lysate**  |  **Ni\(^{2+}\) Beads**  |  **FPLC**
---|---|---
1  | 2.5  | 3.5  | 5  
1  | 2.5  | 3.5  | 1  
2.5 | 3.5  | 5   

**Figure 3.2** Purification of recombinant p27 probe. Samples were Coomassie stained to visualize and compare crude versus purified samples of recombinant p27. A small scale purification using 500 µl of lysate was incubated with Ni\(^{2+}\) chelated beads and was used for comparison to FPLC purified samples.
Figure 3.3 Development of a sensitive ELISA technique to assess recombinant p27 protein. (A) In the indirect ELISA, concentrations of p27 protein in the range between 0-50 µg/ml were confined to the linear range of the assay. (B) Similar to the indirect ELISA, the sandwich ELISA had increasing antigen concentration correlating with a linear increase in absorbance until 50 µg/ml. Data are means ± standard deviation, n= 4. (C) Immunoprecipitation of the p27 probe using anti-p27 or anti-V5 bound to agarose beads and incubated in the presence of recombinant p27 diluted in RIPA buffer for overnight at 4°C. HC= heavy chain of antibody, LC=light chain of antibody.
The ELISAs confirmed that the V5 epitope was both accessible and able to bind to its antibody and thus functional as a detection method on the microarray. It also verified that the p27 probe concentration (50 µg/ml) to be used for the microarray was detectable and that the protein was soluble in the array probing buffers.

An immunoprecipitation assay using either anti-p27 or anti-V5 antibodies bound to agarose beads verified the identity of the p27 probe and the accessibility of the V5 tag respectively, in the native conformation of the protein (Figure 3.3C).

To assess the functionality of the p27 probe in binding to other proteins, well-known targets of p27, cyclinE1 and Cdk2, were also cloned. FPLC purification of these two proteins was unsuccessful however, with very low amounts of target protein present in the purified lysate (data not shown). Furthermore, the BSA assay became an unreliable technique for determining protein concentration due to the presence of contaminating proteins in the purified lysate. As such, a Co-IP assay to verify interactions between p27 and its known targets could not be performed. Thus, the ELISA was used to confirm the functionality of recombinant p27 as a probe within the confines of this project.
3.2 35 novel p27 interacting partners identified

Recombinant p27 protein was used to sequentially probe two ProtoArray® human protein microarrays v4.0, which each consisted of 8222 unique human proteins spotted in duplicate alongside built-in controls (Figure 3.4). The array was scanned with a LS Series Laser Scanner (Tecan Group AG) and then analyzed with GenePix Pro 3 image analysis software. The binding of the p27 probe to a total of four protein spots for each unique protein was analyzed. Statistically significant protein hits were determined based on Z-score values above two which pertains to a 97% confidence level as calculated by Invitrogen’s Protoarray® Prospector software. A positive control chip, with a total of 4224 spots, was probed with a calmodulin kinase control probe prior to the human array to ensure appropriate background, probing conditions, and signal intensities were obtained with the Alexa Fluor® 647 conjugated V5 antibody.

Recombinant p27 displayed 35 statistically significant positive interactions with a Z-score >= 2 out of 8222 unique proteins (0.43% positive hits) (Figure 3.5 A&B). These protein hits mainly consisted of protein kinases (27 out of 35 from 8222 spotted proteins (76%), 27 out of 477 total kinases (6%)). Out of the 35 hits, five proteins were previously established targets of p27 (Cdk2 [56], CK2-α' [67], Pim-1 [105], GSK3β [106], and cdc2 [71]) while five known p27 targets (Cdk4 [55], Cdk6 [55], Lyn [77, 78], CyclinD2 [55], Cyclin D3 [55]) that were present on the chip fell below the significant signal range (Figure 3.5B). As such, 30 novel p27 interactions were identified that have yet to be characterized (Figure 3.5A). Z-score values obtained for all 35 hits were fairly similar in value between both array runs with the exception of vascular endothelial growth factor receptor 2, VEGFR2 (FLK1, KDR) (Figure 3.5A).

A scatter plot of the signal intensities pertaining to each protein spot showed a separation of the positive hits (5,000 to 64,000 signal range) from all the background signals (<5000) present on the microarray (Figure 3.6). For all the positive hits obtained and for the majority of proteins present on the array, both protein spots A and B were significantly similar in their signal intensity as indicated by similar Z-score values. However, the plot also served to highlight the presence of outliers which must be taken into consideration.
**Figure 3.4**

**ProtoArray® Human Protein Microarray**

![Image of ProtoArray® Human Protein Microarray. A close up image of block 38 is shown where recombinant p27 bound to glycogen synthase kinase, GSK3β.]

4 Columns x 12 Rows = 48 Blocks
20 Rows x 20 Columns / Block

**GSK3β**
Z-score = 27.2
(Row 19, Columns 17, 18)

**Figure 3.4** Image of ProtoArray® Human Protein Microarray. A close up image of block 38 is shown where recombinant p27 bound to glycogen synthase kinase, GSK3β.
Figure 3.5

Thirty-five significant hits for p27 with Z-Scores >= 2 were identified on the microarray. (A) Comparison of Z-score values for 30 novel p27 hits derived from two independent microarray analyses. Acronyms are defined in appendix. (B) Comparison of Z-score values obtained for known targets of p27. Proteins hits were identified as either statistically significant (Z >= 2) or insignificant (Z < 2) from both microarray runs.
**Figure 3.6**

Scatter plot of Spot A vs. B signals of each protein on Microarray.

*Figure 3.6* Scatter plot of signals pertaining to both spots of each microarray protein. Significant positive interactions appear isolated from the other spots at the region of highest signal intensity (*). Data excluded the Alexa Fluor control signals.

**Figure 3.7**

Histogram of Protein Signals

*Figure 3.7* Histogram of microarray protein signals. The high frequency of proteins present at low signal intensity corresponds to background signals. Distribution of protein interactions deemed statistically significant are present in a broad range of higher signal intensities.
An outlier involved one spot having a high signal intensity (i.e. >10,000 for protein spot A only) compared to the second spot, thus counting as an insignificant hit. The greatest outlier, corresponding to a signal intensity of 15,227 for protein spot A with a signal intensity of only 69 for protein spot B corresponded to v-raf-1 murine leukemia viral oncogene homolog 1 (RAF-1) (Figure 3.6). RAF-1 functions downstream of the Ras family of membrane-associated GTPases to phosphorylate mitogen-activated protein kinase kinase 1 (MEK1) and MAP2K2 (MEK2, another protein target obtained from the screen), which in turn phosphorylates the extracellular signal–regulated kinases, ERK1 and ERK2. Activation of ERK1/2 has been implicated in G0/G1 control of p27 through phosphorylation on Ser10, thus inducing the translocation of the protein from the nucleus to the cytoplasm and targeting p27 for eventual proteasomal degradation [57]. p27 regulation of either RAF-1 or MEK2 would serve as a level of control in this pathway that has yet to be established. Thus, identifying RAF-1 highlighted the value for investigating the outliers identified on the scatter plot (Figure 3.6).

A histogram for both arrays served to depict the signal intensity distribution of both positive hits and background signals on the chip (Figure 3.7). Positive hits were lower in frequency and ranged in signal intensity from 5,000 - 64,000. In contrast, a high frequency of background signals were present on the array, which corresponded to signal intensities below 5000 (Figure 3.7). The histogram revealed that signal intensities from microarray #1 were present in the 6,000-16,000 range and the 32,000-64,000 range while microarray #2 had signals present in the 18,000-30,000 range. This was indicative of an overall lower signal intensity corresponding to positive hits obtained on array #2 (Figure 3.7).

Positive hits were further categorized based on their associated physiological function as provided by the Gene Ontology Database (www.geneontology.org). Based on the protein function distribution of the hits obtained, 33% of the proteins were associated with a role in cell growth, while the subsequent largest categories were associated with distinct yet overlapping processes of cell cycle regulation (21%) and hypertrophy (16%) (Figure 3.8). As such, most of the targets were associated with cell growth and/or proliferation, which complemented the role of p27 as a tumour suppressor. Furthermore, a number of targets were associated with a breast cancer role (12%), which has already been well-established to involve specific p27 regulation [78, 79] (Figure 3.8).
Figure 3.8

Protein Function Distribution

- Cell growth: 33%
- Cell Cycle: 21%
- Hypertrophy: 16%
- Breast Cancer: 12%
- Cell differentiation: 9%
- Cell Death: 5%
- Angiogenesis: 2%
- VSM proliferation: 2%

Figure 3.8 Protein function distribution for significant hits obtained.
3.3 Examining the protein targets: linking p27 to G2/M and inflammation

We hypothesized that non-CK2-α' partners of p27 may be involved in regulating the growth restraining activity of p27. Performing a protein interaction screen with a recombinant p27 probe served as a means to capture an array of possible novel targets of p27. Despite our initial basis for performing the protein screen, we did not restrict our investigation to only possible hypertrophic agents, but instead used these results as an opportunity to expand current knowledge of p27 function and regulation. Furthermore, since Z-score values do not correlate with the strength of the interaction, all targets obtained above a Z-score of two were considered equally significant.

