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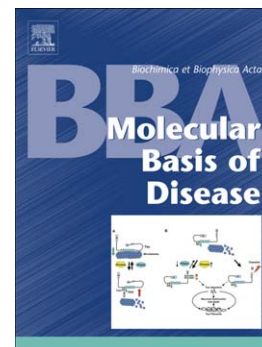
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**Activation of Cardiac Hypertrophic Signaling Pathways in a Transgenic Mouse with  
the Human *PRKAG2* Thr400Asn Mutation**

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## Summary

Human mutations in *PRKAG2*, the gene encoding the  $\gamma 2$  subunit of AMP activated protein kinase (AMPK), cause a glycogen storage cardiomyopathy. In a transgenic mouse with cardiac specific expression of the Thr400Asn mutation in *PRKAG2* ( $TG^{T400N}$ ), we previously reported initial cardiac hypertrophy (ages 2-8 weeks) followed by dilation and failure (ages 12-20 weeks). We sought to elucidate the molecular mechanisms of cardiac hypertrophy.  $TG^{T400N}$  mice showed significantly increased cardiac mass / body mass ratios up to ~ 3-fold beginning at age 2 weeks. Cardiac expression of ANP and BNP were ~2- and ~5-fold higher, respectively, in  $TG^{T400N}$  relative to wildtype (WT) mice at age 2 weeks. NF- $\kappa$ B activity and nuclear translocation of the p50 subunit were increased ~2 to 3-fold in  $TG^{T400N}$  hearts relative to WT during the hypertrophic phase. Phosphorylated Akt and p70S6K were elevated ~2-fold as early as age 2 weeks. To ascertain whether these changes in  $TG^{T400N}$  mice were a consequence of increased AMPK activity, we crossbred  $TG^{T400N}$  with  $TG^{\alpha 2DN}$  mice, which express a dominant negative, kinase dead mutant of the AMPK  $\alpha 2$  catalytic subunit and have low myocardial AMPK activity. Genetic reversal of AMPK overactivity led to a reduction in hypertrophy, nuclear translocation of NF- $\kappa$ B, phosphorylated Akt, and p70S6K. We conclude that inappropriate activation of AMPK secondary to the T400N *PRKAG2* mutation is associated with the early activation of NF- $\kappa$ B and Akt signaling pathway, which mediates cardiac hypertrophy.

## 1. Introduction

Mutations in the gene *PRKAG2*, encoding the  $\gamma 2$  subunit of the adenosine monophosphate-activated protein kinase (AMPK), cause an unusual glycogen storage cardiomyopathy in humans, characterized by cardiac glycogen overload, ventricular preexcitation (Wolff-Parkinson-White syndrome) and cardiac hypertrophy [1-3]. This recently recognized cardiomyopathy is often misdiagnosed as classical hypertrophic cardiomyopathy (HCM), which is caused by sarcomere protein mutations [4]. Cardiac hypertrophy occurs in up to 80% of individuals with *PRKAG2* mutations [5] and is usually progressive, evolving to left ventricular dysfunction and dilatation [3, 6]. Histopathology shows an absence of myofiber disarray and less fibrosis than is usually found in HCM [7], suggesting that the signaling pathways and molecular mechanisms underlying progression of cardiomyopathy secondary to *PRKAG2* mutations may be different from HCM. Our recent genotype-phenotype studies in mice have characterized the evolution of morphological changes of the left ventricle [4, 8].

In an earlier report [8], we constructed and characterized a transgenic mouse overexpressing human *PRKAG2* with the previously described [1] Thr400Asn (T400N) mutation (TG<sup>T400N</sup>). This mouse recapitulates the human phenotype, developing left ventricular hypertrophy at an early age which is maximal at age 8 weeks, progressing to left ventricular dilation and impaired contractility by age 12 weeks, and end-stage heart failure at age 20 weeks. The massive cardiac hypertrophy seen in this cardiomyopathy

secondary to *PRKAG2* mutations is not merely a reflection of excess glycogen. Direct measurements of glycogen content suggest that glycogen is responsible for only ~4% of the increase in cardiac mass in  $TG^{T400N}$  mice. However, the molecular pathways that contribute to hypertrophy have not been previously studied.

One of the cardinal features of heart failure of many different etiologies is left ventricular (LV) hypertrophy. Therapies that reduce mortality in patients with heart failure invariably produce favorable effects on the hypertrophy process and limit disease progression [9]. Therefore, understanding the molecular basis of hypertrophy in glycogen storage cardiomyopathies may be essential in the development of therapeutic strategies. The aims of this study were to determine the early molecular signaling events that are associated with cardiac hypertrophy, focusing on pathways known to be modulated by AMPK.

In the present study, we demonstrate in the  $TG^{T400N}$  murine model of glycogen storage cardiomyopathy that inappropriate activation of AMPK triggers hypertrophic signaling at a very early age with the activation of the Akt signaling pathway and the transcription factor, nuclear factor  $\kappa$ B (NF- $\kappa$ B). Genetic reversal of AMPK overactivity leads to a reduction in hypertrophy by reducing NF- $\kappa$ B nuclear translocation, phosphorylated Akt, and p70S6K.

## 2. Methods

### 2.1. Construction of $TG^{T400N}$ transgenic mice

The construction and initial characterization of the transgenic mouse with the human T400N mutation in *PRKAG2* ( $TG^{T400N}$ ) have been previously described [8]. Cardiac-specific expression of the transgene was achieved with the  $\alpha$ -myosin heavy chain promoter. Transgenic mice were produced in the FVB background and maintained as heterozygotes by breeding with wildtype (WT) FVB mice. FVB background  $TG^{\alpha 2DN}$  mice, which express a dominant negative, kinase dead mutant of the AMPK  $\alpha 2$  catalytic subunit and have low myocardial AMPK activity, have been previously described [10]. Double transgenic mice ( $TG^{T400N}/TG^{\alpha 2DN}$ ) were obtained by crossbreeding. Mice aged 2-20 weeks were used for the study. This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

### 2.2. Analysis of protein expression

Extraction of nuclear and whole cell protein fractions, immunoblotting, autoradiography, and densitometry were performed as described previously [11-13]. Protein levels were measured by the Bradford method (Bio-Rad). An equal amount (50  $\mu$ g)

of protein was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, protein was transferred to PVDF membranes (Amersham Biosciences). The membranes were then blocked in Tris-buffered saline Tween-20 (TBS-T; 10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) and 5% non-fat dry milk for 1 h, and subsequently washed and incubated with primary antibodies in TBS-T and 2% bovine serum albumin (BSA) at 4 °C overnight. The following antibodies and titers were used: Akt (1:1000 dilution, Cell Signaling, # 9272), phospho-Ser<sup>473</sup> Akt (1:1000 dilution, Cell Signaling, # 9271), phospho-Thr<sup>389</sup> p70S6K (1:1000 dilution, Cell Signaling, # 9205), phospho-Thr<sup>180</sup>/Tyr<sup>182</sup> p38 MAPK (1:1000 dilution, Cell Signaling, #9211), phospho-Thr<sup>202</sup>/Tyr<sup>204</sup> ERK1/2 (1:1000 dilution, Cell Signaling, #9101), phospho-Tyr<sup>705</sup> STAT3 (1:1000 dilution, Cell Signaling, # 9131), calcineurin A (1:500 dilution, Chemicon, #AB1695), NF-κB p50 (1:200 dilution, Santa Cruz, # sc-114), and NFATc3 (1:200 dilution, Santa Cruz, #sc-23814). After washing with TBS-T, membranes were incubated with anti-rabbit (1:10000 dilution, Amersham, #NA934V) or anti-goat (1:2000 dilution, Santa Cruz, sc-2020) horseradish peroxidase conjugated secondary antibody for 1 h. Signal was detected by chemiluminescence using the ECL detection system (Amersham). Gel staining with Coomassie Blue was used as an internal control for equal loading of protein. Quantification of bands on X-ray film was performed using Image J Software (NIH).

### 2.3. RNA isolation and real-time quantitative PCR (QPCR)

Total RNA was isolated from whole heart with TRIzol (Invitrogen). Reverse



transcriptase reactions were performed as described [11, 13], using the Superscript III First-Strand Kit (Invitrogen) for first-strand cDNA synthesis. Primers for real-time quantitative PCR (QPCR) analysis were designed using published sequence information, avoiding regions of homology with other genes, or were purchased as prevalidated sequences from a commercial source (Realtime Primers) (Table 1). For each gene, 10 ng of cDNA were analyzed on an ABI PRISM 7700 using Absolute SYBR Green ROX PCR Master Mix (Thermo Scientific). Fold-change analysis was based on normalizing to cyclophilin transcript levels in each sample. As an initial screen of expression of NF- $\kappa$ B related genes, a commercially available kit with QPCR primer assays on a 96-well plate (Mouse NF $\kappa$ B Signaling Pathway RT<sup>2</sup> Profiler™ PCR Array, SABiosciences) was interrogated according to the manufacturer's instructions. This array profiles the expression of 84 key genes related to NF $\kappa$ B-mediated signal transduction. Differences in expression identified by this array were validated by individual QPCR.

#### 2.4. NF- $\kappa$ B Binding Activity

Electrophoretic mobility shift assays (EMSA) were performed as described [12]. In brief, nuclear proteins were isolated and protein concentrations were measured using BSA as a standard by the Bradford method (Bio-Rad). Protein-DNA binding was carried out in a final volume of 20  $\mu$ l. Four  $\mu$ l of a 5X binding buffer (1X concentration of 5% glycerol, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 40 mM KCl, 40 mM Tris, pH 8.0) along with 800 ng of dIdC and 20  $\mu$ g nuclear proteins was added to each tube. For specific and nonspecific competitor

reactions, 100-fold excess of the appropriate unlabelled double-strand DNA was added: specific, AGTTGAGGGGACTTTCCCAGGC (NF- $\kappa$ B) and non-specific, CGCTTGATGAGTCAGCCGGAA (AP1) (complementary strand not shown; Promega). Two  $\mu$ l of  $^{32}$ P-labeled NF- $\kappa$ B probe (approximately 20,000 cpm/ng) was added to each reaction and incubated for 20 min at room temperature. Four  $\mu$ l of loading buffer (prepared with 0.85 ml 1X binding buffer and 0.15 g Ficoll 400 powder) was added to each reaction, and samples were then placed on ice until loading. Samples were resolved on a 4% (80:1 acrylamide:bis) acrylamide gel in 0.25X TBE buffer. The gel was then exposed to a storage phosphor cassette and analyzed with ImageQuant (Molecular Dynamics).

In addition, NF- $\kappa$ B binding to its consensus binding site was measured using a commercially available colorimetric assay (TransAM<sup>TM</sup> NF $\kappa$ B p50 DNA-binding ELISA kit, Active Motif) according to the manufacturer's instructions.

### 2.5. Statistical analysis

Results are expressed as mean  $\pm$  SE. Differences between WT and TG<sup>T400N</sup> mice at each age were compared by Student's *t* test, whereas differences among WT, TG<sup>T400N</sup>, and TG<sup>T400N</sup>/TG <sup>$\alpha$ 2DN</sup> mice were analyzed by ANOVA followed by *post hoc* Bonferroni correction for multiple comparisons. A *p* value of less than 0.05 was considered significant.

### 3. Results

#### 3.1. The *PRKAG2* T400N mutation causes cardiac hypertrophy

The *PRKAG2* T400N mutation resulted in cardiac hypertrophy in TG<sup>T400N</sup> mice as early as age 2 weeks, which was maximal at age 8 weeks. We previously showed a progressive increase in cardiac mass / body mass ratio in TG<sup>T400N</sup> mice relative to WT littermates from age 1 week, through 2 weeks, to 4 weeks [8]. At age 8 weeks, the cardiac mass / body mass ratio (mg/g) increased further in TG<sup>T400N</sup> mice to 19.4±0.9, relative to 4.1±0.1 in WT mice ( $p<0.01$ ,  $n=6$ /group), consistent with progressive cardiac hypertrophy. To confirm that morphologically evident cardiac hypertrophy was associated with induction of corresponding molecular markers of cardiac hypertrophy, we quantified ANP and BNP by QPCR at an early age ( $n=3$ /group). Cardiac hypertrophy in TG<sup>T400N</sup> mice was accompanied by the upregulation of cardiac ANP and BNP transcripts. There were significant increases in ANP and BNP in TG<sup>T400N</sup> hearts at age 2 weeks (Figs. 1A and 1B).