It is well-established that p27 regulates the G1/S phase of the cell cycle where it interacts with and inhibits cyclinE1-Cdk2, resulting in cell cycle arrest. p27 expression is high during transition from G0/G1 and decreases steadily afterwards as the cell cycle progresses [59, 61]. Interestingly, the microarray screen revealed several interacting kinases (20%) that have specific roles in the G2/M transition phase of the cell cycle. Adult cardiomyocytes display a dual cell blockade, with 85% found in G0/G1 and 15% found in the G2/M phases [54]. Thus, p27 may mediate growth arrest at the G2/M phase of the cell cycle in various organs including the heart. In an effort to explore this novel role for p27, several protein kinases were selected for further investigation including:

1) CHK2 (CHK2 checkpoint homolog (S. pombe), CHEK2)
2) NEK6 (NIMA (never in mitosis gene a)-related kinase 6)
3) PLK1 (polo-like kinase 1 (Drosophila), PLK)
4) AURKA (aurora kinase A, AURA)
5) PBK (PDZ binding kinase, TOPK)

These proteins participate in regulating the centrosome cycle and the formation of the mitotic spindle. They are activated downstream of the ATM (ataxia-telangiectasia mutated) kinase/ATR (ATM-RAD3 related) pathway in the presence of DNA damage (Figure 3.9) [107-111]. Notably, activation of CHK2 has been observed in end-stage human heart failure [112].
Figure 3.9 G2/M checkpoint and its players. In the presence of DNA damage, the ATM / ATR pathway is activated, leading to phosphorylation of Chk1 and Chk2 followed by Cdc25 family members. Phosphorylated Cdc25 is sequestered in the cytoplasm by 14-3-3 proteins, which prevents activation of cyclin B/Cdk1 and results in G2/M arrest. In the absence of DNA damage, Cdc25 is activated by Plk1 and Aurora A (AURKA). IKKα also regulates M phase through phosphorylation and activation of Aurora A [109].
NEK6 is of interest since it is highly expressed specifically in the heart and skeletal muscle [113]. Inhibition of Nek6 function, either by expression of an inactive mutant or by siRNA knockdown, induces mitotic arrest [114]. As such, p27 may possibly inhibit NEK6 and cause G2/M arrest of the cell cycle. NEK6 has also been implicated as a possible activator of NF-κB and thus may serve a role in the inflammatory NF-κB pathway [115] (Figure 3.9). Reportedly, the regulation of G2/M is under control of the NF-κB pathway as NF-κB serves as a transcription factor for several G2-M specific genes, including cyclin B1, cyclin B2, PLK1, and cdc25B. Selective inhibition of NF-κB by ERK 5 during the G2–M phase substantially delays mitotic entry [116]. In addition, siRNA-mediated knockdown of IKKα, a NF-κB activator and microarray p27 target, leads to accumulation of cervical cancer cells in the G2/M phase of the cell cycle by modulating Aurora A phosphorylation and activation [109] (Figure 3.9). Based on these findings, there are multiple pathways and possible downstream targets that may interact with p27 to cause G2/M cell cycle arrest.

Alternative to its role in G2/M, the microarray target IKKα is involved in several other important physiological processes. IKKα is a serine/threonine protein kinase that exists as a component of a cytokine-activated protein complex, IKK, along with the catalytic subunit IKKβ, and a regulatory subunit IKKγ (NEMO). This cytoplasmic complex is responsible for directly activating NF-κB, an essential transcription factor involved in inflammation, cell cycle regulation and numerous cardiac pathologies [117].

IKKα KO mice develop to term but die within hours after birth [17, 118]. The cause of death was attributed to a cardiovascular malfunction upon observance of mutant placentae severely congested with bulging vessels and blood sinuses, though the cardiac muscle itself was morphologically normal [118]. Systemic IKKα KO mice exhibit several physical abnormalities including a lack of extremities (limbs, tails and ears) along with severe craniofacial deformities. These animals have a smooth, taut, shiny skin and an epidermis that was <5-10 fold thicker due to excessive proliferation of the basal layer when compared to wild-type controls, thus implicating IKKα in epidermal differentiation [17, 118].

In contrast, the second catalytic subunit of IKK, IKKβ, displayed very different KO phenotypes in mice with embryos dying at embryonic day 12.5-14.5 as a result of massive liver apoptosis
Identical phenotypes of embryonic lethality and severe liver degeneration were reported for KOs of NF-κB components p65/RelA [121] and in p65 and p50 double KOs [122]. Both RelA-/- and IKKβ-/- embryonic fibroblasts displayed an increased sensitivity to TNF-α-induced apoptosis. Hence, NF-κB activation is absolutely required to protect hepatocytes from TNF-α-induced apoptosis [119-121].

Interestingly, IKKα-/- embryonic fibroblasts, treated with TNF-α, IL-1 or LPS, revealed normal stimulation of IKK activity. Furthermore, IKKα KO mice revealed that IKKα was not required for classical NF-κB activation to proinflammatory signals. In contrast, IKKβ-deficient embryonic fibroblasts exhibited little IKK or NF-κB activation in response to inflammatory cytokines TNF-α or IL-1 compared to wildtype [119, 120]. Differences in KO phenotype reveal that IKKβ cannot compensate for the lack of IKKα in IKKα-/- mice, but can effectively substitute for IKKα in activating NF-κB in response to inflammatory stimuli.

More importantly, in failing human hearts, the enhanced activity of IKKβ in particular was seen [8]. IKKβ activation of NF-κB was demonstrated downstream of LPS stimulation of cultured neonatal and acutely dissociated adult cardiac myocytes [14]. Thus, the significance of IKKβ specifically in heart inflammation was shown. In addition to inflammation, studies have shown that patients with HF have significantly higher levels of active NF-κB and that its inhibition prevents cardiac hypertrophy [6, 7]. The importance of IKKβ in cardiac growth was specifically demonstrated when a dominant-negative IKKβ mutant blocked hypertrophy in cardiomyocytes [43]. Collectively, distinct roles for each of the IKKα and IKKβ components can be seen.

Despite having separate functions, IKKα and IKKβ, share highly homologous sequences. They are of comparable size with IKKβ having a slightly longer length of 756 amino acids (87 kDa) compared with the 745 amino acid length (85 kDa) for IKKα (Figure 3.10). In addition, both proteins share a high degree of sequence homology, 52% sequence identity (the extent to which two nucleotide or amino acid sequences are invariant) and >70% sequence similarity (the extent to which nucleotide or protein sequences are related) [17]. Their structure consists of a single NH2-terminal catalytic domain, a centrally positioned leucine zipper (LZ) motif through which they heterodimerize, and COOH-terminal helix-loop-helix (HLH) domain that may function to regulate the catalytic activity of the IKK complex [123] (Figure 3.10).
**Figure 3.10** Schematic diagram of IKK and their putative functional and structural motifs. CC, coiled coil; Helix, a-helix; HLH, helix-loop-helix; LZ, leucine zipper; ZF, zinc finger [11].
The different physiological functions for IKKα and IKKβ, were attributed to differences in their HLH domains [123]. Binding of IKKβ (and probably that of IKKα) to the regulatory IKK component NEMO (IKKγ) is conferred by a C-terminal decapeptide motif termed the NEMO binding domain (NBD) that recognizes amino acid residues, 135-235, located in the N-terminal coiled-coil (CC) motif of IKKγ. IKKγ is only 48 kDa and is predominantly helical with large stretches of CC structure, including an LZ motif and zinc finger (ZF) domain at the C-terminus (Figure 3.10).

Based on the strikingly similar structures of IKKα and IKKβ, as well as the well-established importance of IKKβ in activating NF-κB particularly in the heart, the target IKKβ was also included in the investigation. This was further justified based on our results that 50% (5 out of 10) known p27 interacting proteins failed to appear as significant hits from the screen.

Our laboratory has previously demonstrated that p27 exhibits an anti-hypertrophic role through its interaction with CK2-α', a cytoplasmic protein kinase [67]. Identifying that p27 inhibits the IKK/NF-κB pathway may provide an alternate mechanism through which p27 facilitates its anti-hypertrophic role. Furthermore, establishing a role for p27 regulation of IKK would provide a novel link between both the inflammatory and cell cycle regulating pathways. Thus, the microarray protein IKKα and its catalytic partner IKKβ pose as very interesting targets due to their involvement in all three processes, namely G2/M regulation, hypertrophy, and inflammation.

Amongst the remaining kinases obtained from the screen, MAP2K2 (MEK2) [124] and the protein kinase GSK3β [106] have also an established role in cardiac hypertrophy. Hypertrophic growth is reduced by overexpression of GSK3β in cultured cardiomyocytes and in the hearts of transgenic mice [106]. Recently, GSK3β has been shown to interact with p27 and phosphorylate p27 on two C-terminal serine residues leading to its stabilization [125]. As such, this kinase served as a positive control for our experiments.

Based on the analysis of these available experimental data, we chose to pursue 11 out of the 35 targets obtained from the microarray screen for further preliminary experiments (Figure 3.11).
**Figure 3.11**

Eleven selected microarray targets and their putative functions. Each target was selected based on their roles in regulating the G2/M transition phase, hypertrophy and inflammation. Note that the microarray target and NF-κB activator IKKα has defined roles in all three physiological processes. Arrows link the specific targets obtained on the chip to their biological roles.
3.4 Cloning and ectopic expression of recombinant p27 and target proteins in mammalian cells

Eleven protein targets were selected for further investigation and were cloned into lentiviral plasmids (with the exception of IKKα/β, which will be addressed shortly). All cDNAs were cloned with a C-terminal V5 tag to facilitate their detection in both Western blot and Co-IP assays. These lentiviral plasmids were transfected into HEK293FT cells to produce lentiviruses which were then collected and transduced into various cell lines to obtain ectopic mammalian protein expression. Interestingly, in the mouse and human fibroblast cell lines, protein production of the nine lentivirally cloned targets were only at a 1 in 9 (11%) success rate while the GP2 293 human embryonic kidney cells and the HT1080 fibrosarcoma cells yielded a 78% and a 100% success rate respectively (Table 3.1). Ectopic protein levels in the HT1080 cell line were subject to variation however, as there was an inconsistent production of the target proteins with an average of only 6/9 (67%).

To obtain sufficiently reproducible expression of ectopic proteins, the lentiviral plasmids were used to drive protein expression rather than using the lentiviruses themselves. Lentiviral plasmids were transfected at a concentration of 2.5 µg into both GP2 293 and HT1080 cell lines to test for protein production. Using plasmids to drive protein expression rendered the tedious virus production dispensable. The other cell lines were not used for plasmid expression testing due to previously established results showing poor transfection efficiencies (data not shown). Ectopic protein expression was assessed by immunoblot analysis employing the anti-V5 antibody (Figure 3.12A). We found that all transfected plasmids were productive for protein production in GP2 293 cells. In contrast, expression of ectopic proteins was virtually absent in HT1080 cells (Table 3.1).

IKKα and IKKβ human cDNAs were purchased as mammalian expression vectors containing an N-terminal FLAG tag to facilitate protein detection. Both the IKK mammalian expression plasmids were functional only within the GP2 293 cell line as analyzed by immunoblotting using an anti-FLAG antibody (Figure 3.12A).
The lentiviral construct, pS1-p27, cloned with an N-terminal His tag, was also transfected into HEK293FT producer cells followed by viral transduction into various cell lines to obtain protein expression. This particular lentiviral construct had a different cloning site compared to the other target clones and had failed to produce the viral p27 protein in all the cell lines tested. Sequencing of this construct identified a mutation present in the 3’LTR (long terminal repeat) region of the lentiviral vector (data not shown). The LTRs flank the functional genes and facilitate the integration of viral DNA into the host genome to allow for transcription and eventual protein production. Use of the lentiviral plasmid for direct mammalian protein production avoided the viral DNA integration step and allowed for successful p27 protein production to occur in the GP2 293 cell line. When examining protein expression in each cell line and comparing either the viral transduction or plasmid transfection methods, we observed that the most reliable means to produce the proteins for both p27 and the targets was to drive protein expression using the lentiviral plasmids transfected in GP2 293 cells.

Table 3.1 Expression of recombinant proteins in mammalian cells. Ratios of success rate for target protein expression are shown and are categorized based on whether lentiviruses or the lentiviral plasmids themselves were used to drive protein expression. Highlighted entries correspond to successful expression of proteins produced directly from lentiviral plasmids. **Note:** BJ and NIH 3T3 cells were not transfected with plasmids due to previously established results showing low transfection efficiency in these two cell lines.
**Figure 3.12** Recombinant p27 and target proteins interact *in vitro*. Ectopically expressed p27 generated from pCMV5-p27 (no tags) was incubated with V5- or FLAG-conjugated protein microarray targets immobilized on Protein G agarose beads using anti-V5 or anti-FLAG antibodies as appropriate. Samples were immunoblotted with (A) anti-V5 and anti-FLAG to detect microarray targets and with (B) an antibody against p27, anti-p27. HC= heavy chain of antibody, LC=light chain of antibody.
3.5 Interaction of p27 and its targets as recombinant proteins *in vitro*

To this point, we established the ectopic expression of the putative targets in human GP2 293 cells (transfection efficiency >90%; data not shown). Next, Co-IP assays were performed in an effort to critically evaluate the interactions that were obtained from the microarray results. An immunoblot of the assay verified that an interaction between the target proteins and p27 occurred (Figure 3.12B). Thus, the interaction between p27 and the selected protein targets were replicated in an *in vitro* setting using both microarray and Co-IP assay techniques.

When performing an immunoprecipitation, the buffer environment (i.e. pH, ionic strength, detergent identity and/or concentration) is very critical for protein interaction. Several buffers were tested including a HEPES lysis buffer, NETN lysis buffer, microarray probing buffer, and RIPA buffer using both 150 mM and 250 mM NaCl concentrations to determine whether a higher salt concentration can strengthen the protein interaction. The Co-IP was successful only in RIPA buffer and at a salt concentration of 150 mM NaCl (data not shown).

3.6 p27 and IKKβ interact as recombinant proteins *in vitro* and endogenously as a cytoplasmic complex

As discussed in Section 3.3, IKKα/β served as particularly interesting targets due to their involvement in regulating several physiological processes including the G2/M phase, hypertrophy and inflammation. A possible role for p27 in attenuating one of the mentioned processes focused our efforts in characterizing the interaction between p27 and IKKα/β.

To identify the binding specificity between p27 and IKKα as well as p27 and IKKβ, we performed a Co-IP assay using truncated p27 mutants. The affinity assay revealed that both recombinant IKKα and recombinant IKKβ bound to the carboxy terminus of p27 independent of the cyclinE1/Cdk2 interaction site of p27 (Figure 3.13). C-terminal binding of p27 was also seen with our recently established target of p27, cytoplasmic kinase CK2-α' [67]. To establish whether p27-IKK interaction occurred in the heart, a Co-IP using rat neonatal cardiomyocyte extract was performed.
Figure 3.13 IKKα and IKKβ bind to the C-terminus of p27. Ectopically expressed full-length p27 generated from pS2-p27 or p27 fragments was incubated with either FLAG-conjugated IKKα or IKKβ immobilized on agarose beads using an antibody against the FLAG tag. Samples were immunoblotted with anti-FLAG and anti-V5 to detect IKKα/β and p27 respectively. Note: The p27 expression constructs were cloned with C-terminal V5 tags, which differed from the p27 construct used in the Co-IP shown in Figure 3.12. HC= heavy chain of antibody, LC=light chain of antibody.
The Co-IP verified that p27 interacts with both IKKα and IKKβ as endogenous proteins in cardiomyocytes (Figure 3.14). Interestingly, the immunoprecipitation of IKKα was originally unsuccessful as indicated by the absence of both the heavy chain and light chain of anti-IKKα on the western. Further investigation revealed that rabbit IKKα antibody had a higher affinity for protein G rather than protein A agarose beads as opposed to rabbit IKKβ which displayed a higher affinity with protein A (data not shown).

Immunocytochemistry confirmed the co-localization of p27 and IKKβ in the cytoplasm of isolated rat neonatal cardiomyocytes (Figure 3.15). Unfortunately, the antibody against IKKα was not suited for immunocytochemistry as it falsely stained the sarcomeric structures of the cardiomyocytes. Thus, only the co-localization of IKKβ and p27 was confirmed.

**Figure 3.14**

![IP p27 IKKα IKKβ](image)

**Figure 3.14** p27 and IKKα/β interact in rat neonatal cardiomyocytes. Cardiomyocyte extracts were subjected to an IP with anti-p27 or anti-IKKβ immobilized on agarose beads. Samples were immunoblotted with IKKα or IKKβ.
**Figure 3.15**

IKKβ and p27 co-localize in the cytoplasm of cardiomyocytes. Isolated rat neonatal cardiomyocytes were co-stained with specific antibodies as indicated.

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*Figure 3.15* IKKβ and p27 co-localize in the cytoplasm of cardiomyocytes. Isolated rat neonatal cardiomyocytes were co-stained with specific antibodies as indicated.
3.7 p27 protein levels decline in response to LPS stimulation

To elucidate if p27 plays a role in the inflammatory pathway, Hela cells were stimulated with the well-established NF-κB activator and sepsis-inducer, LPS. LPS is a constituent of the outer membrane of gram-negative bacteria that triggers an inflammatory response by activating the classical pathway of NF-κB through the IKKβ component of IKK [14]. By activating NF-κB, LPS promotes the secretion of pro-inflammatory cytokines from macrophages, and many other cell types, such as cardiomyocytes.

Prior to LPS stimulation, Hela cells displayed both a cytoplasmic and nuclear fraction of p27 protein (Figure 3.16). Interestingly, p27 protein was seen to be concentrated within the euchromatic regions inside the nucleus. Within 30 minutes of LPS stimulation, p27 protein levels drastically declined in both the cytoplasmic and nuclear compartments of the cell. After one hour of LPS stimulation, cytoplasmic p27 protein levels were seen to rise. However, the nuclear p27 protein fraction remained very low to almost absent in cells (Figure 3.16).

3.8 Ectopic p27 protein expression blocks LPS-induced activation of NF-κB

In nonstimulated cells, NF-κB is sequestered in the cytoplasm by an inhibitory complex IκB, which masks the nuclear localization signal of NF-κB thereby inhibiting its nuclear uptake and transcriptional activity [11]. In the presence of LPS, IKKβ becomes activated and phosphorylates the inhibitory molecule IκB. This targets IκB for degradation, freeing NF-κB to translocate to the nucleus [11].