Because TG<sup>T400N</sup> cardiac myocytes are laden with glycogen [8], we determined the contribution of glycogen to the increased cardiac mass. In TG<sup>T400N</sup> mice, the average cardiac mass was increased 221±14% and 352±51% relative to WT littermates at ages 4 weeks and 8 weeks, respectively ( $n=5$ /group). However, the contributions of glycogen to increased cardiac mass at ages 4 weeks and 8 weeks were only 4.4% and 3.5%, respectively (Table 2). Therefore, most of the increased cardiac mass observed in TG<sup>T400N</sup>

mice appears not to be the direct result of glycogen accumulation, prompting us to examine signaling pathways regulated by AMPK and known to be associated with cardiac remodeling.

### *3.2. Early activation of the NF- $\kappa$ B and Akt signaling pathways contribute to cardiac hypertrophy in TG<sup>T400N</sup> mice*

To determine which hypertrophic signaling pathways are associated with cardiac hypertrophy in TG<sup>T400N</sup> mice, we focused on those pathways which are known or suspected to be direct or indirect targets of AMPK. Specifically, AMPK has been shown to activate nuclear factor  $\kappa$ B (NF- $\kappa$ B) [14, 15], p38 mitogen-activated protein kinase (p38 MAPK) [16-20], and Akt [21-26].

NF- $\kappa$ B is a transcription factor which regulates genes mediating inflammation and hypertrophy, and it is activated during oxidative stress. NF- $\kappa$ B activity as measured by EMSA was upregulated in TG<sup>T400N</sup> hearts from age 2 through 20 weeks (Fig. 2A). We confirmed elevation of NF- $\kappa$ B binding activity to its consensus binding site up to 2.7-fold in TG<sup>T400N</sup> hearts at ages 2, 8, and 20 weeks using a commercially available colorimetric assay (Fig. 2B). Since NF- $\kappa$ B activation involves the nuclear translocation of its subunits, we assessed the expression of the p50 subunit in nuclear protein extracts from TG<sup>T400N</sup> and WT hearts by immunoblot. Elevated levels of p50 were observed in the nucleus in TG<sup>T400N</sup> mice at ages 2 and 8 weeks (Fig. 2C).

To screen for changes in mRNA expression of a wide range of genes related to NF- $\kappa$ B mediated signal transduction, we performed QPCR using the Mouse NF $\kappa$ B Signaling Pathway RT<sup>2</sup> Profiler™ PCR Array (SABiosciences) that quantifies 84 genes. Genes showing upregulation of at least 2-fold in TG<sup>T400N</sup> relative to WT hearts on the array were further validated with individual QPCR in TG<sup>T400N</sup> and WT hearts at ages 2 and 8 weeks ( $n=3$ /group) (Table 3). Fas ligand (*FasL*) was upregulated at ages 2 and 8 weeks, whereas toll-like receptor 2 (*Tlr2*) and tumor necrosis factor (ligand) superfamily, member 14 (*Tnfsf14*) were upregulated only at age 8 weeks. All three genes are known to activate NF- $\kappa$ B and have been associated with cardiac remodeling [27-29]. Furthermore, interleukin 6 (*Il6*), upregulated at age 8 weeks, is an NF- $\kappa$ B responsive gene that is known to mediate cardiac hypertrophy [30, 31]. Interleukin 10 (*Il10*), upregulated at ages 2 and 8 weeks, is also involved in the NF- $\kappa$ B signal transduction pathway and has been shown to be associated with cardiac hypertrophy [32]. The finding that toll-like receptor 9 (*Tlr9*) is upregulated at age 8 weeks is intriguing because it impairs cardiac contractility [33], and TG<sup>T400N</sup> hearts develop progressive dysfunction beyond age 8 weeks [8]. In contrast, interferon gamma (*Ifng*), upregulated at 2 and 8 weeks, is known to inhibit cardiac hypertrophy [34], and may represent a compensatory effect in response to pathological hypertrophy in TG<sup>T400N</sup> hearts.

We next examined whether increased NF- $\kappa$ B activity in TG<sup>T400N</sup> hearts was associated with activation of hypertrophic signaling pathways. We observed that the Akt-

mTOR signaling pathway, which upregulates protein synthesis, was activated. We quantified the active, phosphorylated forms of Akt and another downstream signaling protein, p70S6K, by immunoblot. There were significant increases in phosphorylated Akt and p70S6K in TG<sup>T400N</sup> relative to WT hearts at ages 2 and 8 weeks ( $n=3$ /group) (Fig. 3). No difference in total Akt levels was observed.

### *3.3. The calcineurin and MAPK pathways do not contribute to cardiac hypertrophy in TG<sup>T400N</sup> mice*

We systematically analyzed other known mediators of cardiac hypertrophy [35, 36]. To assess whether activation of the calcineurin-NFAT signaling pathway is associated with hypertrophy in TG<sup>T400N</sup> hearts, we quantified calcineurin A by immunoblot ( $n=3$ /group). There were no increases in calcineurin A levels and cytoplasmic NFATc3 levels in TG<sup>T400N</sup> hearts at ages 2 and 8 weeks (Fig. 4). Instead, a significant decrease in calcineurin A was observed in TG<sup>T400N</sup> hearts at age 8 weeks, suggesting that activation of calcineurin-NFAT signaling pathway was not responsible for early cardiac hypertrophy in TG<sup>T400N</sup> mice.