To assess if p27 could inhibit the NF-κB pathway, Hela cells containing lentivirally-mediated overexpression of p27 were stimulated with LPS. Prior to LPS exposure, both IκB and NF-κB were highly expressed in the cytoplasmic fraction of the cell (Figure 3.17). Upon 30 minutes of LPS stimulation, endogenous p27 and IκB protein levels were drastically decreased. As previously established [154], a marked increase in nuclear NF-κB–p65 protein content occurred, suggesting its translocation from the cytosol to the nucleus (Figure 3.17). When p27 was overexpressed in this cell line, IκB remained at high levels in the cytoplasm despite LPS stimulation. Furthermore, NF-κB was prevented from translocating to the nucleus and instead was restricted to the cytoplasmic fraction of the cell (Figure 3.17).
Figure 3.16 p27 protein expression declines in the presence of LPS. Hela cells were exposed to LPS (1 µg/ml) for 0, 10, 30 and 60 min and stained with an antibody against p27 (green) and with Hoechst 33342 (blue) for DNA.
Figure 3.17

Figure 3.17 p27 overexpression prevents IκB degradation and NF-κB translocation. Hela cells were transduced with lentivirus encoding p27 (pS2-p27) for 60 hrs prior to stimulation with LPS for 30 minutes. Cells were stained with Hoechst 33342 (blue) for DNA and with specific antibodies as indicated.
3.9 TAT-p27 attenuates the LPS inflammatory response in mice

LPS activates signaling pathways through toll-like receptor 4 (TLR4), leading to the expression of proinflammatory cytokines that have been implicated in myocardial dysfunction in murine models of endotoxin-induced sepsis [133, 155, 156]. To elucidate if p27 plays a role in inflammation in vivo, we treated 10-12 wk old adult male C57BL6/J mice with LPS followed by assessment of cardiac function by echocardiography.

Initially, a dose response curve was generated by administering 0, 2, 5, 10 and 20 mg/kg of LPS intraperitoneally in C57BL6/J mice (22-25g, n=3 per group). In accordance with previous studies modeling sepsis [154], LPS-challenged mice exhibited septic symptoms such as reduced mobility, conjunctivitis, fur ruffling and diarrhea. Echocardiographic measurements revealed LPS administration resulted in significant cardiac dysfunction with a reduction in FS by day 7 of 28% with 2 mg LPS, 30% with 5 mg LPS, 34% with 10 mg LPS and the greatest reduction in FS at day 3 of 37% with a 20 mg LPS dose when compared to saline-injected mice (Figure 3.18A). The administration of 20 mg of LPS however, resulted in a mortality of 100% for mice in this group by day 3. The body weight (BW), as an anticipated response to the infection [126], also underwent a significant decline of approximately 18% with 2 mg LPS at day 1 compared to saline controls. At day 3, a significant weight loss was seen of 21% with 5 mg of LPS, 19% in the 10 mg LPS, and 25% in the 20 mg LPS group compared to the saline controls. BW recovery occurred in the 2 mg group after day one and the surviving two groups, 5 mg and 10 mg LPS, after day 3. In addition, higher LPS doses correlated with a more gradual recovery of BW compared to the more rapid recovery at lower doses (Figure 3.18B). Based on these findings, we determined that the best LPS dose to administer would be 10 mg of LPS which results in both significant cardiac dysfunction as well as ensuring mice survival during the duration of the study in order to measure FS.

To investigate the effect of p27 on inflammation, LPS-treated mice (10 mg/kg body weight; n=5 per group) were IP injected after 24 hours with either 200 µl of recombinant TAT-p27 protein (8.5mg/kg) or 200 µl of saline as a control. We have previously demonstrated that TAT-p27 fusion protein inhibits cardiac hypertrophy in mice with a half-life of 6-8 hours in the heart [67]. Echocardiography was measured on days 1, 2, and 5.
**Figure 3.18**

**Saline or LPS (2, 5, 10, or 20 mg/kg)**

<table>
<thead>
<tr>
<th>Day 0</th>
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**A**

**Fractional Shortening**

- **saline**
- **2mg**
- **5mg**
- **10mg**
- **20mg**

**Time (days)**

- 0
- 1
- 3
- 7
Figure 3.18 Higher LPS doses correlate with a greater cardiac dysfunction and reduction in body weight. Higher LPS dose correlates with a greater reduction in (A) FS and (B) BW. Data are means ± SEM, n=3. *p<0.05, **p<0.01, ***p<0.001 versus saline.
LPS administration resulted in significant cardiac dysfunction with a reduction in FS of 30% by day 5 in saline-injected mice compared to baseline levels at day 0 (Figure 3.19A). In contrast, administration of TAT-p27 significantly improved left ventricular (LV) function in mice as measured by echocardiography at 5 days post LPS injection. TAT-p27-treated mice displayed an improved FS of 33% when compared to saline controls (Figure 3.19A). These findings indicate that TAT-p27 treatment is able to significantly attenuate the LPS response and improve cardiac function. This difference in fractional shortening of the LV between both treatment groups is clearly visualized in the M-mode tracings (Figure 3.19B).

Echocardiographic measurements of EDD and ESD did not significantly differ between both TAT-p27 treated mice and saline controls (Table 3.2). This indicated that a significant dilation of the LV did not occur in both treatment groups. The heart rate (HR) decreased after LPS injection and recovered by day 2 and 5 with no significant difference in both treatment groups (Table 3.2). Weight loss between both TAT-p27 and saline treated mice did not significantly differ. Both groups consistently displayed a similar weight loss trend with a weight decline of 16% by day 2 followed by a period of recovery in both groups after day 2 (Figure 3.20A). Similarly, the heart weight (HW)/ BW ratios of both treatment groups did not significantly differ (Figure 3.20B).

Based on the significant attenuation of LPS-induced cardiac dysfunction by TAT-p27, we also investigated if TAT-p27 treatment would increase the survival of LPS-induced septic mice. Based on the dose response curve (Figure 3.18A), we determined that a dose of 30 mg/kg would be appropriate to assess survival. Treatment was administered 6 hrs after LPS injection based on the first signs of visible distress. Treatment consisted of 200 µl of recombinant TAT-conjugated p27 protein (8.5 mg/kg, n=15) or 200 µl of either saline (n=15) or TAT-LacZ (8.5 mg/kg, n=5) as controls. Interestingly, TAT-p27 treated mice displayed a distinct survival advantage with 87% survival compared to 6% survival in the saline group and 0% survival in the LacZ treated mice at day one post injection of LPS and treatment (Figure 3.21). The TAT-p27 treated mice remained at 53% survival for days 2 and 3 while the saline treated mice remained at a 6% survival (Figure 3.21). Mice that died exhibited signs of disseminated intravascular coagulation (DIC) as evidenced by visible respiratory distress and hemorrhaging from blood vessels inside the body as well as through external orifices such as the nose and ears.
Figure 3.19

LPS (10 mg/kg) or Saline

Day 0 | Day 1 | Day 2 | Day 5
--- | --- | --- | ---
Echo

200 µl TAT-p27 (8.5 mg/kg)

A Fractional Shortening

FS [%]

day 0 | day 1 | day 2 | day 5
--- | --- | --- | ---

Saline | TAT-p27
Figure 3.19 TAT-p27 improves cardiac left ventricular cardiac function. LPS was administered at a dose of 10 mg/kg body weight followed by IP injection after 24 hours of either 200 µl of TAT-p27 (8.5 mg/kg) or 200 µl of saline as a control. (A) FS was improved in TAT-p27 treated mice compared to the saline controls in LPS challenged mice. Data are means ± SEM, n=10. ***p<0.001 versus day 0 (baseline). ##p<0.01. ###p<0.001. (B) Representative M-mode echocardiograms in saline or TAT-p27 treated mice.
Table 3.2

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 5</th>
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<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>TAT-p27</td>
<td>Saline</td>
<td>TAT-p27</td>
</tr>
<tr>
<td>EDD (mm)</td>
<td>2.94 ± 0.08</td>
<td>3.02 ± 0.09</td>
<td>3.22 ± 0.16</td>
<td>3.22 ± 0.11</td>
</tr>
<tr>
<td>ESD (mm)</td>
<td>1.59 ± 0.04</td>
<td>1.63 ± 0.05</td>
<td>2.12 ± 0.11</td>
<td>2.08 ± 0.08</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>511 ± 19</td>
<td>494 ± 18</td>
<td>455 ± 19</td>
<td>456 ± 12</td>
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</tbody>
</table>

Table 3.2 Echocardiographic measurements of end-diastolic diameter, end-systolic diameter, and heart rate. Data obtained from transthoracic M-mode tracings at baseline (Day 0), LPS injection (Day 1) and after saline or TAT-p27 treatment (Day 2 and 5). EDD, end-diastolic diameter; ESD, end-systolic diameter; HR, heart rate (beats per minute, bpm). Data are means ± SEM, n=10.
Figure 3.20 TAT-p27 treatment does not affect weight loss or heart weight. (A) BW of TAT-p27 treated mice and saline controls followed a similar trend of decreasing post LPS and increasing after day 2. Treatment groups did not significantly differ. Data are means ± SEM, n=10. (B) HW/BW ratios of both TAT-p27 (n=7) and saline (n=8) treatment groups did not significantly differ. Data are means ± SEM.
Figure 3.21

*Figure 3.21*: TAT-p27 offers a survival advantage in LPS-challenged mice. LPS was administered at a dose of 30 mg/kg body weight followed by IP injection after 6 hours of either 200 µl of recombinant TAT-conjugated p27 protein (8.5 mg/kg, n=15), or 200 µl of either saline (n=15) or TAT-LacZ (8.5 mg/kg, n=5) as controls. p<0.05.
Chapter 4  Discussion

HF remains as the number one cause of morbidity and mortality in North America. Further advances in therapy however, will be critically dependent on an improved understanding of the mechanisms underlying its pathophysiology. The cell cycle inhibitor p27 plays an important role in tumourigenesis and the regulation of cardiomyopathy. Within cardiomyocytes, ectopic expression of p27 has been shown to abrogate the development of cardiac hypertrophy. In the presence of Ang II, phosphorylation and turnover of p27 in hypertrophied cardiomyocytes is initiated by CK2-α'. The silencing of CK2-α’ abolished cardiac hypertrophy, while overexpression of siRNA resistant CK2-α' or the silencing of p27 induced cardiomyocyte hypertrophic growth in the absence of Ang II stimulation. The dominant-negative form of CK2-α' however, failed to inhibit hypertrophy in p27−/− mice [67]. Therefore we surmised that the growth restraining activity of p27 may be through interaction with non-CK2-α' proteins.

4.1 Assessment of bacterially generated recombinant p27 protein as a probe for a protein microarray

To identity novel targets of p27, we screened a human protein microarray using a recombinant p27 probe synthesized with a C-terminal V5 epitope tag. The use of an antibody against a specific tag rather than the protein itself minimized any nonspecific interactions that may arise as a result of its use. The p27 probe was synthesized in E. coli despite E. coli having different translational folding mechanisms which become less efficient with increasing protein size. However, due to its small 27 kDa size and its intrinsically unstructured nature [57], use of bacterial-synthesized p27 was validated. This also implied that p27 was not post-translationally modified since it was generated in a prokaryotic system. Thus commonly phosphorylated residues including Thr187, Ser83, Tyr88 and Tyr89, all of which influence p27 interaction with its target proteins [71], were not phosphorylated. The use of unmodified p27 could facilitate interactions with a wider range of proteins that may have been masked by its phosphorylation. However, the possibility that the lack of p27 phosphorylation may limit its interactions with certain proteins cannot be excluded. This scenario is less likely however, since it has been extensively shown that p27 phosphorylation largely serves to negatively influence protein...
binding [71]. One must also take into consideration that the spotted proteins on the chip were produced in Sf6 insect cells and Chinese hamster ovarian (CHO) cells, both of which are capable of performing post-translational modifications of their proteins. This fact along with the knowledge that 20% of the spotted proteins expressed on the chip were non-full length proteins could also impact the binding of p27 to certain targets.

4.2 ELISA assay as a surrogate technique to the microarray

To ensure the functionality of p27 as a probe within the experimental conditions of the chip, an indirect ELISA was performed. This confirmed that our p27 probe was soluble in the probing buffers, and detectable by the V5 antibody both in the native confirmation of p27 and at the probe concentration recommended for use on the chip. To mimic p27 binding to a molecule as opposed to the surface of a plate, a sandwich ELISA was performed using bound p27 antibody prior to p27 protein incubation. This method generally serves to increase the sensitivity of the assay when compared to an indirect ELISA. Absorbance values obtained were similar to the indirect ELISA, providing evidence that the recombinant p27 protein within its native conformation was functional in its binding capacity. However, increased sensitivity provided by a sandwich ELISA was not observed. This result may be a reflection of the type of lysate used, as generally, when performing an ELISA with whole cell extracts, the lysate contains multiple proteins. The use of pre-bound antibody would then help to concentrate one specific protein and increase the absorbance value. As a consequence, the use of purified protein no longer supplied the advantage that a sandwich ELISA would provide, explaining at least partially why both assays yielded similar results.

It is also important to consider that the ELISA assays failed to account for the difference in affinity between the protein-protein interactions that happen on a chip and the protein-antibody interactions occurring in an ELISA. As such, despite displaying maximal absorbance at the recommended probe concentration for the chip and plateauing for higher protein concentrations, the ELISA did not exclude the possibility of using higher p27 protein concentrations to probe the array.
4.3 Limitations of the protein microarray analysis

Having confirmed the integrity and functionality of the recombinant p27 protein as a probe in the confines of our experimental protocol, we then proceeded to use p27 to sequentially probe two high-density protein microarrays. The use of two arrays allowed for comparison and confirmation of the protein interactions observed. This protein screen served to provide a list of candidates which can then be further investigated and confirmed using other experimental techniques.

In the microarray analysis, positive interactions were defined as statistically significant when the Z-score values were greater than two which correlated with a 97% confidence level. 35 protein targets were identified as statistically significant hits, which mainly consisted of protein kinases (27 out of 35 from 8222 spotted proteins (76%), 27/477 total kinases (6%)). As p27 is a kinase inhibitor with limited promiscuity, obtaining mostly kinase interactions and having a confined number of hits compared to the total number of kinases and other proteins present on the array, added further validity to the results obtained.

Interestingly, when observing interactions with a Z-score of three or higher (23 hits, CI of 99%), zinc binding alcohol dehydrogenase domain containing 2 (ZADH2) was the sole non-kinase target obtained. As in most protein-protein interaction studies, there is always an expected proportion of false positives among the declared significant results. A complete absence of false positives would come at the cost of a higher percentage of false negatives [127]. ZADH2 is well known for producing false positive results from other interaction assays as it tends to display promiscuous binding with proteins that it might not necessarily interact with in vivo. p27 interaction with ZADH2 however, did imply that other false positives might be present within our target set. False positive interactions might be due to factors such as improper folding of full-length or partially full-length proteins, thus exposing normally hidden domains which may be conducive to p27 binding. In addition, an interaction with p27 on the chip may be absent in vivo due to differing subcellular localization. As such, each protein hit was further analyzed based on their physiological function and was pursued if identified as a promising target of p27.
The microarray employed in the present study contained at least 10 known p27 interacting kinases, which we expected to give rise to statistically significant interactions. Only 5 out of the 10 (50%) kinases, namely GSK3β, CK2-α', Cdk2, Pim-1, and cdc2, were identified as significant hits. In depth analysis of the microarray results revealed that the p27 probe failed to exhibit an interaction with certain kinases that were previously shown to do so. We classified the lack of p27 binding to these targets to be false negative interactions present in the microarray analysis. Unfortunately, we obtained five false negative interactions (out of 10 known p27 targets), namely the cell cycle regulators Cdk4, Cdk6, CyclinE1 and CyclinE2 as well as the Src-family tyrosine kinase Lyn. Interestingly, all five proteins that failed to bind to p27 have been reported to interact with the N-terminus of p27 [57]. In contrast, out of the five known p27 interacting partners that bound to the p27 probe, only two proteins (cdc2 and Cdk2) bind to the N-terminus of p27. The remaining kinases, with the exception of PIM-1 whose binding specificity remains to be characterized, bind to the C-terminus of p27. As such, there was no clear distinction between targets failing to bind to either the amino- or carboxy-terminus of p27. Therefore, possible interference of either the N-terminal His tag or the C-terminal V5 epitope for p27 binding to these targets cannot be ignored.

Obtaining false negatives in a protein interaction assay was not unexpected; however, it provided evidence that there may be proteins present on the chip which failed to interact with p27. False negatives (Z-score value <2) may arise due to the possibility of having an insufficient amount of protein spotted on the array. This may prevent significant p27 binding, thus producing a lower signal that corresponds to a negative hit. Unfortunately, information pertaining to the amount of protein spotted on the chip (as determined by relative fluorescent units using an antibody against either the GST or His tag) was absent for over 600 proteins (out of 8222 proteins) including the majority of protein kinases. As such, an analysis on signal intensity and amount of protein could not be performed. Therefore, it could not be determined whether a low amount of spotted protein may have contributed to a false negative result when compared to the positive hits. Furthermore, Z-score values do not correlate with the strength of the interaction since they are measure of how much antibody is bound to a spot on the array. Therefore, differentiating between a strong or weak interaction as a means to explain false negative results was not possible within this experimental means.
It is possible that a protein on the array may have only one spot that is functional for binding to the probe. As a result, an interaction with p27 would be considered insignificant since the statistical analysis takes into account the signal intensity arising from both protein spots. When examining a scatter plot of signal intensity of protein spot A vs. B (Figure 3.6), it allowed us to identify proteins that, for example, had a high signal intensity for protein spot A while the second protein spot B, present in far less an amount, would result in a readout of very low intensity. These outlier proteins were identified and also investigated as possible targets of p27.

One must also take into account that the microarray presented an artificial environment for protein interactions. Thus, false negative hits may also be due to the absence of specific binding factors or conditions that may be required to mediate an interaction. Furthermore, the presence of non-full length or the nonfunctional conformation of proteins on the chip may also prevent proper binding of p27 to its target.

Based on these obstacles, increasing the probe concentration on the array may not necessarily reduce the false negative pool. Furthermore, a higher probe concentration would also yield a higher background, thus raising the overall signals arising from the chip without necessarily increasing how much p27 binds to a protein spot. As a consequence, signals arising from positive hits may not be substantially above the background signals, thus also increasing the risk of reducing the number of significant hits obtained. Further experimentation is necessary however, to properly assess if a beneficial outcome would result by increasing the probe concentration.