We also examined whether increased MAPK activity was associated with hypertrophy in TG<sup>T400N</sup> hearts. We quantified the active, phosphorylated forms of p38 MAPK and ERK1/2, and another downstream signaling protein, phosphorylated STAT3, by immunoblot ( $n=3$ /group). There was no increase in levels of phosphorylated p38

MAPK (Figs. 5A and 5B), phosphorylated ERK1/2 (Figs. 5C and 5D), and phosphorylated STAT3 levels (Fig. 5E) at ages 2 and 8 weeks. These data suggest the absence of a role for MAPK signaling pathway in cardiac hypertrophy in TG<sup>T400N</sup> mice.

*3.4. Normalization of AMPK activity leads to normalization of NF-κB levels in the nucleus, normalization of Akt and p70S6K signaling, and attenuation of cardiac hypertrophy in TG<sup>T400N</sup> mice*

To ascertain whether attenuation of inappropriate AMPK activity in TG<sup>T400N</sup> hearts would lead to attenuation of the associated upregulation in NF-κB, Akt, and p70S6K signaling that we observed, we crossbred TG<sup>T400N</sup> mice with TG<sup>α2DN</sup> mice, which express a dominant negative, kinase dead mutant of the AMPK α2 catalytic subunit and have low myocardial AMPK activity [10]. Thr<sup>172</sup> phosphorylation of the α subunit of AMPK, which reflects AMPK activity, was assessed by immunoblotting and was found to be reduced in TG<sup>T400N</sup>/TG<sup>α2DN</sup> hearts relative to TG<sup>T400N</sup> hearts (22 ± 5 versus 100 ± 22 in arbitrary units,  $n=3/\text{group}$ ,  $p<0.05$ ). As we have previously reported [8], TG<sup>T400N</sup>/TG<sup>α2DN</sup> mice exhibited an attenuation in cardiac hypertrophy as measured by cardiac mass / body mass ratios and by echocardiography. Specifically, at age 2 weeks, cardiac mass / body mass ratios (mg/g) in WT, TG<sup>T400N</sup> mice, and TG<sup>T400N</sup>/TG<sup>α2DN</sup> were 4.9 ± 0.2, 7.4 ± 0.2, and 6.1 ± 0.2 respectively ( $n=3/\text{group}$ ,  $p<0.001$  for TG<sup>T400N</sup> versus WT, and  $p<0.01$  for TG<sup>T400N</sup>/TG<sup>α2DN</sup> versus TG<sup>T400N</sup>). Corresponding to these changes, significantly reduced levels of the p50 subunit of NF-κB in the nucleus were observed in TG<sup>T400N</sup>/TG<sup>α2DN</sup> hearts relative to TG<sup>T400N</sup> hearts

at age 2 weeks ( $n=3$ /group) (Figs. 6A and 6B). Similarly, phosphorylated Akt and p70S6K were significantly decreased in  $TG^{T400N}/TG^{\alpha2DN}$  hearts relative to  $TG^{T400N}$  hearts at age 2 weeks ( $n=3$ /group) (Figs. 6C, 6D and 6E). These data confirm that attenuation of inappropriate AMPK activity in  $TG^{T400N}$  hearts leads to an attenuation of increased NF- $\kappa$ B, Akt, and p70S6K signaling associated with cardiac hypertrophy.



#### 4. Discussion

*PRKAG2* mutations in humans cause cardiac hypertrophy and sudden cardiac death [1-3]. Previously, in a transgenic mouse model with cardiac specific expression of the T400N mutation in *PRKAG2* (TG<sup>T400N</sup>), we have shown that the mutation leads to early inappropriate activation of cardiac AMPK and glycogen deposition [8]. Concurrently, the heart manifests cellular and ventricular wall hypertrophy, only a small proportion of which is attributable to glycogen deposition. The specific mechanisms whereby *PRKAG2* mutations activate hypertrophic signaling pathways and result in cardiac dilation and failure are unknown. In this study, we analyzed the TG<sup>T400N</sup> model to dissect the downstream effects of this mutation on signaling pathways mediating cardiac hypertrophy. We demonstrated early changes in hypertrophic signaling pathways that are targets of AMPK, including Akt and the transcription factor NF- $\kappa$ B.

We analyzed known or suspected AMPK targets that could potentially activate hypertrophic pathways, including the transcription factor NF- $\kappa$ B [14, 15], p38 mitogen-activated protein kinase (p38 MAPK) [16-20], and Akt[21-26]. Additionally, we systematically analyzed other pathways known to mediate cardiac hypertrophy and which may be active in TG<sup>T400N</sup> hearts. Our analysis showed that NF- $\kappa$ B was activated in TG<sup>T400N</sup> hearts. NF- $\kappa$ B is a redox-sensitive transcription factor that can be activated during oxidative stress [37]. Interestingly, elevated intracellular glucose stimulates the translocation of NF- $\kappa$ B [35]. Increased cardiac glucose uptake has been documented in

both the TG<sup>T400N</sup> model (Banerjee and Ahmad, unpublished data), and in a transgenic mouse model with the N488I mutation [36]. Therefore, NF- $\kappa$ B may be activated not only by direct AMPK effects but also by indirect effects of perturbations in intracellular glucose. We also observed early activation of the Akt-mTOR signaling pathway. Increased levels of phosphorylated Akt and another downstream mediator, phosphorylated p70S6K, were present. However, we found no activation of the calcineurin and MAPK (phosphorylated ERK, phosphorylated p38 MAPK and phosphorylated STAT3) pathways in TG<sup>T400N</sup> mice. Our results suggest that both NF- $\kappa$ B activation and Akt-mTOR signaling pathway participate in the development of cardiac hypertrophy in the TG<sup>T400N</sup> mouse.

We speculated that initial inappropriate activation of AMPK, secondary to the T400N mutation in *PRKAG2*, triggers a hypertrophic remodeling program through its targets, NF- $\kappa$ B and Akt. Therefore, we hypothesized that early reduction of AMPK activity in the TG<sup>T400N</sup> heart by genetic means would attenuate this remodeling program. We constructed a double transgenic mouse (TG<sup>T400N</sup>/TG <sup>$\alpha$ 2DN</sup>) by crossbreeding TG<sup>T400N</sup> mice with TG <sup>$\alpha$ 2DN</sup> mice, which express a dominant negative, kinase dead mutant of the AMPK  $\alpha$ 2 catalytic subunit and have low myocardial AMPK activity [10]. Consistent with our hypothesis, TG<sup>T400N</sup>/TG <sup>$\alpha$ 2DN</sup> mice exhibited attenuation of hypertrophy and reductions in nuclear translocation of NF- $\kappa$ B and expression of phosphorylated Akt and p70S6K. However, we have previously shown that AMPK activity is biphasic in the TG<sup>T400N</sup> heart, with early activation, followed by depression, and then recovery to WT levels [8]. It is possible that that the initial enhanced AMPK activity is required to establish the primary

defect, but that with time enhanced glucose uptake and glycogen deposition is a stimulus for continued NF- $\kappa$ B activation that subsequently drives cardiac hypertrophy and eventually dilation [38, 39].