This experiment was also limited to the 8222 proteins spotted on the microarray. The human genome consists of an estimated 30,000 genes with some genes coding for more than one protein. Thus, not every possible gene product was investigated, making it likely that more protein interactors for p27 still remain. However, factors such as cost and current protein microarray technology place restrictions on the study of every single protein. Microarrays are still in their infancy and technical challenges associated with producing functional protein chips still remain. Even after performing this experiment, in the span of a few months, Invitrogen currently markets an array with 9483 protein spots. As such, time will allow for more inclusive investigations to occur in the future.
Despite its limitations, microarrays allow for the ease of simultaneously screening thousands of proteins in one day. Moreover, having known targets of p27 present as hits, as well as obtaining a high proportion of protein kinases, suggests that the protein microarray run was successful in identifying p27 specific interactions. The consistency of our results and the validity of the targets acquired provide evidence that the Invitrogen Protoarray® Human Protein Microarray can be useful and reliable as an experimental tool to investigate protein-protein interactions.

4.4 Ectopic expression of target genes in mammalian cell lines

Eleven protein targets were selected for further investigation on the basis of their physiological roles in the G2/M phase of the cell cycle, hypertrophy or inflammation. To verify the chip interaction in vitro, each target was expressed in mammalian cells to facilitate a Co-IP assay. Target cDNAs were cloned into lentiviral vectors to enable the production of lentiviruses which can then be used to achieve stable expression of the gene of interest in both dividing and nondividing cells. The viruses however, yielded variable protein production in each cell line. Both BJ and NIH3T3 fibroblast cell lines had only one target express the protein, which may be due to their tendency to switch in between G₀ and G₁ phases. Cells present in G₁ or S/G₂/M are three to four more times transducible compared to cells arrested in G₀ [128]. However, such a low expression of ectopic proteins in lentiviral-transduced BJ and NIH3T3 was surprising since differentiated isolated cardiomyocytes are transducible. Serum withdrawal followed by its reintroduction prior to viral transduction may have served to improve these ratios. The variable nature of protein production between cell lines can be due to several other factors however, including the charge on the cellular membranes which could prevent efficient viral integration and the possibility of creating virus particles that were less efficient at protein production compared to the previous viruses generated between each experimental run.

These unanticipated results created the need for a reliable protein expression method in dividing mammalian cells. To accomplish this goal, lentiviral plasmids were directly used for transient protein production and were transfected into HEK and HT1080 cells. BJ and NIH3T3 cells were not transfected due to previous results showing low transfection efficiencies. However, HT1080
cells were sensitive to the cytotoxicity of the employed liposome formulation (lipofectamine) in the transfection mixture and were therefore omitted from the experiment.

4.5 Critical evaluation of the co-immunoprecipitation analysis

Once stable protein production was achieved with plasmids in GP2 293 cells, a Co-IP assay was performed. It must be noted that as opposed to bacterial-synthesized p27 used to probe the microarray, the protein-protein interaction was now investigated using ectopically expressed proteins from a mammalian system, which involved proper post-translational modifications of p27. As such, the interactions were verified using bait (p27) and prey (target proteins) that were more reflective of in vivo conditions.

In a Co-IP, the quality of the antibody often determines the validity and sensitivity of the results in the assay. An in vitro IP has the advantage of being able to utilize an antibody against a tag as opposed to the native protein itself. As such, high specificity with use of an anti-tag antibody can be achieved. In addition, the same tag and antibody can be used for investigating all target interactions, thus making the use of tagged proteins attractive. While the use of a tag is convenient, it raises some concerns regarding biological relevance since the tag itself may either obscure native interactions or introduce new and unnatural ones. This possibility was tested by incorporating a positive control, GSK3β, in our Co-IP assay (Figure 3.12). Our results indicated that p27 successfully bound to GSK3β, thus confirming that proper binding conditions were established and that interference of the C-terminally cloned V5 tag on GSK3β did not prevent its interaction with p27. This added confidence that the other protein targets included in the IP investigation were under conditions for optimal binding to p27.

Endogenous immunoprecipitation experiments reveal both direct and indirect interactions. Thus, positive results may indicate that two proteins interact directly or may interact via a bridging protein. Ectopic expression of proteins reduces the chance of detecting indirect interactions. It also facilitates the detection of weak protein interactions or interactions that may go undetected due to low endogenous proteins levels. However, like most interaction assays, transient interactions cannot be readily detected. Furthermore, ectopic expression of proteins may yield
false positive interactions that are not physiologically relevant. Forced interactions may also occur between proteins present in the same subcellular compartment, which is not applicable to endogenous proteins. Thus, using endogenous proteins avoids protein overexpression and tagging that can lead to artifacts.

All eleven p27-target interactions were successfully reproduced by Co-IP analysis including the constituents of the IKK complex, IKKα and IKKβ. Full length IKKα and IKKβ interacted with the carboxy-terminus of p27 independently from the N-terminal cyclin E–Cdk2 interaction site of p27. This C-terminal interaction was also seen with our recently established target of p27, CK2-α’ [67]. The majority of p27 cytoplasmic interacting factors have been established as interacting with the carboxy terminus of p27 [57]. As such, identifying that IKKα and IKKβ had a specific carboxy terminal interaction provided evidence that IKK and p27 may interact in the cytoplasm of the cell. This cytoplasmic interaction was further confirmed by immunocytochemistry revealing the colocalization of p27 and IKKβ in the cytoplasm of cardiomyocytes. This also indicated that p27 may play a role in the classical pathway of NF-κB activation which occurs primarily through IKKβ [14]. However, failure to visualize p27 and IKKα colocalization due to antibody issues makes p27 involvement in the classical pathway alone an inconclusive assumption.

4.6 Assessing p27 protein levels in response to LPS stimulation

The endotoxin LPS is a well known activator of the classical IKKβ/NF-κB pathway through interaction with the receptor TLR4. Activation of NF-κB mediates the production of pro-inflammatory cytokines present within sepsis [14]. Hela cells stimulated with LPS were used to assess the response of p27 in the inflammatory pathway. Our results indicated that within 30 minutes of LPS treatment, p27 protein levels were drastically reduced both within the cytoplasm and the nucleus of the cell. Interestingly, this length of time also corresponded to the decrease in IκB levels and translocation of NF-κB to the nucleus. Lentivirally-mediated overexpression of p27, on the other hand, prevented the degradation of IκB, which also corresponded to the
retention of NF-κB in the cytoplasm of the cell. Based on our results, p27 plays a role in LPS-induced inflammation possibly through direct inhibition of the NF-κB pathway.

To alleviate p27 inhibition, it is possible that previously established mechanisms whereby NF-κB promotes cell proliferation through regulating a key p27 transcription factor, may also occur in the myocardium. Studies have shown that LPS and bacterial infection inhibit p27 transcription factor, forkhead box O3A (FOXO3a) [152, 153], through IKK-mediated phosphorylation and resultant tethering of FOXO3a to 14-3-3 proteins in the cytoplasm. Furthermore, silencing FOXO3a reduced the amount of NF-κB inhibitor IκBα [139, 140]. Our findings indicate that p27 protein levels were still decreased after 1 hour of LPS treatment with near absent levels in the nucleus (Figure 3.16). This result is consistent with the mentioned studies as the prolonged decrease in p27 levels may be mediated through transcriptional regulation. Similarly, a second mechanism of inflammatory regulation of p27 was recently established through receptor-interacting protein 1 (RIP1, RIPK1), an essential signalling component of inflammation-induced NF-κB activation. Interestingly, RIP1 regulates p27 levels by an NF-κB-independent signal that also involves regulating the p27 promoter through the forkhead transcription factors [141].

LPS stimulation may also regulate the stability of p27 at a post-translational level. Given the absence of cytoplasmic and nuclear p27 protein after 30 minutes of LPS exposure, LPS may facilitate the downregulation of p27 by targeting p27 for proteasomal degradation. The mechanism of p27 degradation however, remains to be elucidated.

Another feedback mechanism was just recently described with Pim-1, a p27 interacting partner that was also identified as a target from our microarray screen. Pim-1 kinase was shown to promote cell progression and tumourigenesis by down-regulating p27 expression at both transcriptional and post-translational levels. Pim-1 binds and directly phosphorylates p27 at both T157 and T198 residues, resulting in p27 binding to 14-3-3 proteins thereby promoting its nuclear export and proteasome-dependent degradation. Furthermore, Pim-1 also suppressed p27 transcription through phosphorylation and inactivation of FOXO3a, which resulted in the transcriptional repression of the p27 gene [105].

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The exact mechanism of how p27 inhibits the activation of NF-κB remains unclear however. LPS stimulation results in the activation of both Myd88 dependent and Myd88-independent pathways. As such, inhibition of any number of proteins upstream of the NF-κB activation cascade could result in the inhibition of NF-κB activity. It is also possible that ectopic p27 protein may have saturated the proteasomal degradation pathway components (E1, E2 or E3 enzymes) that are activated upon LPS stimulation. As a result, IκB degradation may have been attenuated, which would have prevented NF-κB from translocating to the nucleus. Thus, further experimentation, such as measuring IKKβ kinase activity in the presence of p27 would be necessary to help identify the mechanism.

One central concern in this experiment was the use of lentiviral infection to mediate overexpression of p27 in the cells. Use of lentiviruses presented several disadvantages in this study. First of all, it resulted in a large accumulation of p27 protein in the nucleus of the cell due to its nuclear localization signal. It was important to obtain a greater expression of cytoplasmic protein, since this is where the main components of the NF-κB pathway reside. A lower viral titer would have allowed for a greater cytoplasmic fraction of p27 to exist. Furthermore, a p27 construct with a nuclear export signal would have been more optimal for this study. It is also plausible that p27 may interfere with the NF-κB pathway using an alternative mechanism other than through direct inhibition of IKKβ. As previously mentioned, a similar scenario was seen with the salicylamide compound IMD-0354 which significantly reduced LPS-induced TNF-α expression without inhibiting IKK [47].

A second concern associated with lentiviral use is that an infection of cells with viral particles disrupts the normal physiology of the cell, which may correspond to an interruption in the LPS-induced activation of NF-κB. As such, use of a lentiviral control coding for another protein would have narrowed down whether it was the presence of p27 in particular or if it was the result of a general viral infection that blocked the NF-κB pathway. The possibility that the viral infection alone disrupted NF-κB activation remains unlikely however, since NF-κB is also activated upon viral infections [18, 160]. Furthermore numerous NF-κB studies employ viral means to overexpress proteins to either block or activate the NF-κB pathway [14, 18].
4.7 TAT-p27 and IKK interaction in the heart

More than 75% of severe sepsis and septic shock cases result in cardiovascular collapse with associated myocardial dysfunction [129-130]. Attenuation of cardiac dysfunction has been shown to result in a significant beneficial effect for survival [156]. Therefore, developing effective approaches for preventing cardiac dysfunction during sepsis/septic shock has become a great interest in clinical and basic research [156].

Numerous studies have confirmed the transcription factor NF-κB as responsible for inducing the expression of proinflammatory cytokines that have been implicated as the major mediators of myocardial depression in sepsis [131, 132]. As such, inhibition of the IKK/NF-κB pathway may hold great promise for HF therapy. Pharmacological means to inhibit various parts of the canonical pathway are currently being investigated including pharmacologic inhibition of NF-κB activator, IKK. However, developing effective therapies will require more precise knowledge of the regulation of IKK and NF-κB in the myocardium. Our lab has already established that recombinant p27 protein is effective in preventing the development of cardiac hypertrophy [71]. The discovery that p27 can inhibit IKK and eventual NF-κB activation could provide a novel therapeutic approach for the treatment of HF or various conditions associated with inflammation.

To investigate if p27 plays a role in attenuating cardiac inflammation, LPS-challenged mice were treated with TAT-p27 and LV function was assessed by echocardiography. TAT-p27 treatment displayed a significant improvement in FS compared to saline controls. A lack of a significant difference in EDD and ESD between both treatment groups indicated that LV dilation was not associated with the decrease in systolic function. This finding was not surprising as the initial response seen in cardiac dysfunction is an alteration in contractility with dilation occurring as a later event [146].

The heart rate, on the other hand, was seen to decrease slightly as a result of sepsis but then increase in both treatment groups. This increase in heart rate was a normal physiologic response seen in septic patients to help increase cardiac output. However, even in the presence of high
cardiac output, echocardiographic studies consistently confirm decreased left ventricular systole in patients with sepsis [161].

The HW/BW ratio remained the same between both TAT-p27 and saline treatment groups. However, an increase in HW/BW as a result of cardiac dysfunction is typically seen after a greater time lapse than was undertaken in this study [67, 146]. The similar heart weights provided evidence that the observed cardiac dysfunction may result from more of a functional rather than structural change. Several studies currently attribute reduced myocardial contractility seen in sepsis to functional alterations over structural adaptations. Endotoxin and cytokines were shown to alter and suppress L-type calcium currents in isolated rat cardiomyocytes, which resulted in reduced systolic intracellular calcium concentration and diminished cell contraction [161]. Thus, p27 may attenuate the endotoxin LPS action through inhibiting cytokine production by NF-κB and aid in preserving cytosolic calcium levels necessary for myocardial contraction.

Finally, it is important to consider that myocardial depression could serve as a protective mechanism of the heart. Due to microcirculatory abnormalities, energy generation is impaired which can result in a decrease of heart function to reduce cellular energy expenditure. This physiological response can be seen in patients with ischemic heart disease to maintain myocardial integrity and viability [161]. Cellular changes resulting in diminished cardiac performance seen during animal hibernation have also been observed in septic animals [161]. Thus, further investigation into sepsis-induced myocardial depression is necessary before clinical applications can be pursued.

### 4.8 Implications of TAT-p27 effect on survival in LPS-injected mice

To further assess the role of p27 in inflammation, a survival study was performed on LPS injected mice treated with TAT-p27, saline or TAT-LacZ. Surprisingly, a large survival advantage was seen with the TAT-p27 group compared to both TAT-LacZ and saline treated controls. All the TAT-LacZ controls had died by day one, which could have been caused by TAT-LacZ inducing a stronger immune response than LPS alone. In addition, the molecular
weight of TAT-LacZ is 125 kDa, which is larger than the 27 kDa TAT-p27. This difference in size may have also contributed to a greater immune rejection resulting in the death of these mice. However, since the sample size was smaller (n=5) compared to the saline controls (n=15) and only 6% of the saline controls had survived, nothing conclusive could be drawn. The survival advantage obtained with TAT-p27 strongly suggests that p27 attenuates LPS-induced sepsis in these animals, adding further evidence for its possible role in the inflammatory pathway.

Clinical observations as well as pathological-anatomical studies have clearly shown that circulatory collapse lies at the centre of sepsis. Early events in LPS-induced sepsis are characterized by production of inflammatory cytokines (i.e. TNF-α, IL-1) which in turn activate secondary inflammatory mediators including a substantial amount of cytokines (Figure 4.1). Activation of platelets and increased production of pro-coagulants by endothelial cells results in DIC. DIC involves the formation of small blood clots inside the blood vessels throughout the body which consume all the available coagulation proteins and platelets and thus disrupts normal coagulation processes. As a result, capillary leaks and abnormal bleeding occur, which leads to hypotension and the compromising of blood flow to vital organs, resulting in multiple organ failure and death [142] (Figure 4.1).

Based on the above clinical evidence and the observance of DIC in our animals, death induced by severe sepsis in our model was a consequence of DIC and not necessarily HF. As such, the survival advantage seen with TAT-p27 implies that p27 activity is not restricted solely to the heart. Rather, a systemic role of p27 in counteracting inflammation probably exists. IP injection of biologically active proteins fused to the protein transduction domain from the HIV-TAT protein has been shown to be delivered to all tissues in mice, including the brain [104]. Thus, TAT-p27 does not only target the heart but can transduce any membrane via its TAT domain and act in any number of different organs. As such it is possible that the effect seen on the heart is a secondary effect of p27 action in the systemic circulation.
Figure 4.1 Events in LPS-induced early sepsis and the proinflammatory factors involved. Endotoxin (LPS) triggers production of proinflammatory cytokines (TNF-α, IL-1) and monocyte adherence to endothelial cells. TNF-α and IL-1 activate neutrophils and endothelial cell for increased adherence. Activated cells release secondary inflammatory mediators including cytokines. Activation of platelets and increased production of procoagulants by endothelial cells may trigger microthrombosis, and in most cases life-threatening DIC. Vessel dilation combined with cytokine effects, contribute to low peripheral resistance and oedema along with a capillary leak in wide-spread sepsis, or in some cases, internal bleeding [143].
For example, a loss of intravascular volume due to excessive third space loss results in a decrease in preload which lowers cardiac contractility. p27 attenuation of systemic inflammation may prevent blood loss through capillary leaks and hemorrhaging and thus maintain the pre-load to the heart, thereby improving myocardial function. According to the Frank-Starling law, the greater the blood volume entering the heart during diastole (end-diastolic volume), the greater the volume of blood ejected during systolic contraction (stroke volume). Thus, cardiac output (and a higher ejection fraction) can be increased in the TAT-p27 mice as a result of maintaining cardiac pre-load. As an increase in FS precedes an increase in the ejection fraction of the heart, TAT-p27 treated mice displayed an improvement in FS. Thus, whether p27 improves cardiac function directly at the level of the heart or as a result of its systemic action or even at both levels is currently unknown. However, we propose that the underlying mechanism remains the same; p27 regulates the transcriptional activity of NF-κB through inhibition of its activator IKK. This would result in lower levels of NF-κB-induced TNF-α and IL-1 protein production, which in turn would reduce secondary mediator release and ultimately avoid tissue ischemia due to preventing downstream coagulation processes. Based on our preliminary in vivo data however, a conclusive mechanism cannot be drawn without further experimentation.

Interestingly, one of the targets obtained from our microarray analysis included VEGFR2 (KDR, Flk-1). This particular VEGF receptor was shown to be an important determinant of sepsis morbidity and mortality. In macrophages, VEGF has been shown to be upregulated by the inflammatory mediator, LPS. Yano et al. demonstrated that mice pretreated with neutralizing antibodies against the VEGF vascular endothelial receptor, VEGFR2, prior to LPS stimulation, had significantly reduced mortality [144]. Based on these findings, TAT-p27 may bind VEGFR2 and prevent angiogenic cytokine VEGF activity thus providing a possible alternate mechanism whereby TAT-p27 may attenuate the LPS response. As such, TAT-p27 may inhibit both VEGFR2 and IKK pathways to counteract LPS effects. Despite not clearly defining the underlying mechanism, we have identified a potential role for p27 in regulating the inflammatory pathway, thus providing a novel link between both cell cycle regulation and inflammation.
4.9 Limitations associated with the \textit{in vivo} studies

There are several limitations associated with the \textit{in vivo} studies that need to be taken into consideration. Firstly, a small animal model was used that has a slightly different anatomy of coronary vessels and a higher heart rate of 500-800 beats/minute compared to the average 60-70 beats/minute in humans. Since this study consisted of assessing changes in both the circulatory and cardiac function, these small anatomical variations may deviate the results that would be seen in humans. Despite these differences however, the cardiovascular systems in humans and mice are highly similar and thus mice are considered an appropriate representative model [162].