As detailed elsewhere [40-43], several studies in both *in vitro* cardiac myocyte cultures and *in vivo* mouse models have suggested that the NF- $\kappa$ B signaling pathway is an important intracellular mediator of cardiac hypertrophy. NF- $\kappa$ B has been found to be upregulated in pathological and physiological cardiac hypertrophy. Furthermore, inhibition of NF- $\kappa$ B prevents hypertrophy in response to many of these stimuli, such as phenylephrine, endothelin-1, angiotensin II, and chronic pressure overload. Whereas it is now well established that intracellular calcium stimulates hypertrophy by activation of calcineurin and NFAT, recent evidence suggests that calcium can also alternatively activate NF- $\kappa$ B in a calpain-dependent manner. The mechanisms by which NF- $\kappa$ B mediates cardiac hypertrophy have not been fully elucidated, but include activation of the interleukin 6 (*Il6*) receptor gp130 [30, 31] and transcriptional activation of antiapoptotic genes. Of note, we found *Il6* expression to be increased in TG<sup>T400N</sup> hearts. Although the preponderance of evidence now supports that NF- $\kappa$ B inhibition antagonizes cardiac hypertrophy, it is important to note that there are caveats. NF- $\kappa$ B can mediate both anti- and proapoptotic effects, so that NF- $\kappa$ B inhibition may either protect the myocardium from or predispose it to apoptosis depending on the nature of the stimulus. For example, some studies have shown that NF- $\kappa$ B inhibition promotes apoptosis after TNF- $\alpha$  treatment or hypoxia/reoxygenation, whereas other studies have documented the opposite effects. Our

data suggest that NF- $\kappa$ B is an important mediator of cardiac hypertrophy, and possibly the transition to dilation and heart failure, in *PRKAG2* cardiomyopathy.

Further work will be required for complete elucidation of the pathophysiology of *PRKAG2* cardiomyopathy. For example, echocardiography shows early hypercontractility in TG<sup>T400N</sup> hearts that progresses to normal contractility and eventually impaired contractility [8]. The acute and chronic effects of *PRKAG2* mutations on sarcomere function are unknown. Furthermore, although we have observed increasing cardiac fibrosis with age in TG<sup>T400N</sup> mice, the molecular mechanisms promoting fibrosis are currently under investigation. Finally, the direct molecular electrophysiological effects of *PRKAG2* mutations underlying rhythm and conduction disturbances observed clinically in patients and in TG<sup>T400N</sup> mice require further study [44].

We conclude that the T400N mutation in *PRKAG2* initiates cardiac hypertrophy at an early age, a process associated with activation of the transcription factor, NF- $\kappa$ B, and the Akt-mTOR signaling pathway. Identification of cardiac hypertrophic pathways in the setting of a *PRKAG2* mutation, at early stages in the evolution of disease, suggests possible therapeutic targets, which remain to be tested in further studies.

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**Figure Legends**

**Fig. 1.** The *PRKAG2* T400N mutation was associated with upregulation of the markers cardiac hypertrophy, (A) ANP and (B) BNP, in TG<sup>T400N</sup> myocardium as measured by QPCR. *n*=3/group. Data are expressed as mean ± SE. Open bars, WT; shaded bars, TG<sup>T400N</sup>. \*, *p*<0.05; \*\*, *p*<0.01 relative to WT.

**Fig. 2.** Nuclear factor κB (NF-κB) was activated in TG<sup>T400N</sup> mice. (A) NF-κB activity as measured by EMSA was higher in TG<sup>T400N</sup> hearts at all ages analyzed (2 - 20 weeks). Hearts from transgenic mice overexpressing TNFα (TG<sup>TNFα</sup>) were used as a positive control. (B) NF-κB binding to its consensus binding site was measured using a commercially available colorimetric assay, which confirmed elevation of binding activity in TG<sup>T400N</sup> hearts at ages 2, 8, and 20 weeks. Data are expressed as mean ± SE. Open bars, WT; shaded bars, TG<sup>T400N</sup>. \*, *p*<0.05; \*\*, *p*<0.01; \*\*\*, *p*<0.001 relative to WT. *n*=3-8/group. (C) The p50 subunit of NF-κB was elevated in nuclear protein extracts from TG<sup>T400N</sup> hearts relative to WT littermates at ages 2 and 8 weeks as measured by immunoblot. Coomassie blue staining was used to document the relative quantity of protein loaded.

**Fig. 3.** Activation of the Akt signaling pathway was associated with cardiac hypertrophy in TG<sup>T400N</sup> mice. (A) A representative immunoblot of total cardiac protein extracts showed increased levels of phosphorylated Akt and phosphorylated p70S6K in TG<sup>T400N</sup> hearts

relative to WT at ages 2 and 8 weeks. There was no difference in total Akt level at either age. Coomassie blue staining was used to document the relative quantity of protein loaded for immunoblot. (B) Densitometry analysis of several immunoblots, corrected for protein loading, indicated 3-fold and 2-fold increases in phosphorylated Akt levels in TG<sup>T400N</sup> hearts relative to WT at ages 2 and 8 weeks, respectively. (C) Densitometry analysis of several immunoblots, corrected for protein loading, indicated 2-fold increases in phosphorylated p70S6K in TG<sup>T400N</sup> hearts relative to WT at ages 2 and 8 weeks.  $n=3/\text{group}$ . Data are expressed as mean  $\pm$  SE. Open bars, WT; shaded bars, TG<sup>T400N</sup>. \*,  $p<0.05$ ; \*\*,  $p<0.01$ .

**Fig. 4.** The calcineurin signaling pathway was not activated in TG<sup>T400N</sup> mice. (A) A representative immunoblot of total cardiac protein extracts showed no difference in levels of calcineurin A in TG<sup>T400N</sup> hearts relative to WT littermates at age 2 weeks, but mildly decreased levels at age 8 weeks. Coomassie blue staining was used to document the relative quantity of protein loaded for the immunoblot. (B) Densitometry analysis of several immunoblots, corrected for protein loading, indicated a significant ( $p<0.05$ ) decrease in calcineurin A levels in TG<sup>T400N</sup> hearts relative to WT only at age 8 weeks.  $n=3/\text{group}$ . Data are expressed as mean  $\pm$  SE. Open bars, WT; shaded bars, TG<sup>T400N</sup>. \*,  $p<0.05$ . (C) A representative immunoblot of cytoplasmic cardiac protein extracts showed no change in NFATc3 levels in TG<sup>T400N</sup> hearts relative to WT at ages 2 and 8 weeks. Coomassie blue staining was used to document the relative quantity of protein loaded for the immunoblot.

**Fig. 5.** The MAPK signaling pathway was not activated in TG<sup>T400N</sup> mice. (A) A representative immunoblot of total cardiac protein extracts showed no change in phosphorylated p38 MAPK in TG<sup>T400N</sup> hearts relative to WT at ages 2 and 8 weeks. Coomassie blue staining was used to document the relative quantity of protein loaded for the immunoblot. (B) Densitometry analysis of several immunoblots, corrected for protein loading, indicated no difference in phosphorylated p38 MAPK levels at ages 2 and 8 weeks.  $n=3$ /group. Data are expressed as mean  $\pm$  SE. Open bars, WT; shaded bars, TG<sup>T400N</sup>. (C) An immunoblot of total cardiac protein extracts showed no increased levels of phosphorylated ERK1/2 in TG<sup>T400N</sup> hearts relative to WT at ages 2 and 8 weeks. Coomassie blue staining was used to document the relative quantity of protein loaded for immunoblot. (D) Densitometry analysis of several immunoblots, corrected for protein loading, indicated no increase in phosphorylated ERK1/2 levels in TG<sup>T400N</sup> hearts relative to WT at ages 2 and 8 weeks.  $n=3$ /group. Data are expressed as mean  $\pm$  SE. Open bars, WT; shaded bars, TG<sup>T400N</sup>. (E) An immunoblot of total cardiac protein extracts showed no change in phosphorylated STAT3 levels in TG<sup>T400N</sup> hearts relative to WT at ages 2 and 8 weeks.

**Fig. 6.** Normalization of AMPK activity normalized NF- $\kappa$ B, Akt, and p70S6K signaling in TG<sup>T400N</sup> hearts at age 2 weeks. (A) A representative immunoblot of nuclear protein extracts showed increased expression of the p50 subunit of NF- $\kappa$ B in TG<sup>T400N</sup> hearts relative to WT, an effect which was attenuated in TG<sup>T400N</sup>/TG <sup>$\alpha$ 2DN</sup> hearts. Coomassie blue staining was used to document the relative quantity of protein loaded for immunoblot. (B) Densitometry



analysis, corrected for protein loading, confirmed significantly ( $p<0.01$ ) increased nuclear p50 levels in TG<sup>T400N</sup> hearts relative to WT, but significantly ( $p<0.01$ ) reduced levels in TG<sup>T400N</sup>/TG <sup>$\alpha$ 2DN</sup> hearts relative to TG<sup>T400N</sup>. (C) A representative immunoblot of total cardiac protein extracts showed increased levels of phosphorylated Akt and phosphorylated p70S6K in TG<sup>T400N</sup> hearts relative to WT, an effect which was attenuated in TG<sup>T400N</sup>/TG <sup>$\alpha$ 2DN</sup> hearts. Coomassie blue staining was used to document the relative quantity of protein loaded for immunoblot. (D) Densitometry analysis, corrected for protein loading, confirmed significantly ( $p<0.05$ ) increased phosphorylated Akt levels in TG<sup>T400N</sup> hearts relative to WT, which were normalized in TG<sup>T400N</sup>/TG <sup>$\alpha$ 2DN</sup> hearts ( $p<0.05$ ). (E) Densitometry analysis, corrected for protein loading, confirmed significantly ( $p<0.01$ ) increased phosphorylated p70S6K levels in TG<sup>T400N</sup> hearts relative to WT, but significantly ( $p<0.05$ ) reduced levels in TG<sup>T400N</sup>/TG <sup>$\alpha$ 2DN</sup> hearts relative to TG<sup>T400N</sup>.  $n=3$ /group. Data in bar graphs are expressed as mean  $\pm$  SE. Open bars, WT; shaded bars, TG<sup>T400N</sup>; closed bars, TG<sup>T400N</sup>/TG <sup>$\alpha$ 2DN</sup>. \*,  $p<0.05$  and \*\*,  $p<0.01$  versus WT; †,  $p<0.05$  and ††,  $p<0.01$  versus TG<sup>T400N</sup>.

**Table 1.**

Real-time quantitative PCR primers used to quantify mRNA expression.