Next, the experimental conditions themselves may have contributed to variability in the results obtained. Unavoidable stresses associated with the handling of mice, injections and the changing of cages could affect the normal physiological response of mice to both LPS and treatments. In addition, providing exact dosages of treatment adjusted to each body weight proved difficult. Both TAT-p27 and TAT-LacZ proteins were produced in the lab and quantified using BCA assays. These assays however, can produce variable results due to factors such as technical errors with pipetting and technological errors associated with use of the spectrophotometer. As such, the concentrations of TAT-p27 and TAT-LacZ proteins are approximate values as opposed to exact measurements. Furthermore, slight differences in body weight were adjusted to approximate weights to allow for greater feasibility in administering treatment. In addition to biological differences, these concerns might have contributed to the variability in FS seen among mice in response to LPS and treatment.

Finally, the measurement of cardiac function in the mouse is itself technically challenging due to the small size of the heart and its rapid rate. Despite its size however, transthoracic echocardiography has been used successfully to evaluate the transgenic mouse heart [163]. Unfortunately, echocardiographic measurements obtained can be influenced by several factors including the use of an anesthetic as well as the body temperature of the animal which both affect its heart rate. To control the body temperature, mice were placed on a pad when performing the echocardiography, however the possibility of such influences on the measurements cannot be eliminated. Limitations associated with machine used to perform the
echoes and the differences in training, method of collecting data, and data analysis are all factors that may influence the cardiac parameters obtained.

Despite these limitations however, we believe that this study offers important information on the attenuation of LPS-induced inflammation by the tumour suppressor p27. Furthermore, it provides a novel mechanism that can be further explored for therapeutic purposes for a variety of diseases.
Chapter 5  Conclusions

Growing evidence in recent years has established an important link between inflammatory processes and the development of HF. Proinflammatory cytokines such as TNFα, IL-1 and IL-6 have emerged as significant contributors to myocardial dysfunction after stress, injury or infection [145]. Study of transgenic mice genetically engineered for cardiac-restricted overexpression of TNFα demonstrated that chronic exposure to elevated levels of TNFα led to left ventricular chamber dilation and HF [146]. Central to cytokine production in the heart is the IKK/NF-κB pathway [147]. Consequently, a proper understanding of the signalling cascades involved in regulating NF-κB has great therapeutic potential.

In our search for novel targets of p27, we identified the NF-κB activator IKK as an interacting partner of p27. IKKα/β interaction with p27 was confirmed in vitro based on microarray analysis, Co-IP, and immunocytochemistry. Furthermore, the binding specificity of both IKK components, IKKα and IKKβ, was mapped to the carboxy terminus of p27, independent of the N-terminal binding site of p27 for the cell cycle regulators, cyclins and Cdk5.

To assess a possible role for p27 in inflammation, mice were treated with an inflammatory stimulus, LPS, which activates cytokine production through the IKKβ/NF-κB pathway [14]. Our results show that TAT-p27 treatment significantly improved the left ventricular function in LPS mice compared to saline controls. This highlighted a possible role for p27 attenuation of cardiac inflammation. Furthermore, our preliminary data also indicated that TAT-p27 offered a distinct survival advantage in LPS-treated mice in comparison to both saline and LacZ controls. This finding implies that TAT-p27 has a broader systemic role in attenuating LPS-induced inflammation. In addition, previously established mechanisms of p27 transcriptional regulation by inflammatory processes including IKK, adds further evidence that p27 is involved in the inflammatory pathway. Therefore, we have evidence to support a novel role of p27 in counteracting inflammation both in the heart and at an overall systemic level. Based on our findings, we propose that the underlying mechanism involves p27 inhibition of the cytoplasmic complex IKK, which prevents NF-κB activation and translocation to the nucleus, thus attenuating the inflammatory LPS-induced response (Figure 5.1).
Figure 5.1 Proposed model of p27 and IKK interaction. In the presence of an inflammatory stimulus such as LPS, IKK will phosphorylate NF-κB inhibitor IκB thus targeting it for ubiquitination and proteasomal degradation and freeing NF-κB to translocate to the nucleus. In the presence of p27, the IKK complex is inhibited, primarily through IKKβ, which prevents IκB phosphorylation and inhibits NF-κB activation.
As such, p27 serves as a novel link between both the inflammatory pathway and cell cycle regulation.

Establishing p27 as a molecular link between inflammation and cell cycle regulation has numerous clinical implications. Strong associations between cancer and inflammation have been seen in colon cancer, gastric cancer with *Helicobacter pylori* infections, and hepatocellular carcinoma with viral hepatitis to name a few. These clinical manifestations are further supported by numerous evidence employing mouse models showing inflammation as a critical component of tumour progression [142, 144, 145]. In the heart, diseases such as HF are associated with maladaptive hypertrophic growth and inflammation. In addition, the phenomenon of restenosis after angioplasty of a coronary artery is associated with both vascular inflammation and intimal hyperplasia following the use of stents [151]. Thus, the prevalence of pathological conditions associated with both inflammation and cell proliferative pathways stress the importance of therapeutic targets of both processes. The tumour suppressor properties of p27 are well-established. However, the novel role of p27 in regulating inflammatory pathways makes p27 an even more attractive therapeutic target due to its wider application. The discovery that p27 can inhibit IKK and consequently, the NF-κB pathway, provides a novel mechanism for the development of HF. Thus, p27 serves as a novel therapeutic target for treatment of various diseases or conditions associated with both inflammation and uncontrolled cell proliferation.
5.1 Future directions

Based on our findings, p27 plays a significant role in attenuating LPS-induced sepsis. Further lines of investigation are required however, to assess this novel function of p27.

1) *How does p27 offer a survival advantage?*

It is important to assess how TAT-p27 treatment of LPS-induced septic mice has a distinct survival advantage over the saline-treated counterparts. By measuring specific inflammatory parameters such as circulating TNF-α or IL-1 levels, nitric oxide production by macrophages, LPS-induced T-lymphocyte proliferation, and natural killer cell activity, we can narrow down the specific role that TAT-p27 may have in inflammation. Histological analysis of various tissues including the heart could identify if TAT-p27 treatment may serve to reduce the number of infiltrating macrophages or the extent of apoptosis as a result of LPS-induced inflammation. Furthermore, whether TAT-p27 treatment has a sustained protective effect past three days also remains to be experimentally determined. One can also investigate whether this effect is unique to p27, or whether other cell cycle regulators such as p21 could offer the same survival advantage.

2) *Determining the mechanism of p27 action.*

Based on our findings, p27 plays a significant role in attenuating LPS-induced inflammation, yet the mechanism underlying this pathway still remains unclear. Current experiments in the lab are aimed at determining if NF-κB is activated in both saline and TAT-p27 treated LPS-challenged mice using either the electrophoretic mobility shift assay or the oligonucleotide precipitation techniques. Whether p27 inhibits IKK in particular can be verified by measuring IKKα or IKKβ kinase activity in the presence of p27. Furthermore, identifying and mutating specific p27 binding residues to IKKβ would provide evidence of whether p27 inhibits NF-κB at the level of IKK as opposed to another mechanism.

Through the creation of a p27 construct with a mutation on its nuclear localization signal sequence, it could be more specifically determined whether cytoplasmic or nuclear levels of p27
are contributing to the inhibition of NF-κB. In addition, confirming p27 and KDR interaction would provide another mechanism for p27 attenuation of inflammation that can be explored.

The measurement of p27 levels in various organs and inflammatory cells after LPS challenge will also help to identify where p27 may be targeted and degraded during inflammation. This will also narrow down specific organs that TAT-p27 introduction may be beneficial and whether TAT-p27 may have any specific function in the circulatory system. It is also important to determine the time required for TAT-p27 diffusion into target organs, and the half-life of TAT-p27 in the organism.

3) What other processes are affected in p27-IKK interaction?
Once p27 interaction with IKK-NF-κB is established, it has implications for the role of p27 in regulating other NF-κB-linked processes such as hypertrophy and apoptosis, thus providing another mechanism for p27 action that remains to be explored. The use of transverse aortic banding as a model to induce cardiac hypertrophy can be used to assess if TAT-p27 treatment could prevent hypertrophy through NF-κB inhibition.

4) Protocol modifications
To properly assess the role of p27 in inflammation, we are currently employing a larger sample size of mice in our study. In addition, an alternate method of delivery of TAT-p27 from IP to IV injection is being pursued. Furthermore, the use of alternative controls other than LacZ should be taken into consideration, such as a scrambled peptide that is equivalent in molecular weight to TAT-p27, or the use of amino-terminal fragments of p27 which failed to bind to either IKKα or IKKβ. It also remains to be determined whether multiple injections of TAT-27 treatment will improve the fractional shortening and survival advantage in LPS-challenged mice over that seen when only a single dose of TAT-p27 was administered.
References