<i>Anp</i>	Sense	5'-GAAAAGCAAACCTGAGGGCTCTG-3'
	Antisense	5'-CCTACCCCCGAAGCAGCT-3'
<i>Bnp</i>	Sense	5'-AGGCGAGACAAGGGAGAACA-3'
	Antisense	5'-GGAGATCCATGCCGCAGA-3'
<i>FasL</i>	Sense	5'-CATGGAGTGGTCCTTAATGC-3'
	Antisense	5'-AAGTAGACCCACCCTGGAAG-3'
<i>Ifng</i>	Sense	5'-GAAAAGGATGGTGACATGAA-3'
	Antisense	5'TTGGCAATACTCATGAATGC-3'
<i>Il6</i>	Sense	5'-CAGAGGATAACCACTCCCAACA-3'
	Antisense	5'-CCAGTTTGGTAGCATCCATC-3'
<i>Il10</i>	Sense	5'-CAGAGCCACATGCTCCTAGA-3'
	Antisense	5'-TGTCCAGCTGGTCCTTTGTT-3'
<i>Nlrp12</i>	Sense	5'-TGTAGGCTGCAGATGATCCA-3'
	Antisense	5'-GAACAGAAGCCAGGGATCG-3'
<i>Tlr2</i>	Sense	5'-GGGGCTTCACTTCTCTGCTT-3'
	Antisense	5'-AGCATCCTCTGAGATTTGACG-3'
<i>Tlr9</i>	Sense	5'-GAATCCTCCATCTCCCAACAT-3'
	Antisense	5'-CCAGAGTCTCAGCCAGCACT-3'
<i>Tnfsf14</i>	Sense	5'-CGATCTCACCAGGCCAAC-3'
	Antisense	5'-TCCACCAATACCTATCAAGCTG-3'
<i>Zap70</i>	Sense	5'-CAGACCGACGGCAAGTTC-3'
	Antisense	5'-CCATAGACCAGGGACAGTGC-3'
Cyclophilin	Sense	5'-TGTGCCAGGGTGGTGACTT-3'
	Antisense	5'-TCAAATTTCTCTCCGTAGATGGACTT-3'

**Table 2.**Relative contribution of glycogen to cardiac hypertrophy in TG<sup>T400N</sup> mice.

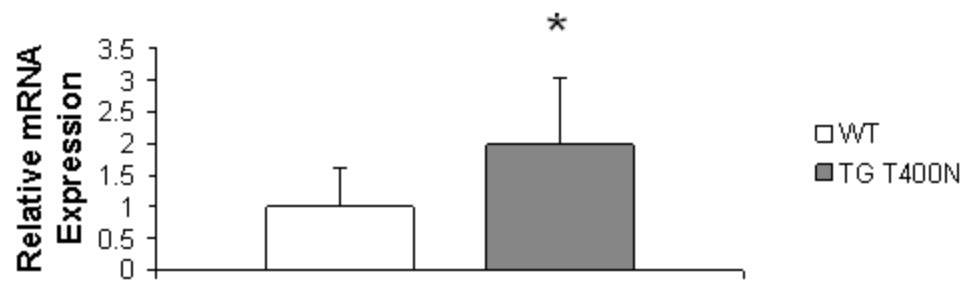
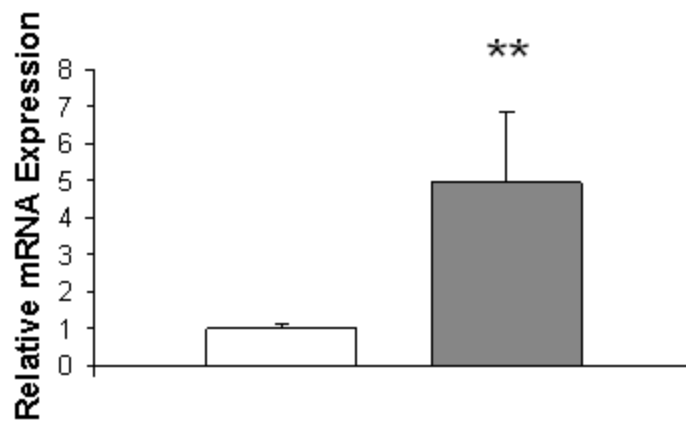
	4 Weeks		8 Weeks	
	WT	TG <sup>T400N</sup>	WT	TG <sup>T400N</sup>
Cardiac mass (mg)	66.7±2.0	214.5±9.6	98.8±8.3	446.5±51.1
Cardiac glycogen content (µg/mg cardiac mass)	0.31±0.05	31.10±4.1	0.30±0.05	28.02±2.64
Contribution of glycogen to increased cardiac mass (%)		4.4±0.6%		3.5±0.3%

*n* = 5 / group.

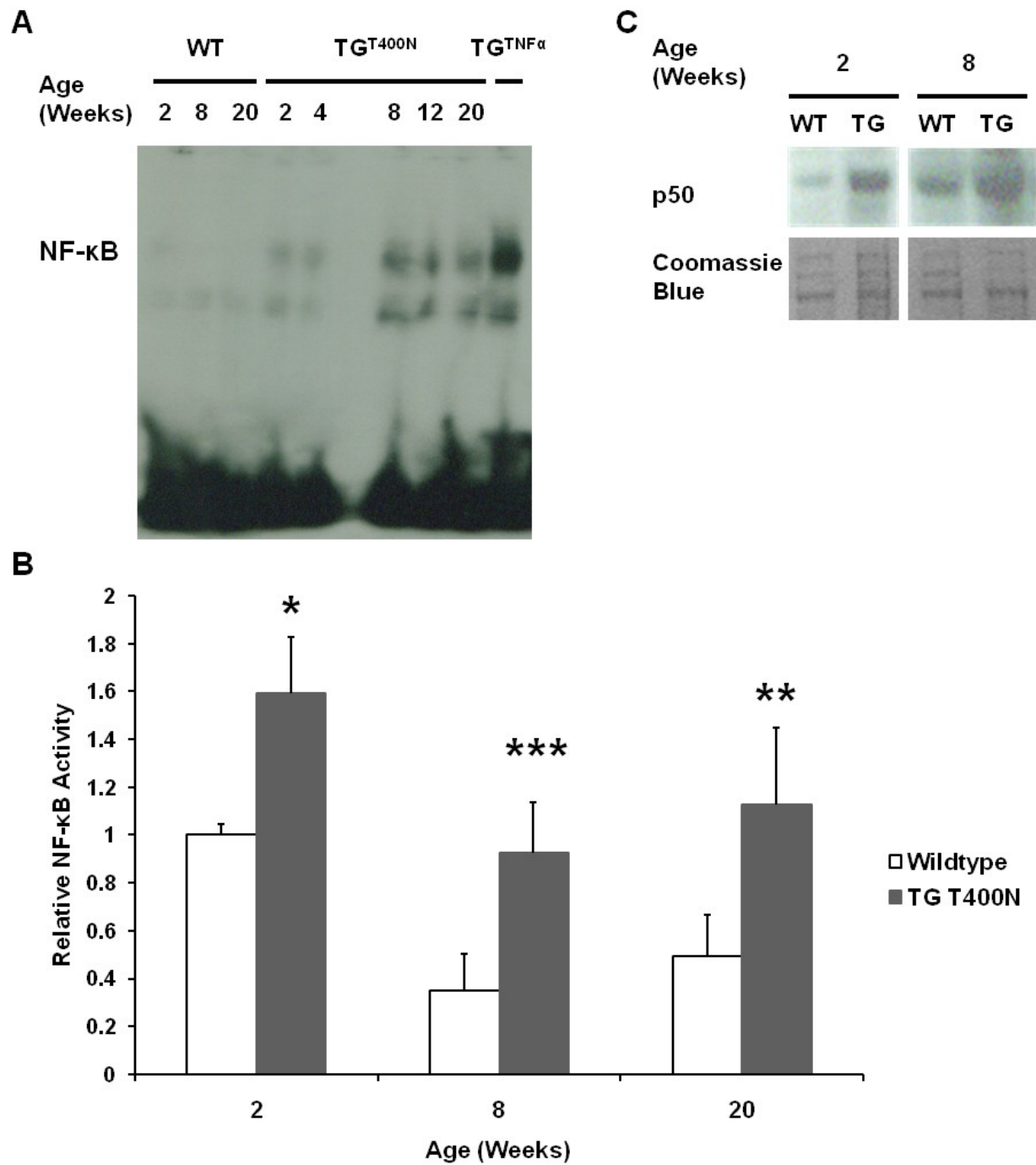
**Table 3.**Upregulation of genes related to NF- $\kappa$ B mediated signal transduction in TG<sup>T400N</sup> hearts.

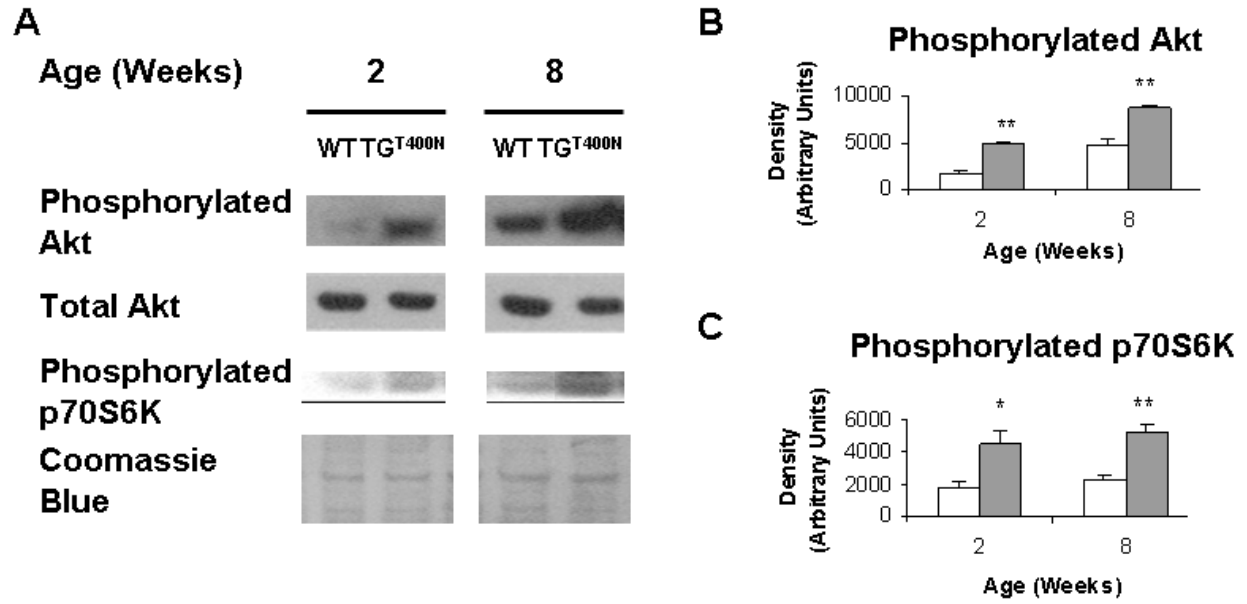
Gene Name	Gene Symbol	Cardiac Expression (Arbitrary Units)					
		2			8		
		TG <sup>T400N</sup>	WT	<i>p</i>	TG <sup>T400N</sup>	WT	<i>p</i>
Fas ligand (TNF superfamily, member 6)	<i>FasL</i>	1.31±0.01	1.00±0.01	<0.001	2.22±0.01	1.00±0.01	<0.01
Interferon gamma	<i>Ifng</i>	2.53±0.06	1.00±0.01	<0.01	2.38±0.02	1.00±0.04	<0.05
Interleukin 6	<i>Il6</i>	0.28±0.01	1.00±0.06	<0.05	4.68±0.15	1.00±0.03	<0.01
Interleukin 10	<i>Il10</i>	2.21±0.05	1.00±0.02	<0.05	4.93±0.02	1.00±0.04	<0.01
NLR family, pyrin domain containing 12	<i>Nlrp12</i>	1.07±0.01	1.00±0.01	<0.01	1.21±0.02	1.00±0.00	NS
Toll-like receptor 2	<i>Tlr2</i>	1.02±0.01	1.00±0.01	NS	4.45±0.01	1.00±0.01	<0.001
Toll-like receptor 9	<i>Tlr9</i>	0.69±0.01	1.00±0.02	<0.05	1.96±0.04	1.00±0.00	<0.01
Tumor necrosis factor (ligand) superfamily, member 14	<i>Tnfsf14</i>	0.67±0.01	1.00±0.01	<0.05	1.82±0.02	1.00±0.01	<0.01
Zeta-chain (TCR) associated protein kinase	<i>Zap70</i>	1.60±0.02	1.00±0.02	<0.01	1.49±0.01	1.00±0.01	<0.01

NS, not significant.

**A****ANP****B****BNP**

AC





ACCEPTED MANUSCRIPT

