THE MOLECULAR PATHOGENESIS OF NOONAN SYNDROME-ASSOCIATED \textit{RAF1} MUTATIONS

by

Xue Wu

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Medical Biophysics
University of Toronto

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2013

ABSTRACT

Noonan syndrome (NS) is one of several autosomal dominant “RASopathies” caused by mutations in components of the RAS-RAF-MEK-ERK MAPK pathway. Germ line mutations in RAF1 (encoding the serine-threonine kinase for MEK1/2) account for ~3-5% of NS, and unlike other NS alleles, RAF1 mutations that confer increased kinase activity are highly associated with hypertrophic cardiomyopathy (HCM). Notably, some NS-associated RAF1 mutations show normal or decreased kinase activity. To explore the pathogenesis of such mutations, I generated “knock-in” mice that express kinase-activating (L613V) or -impaired (D486N) Raf1 mutants, respectively. Knock-in mice expressing the kinase-activated allele Raf1\textsuperscript{L613V} developed typical NS features (short stature, facial dysmorphia, haematological abnormalities), as well as HCM. As expected, agonist-evoked Mek/Erk activation was enhanced in multiple cell types expressing Raf1\textsuperscript{L613V}. Moreover, postnatal Mek inhibition normalized the growth, facial, and cardiac defects in L613V/+ mice, showing that enhanced Mek/Erk activation by Raf1 mutant is critical for evoking NS phenotypes. D486N/+ female mice exhibited a mild growth defect. Male and female
D486N/D486N mice developed concentric cardiac hypertrophy and incompletely penetrant, but severe, growth defects. Remarkably, Mek/Erk activation was enhanced in Raf1_D486N-expressing cells compared with controls. In both mouse and human cells, RAF1_D486N, as well as other kinase-impaired RAF1 mutants, show increased heterodimerization with BRAF, which is necessary and sufficient to promote increased MEK/ERK activation. Furthermore, kinase-activating RAF1 mutants also require heterodimerization to enhance MEK/ERK activation. Our results suggest that increased heterodimerization ability is the common pathogenic mechanism for NS-associated RAF1 mutations.
ACKNOWLEDGMENTS

Foremost, I would like to thank my supervisor Dr. Benjamin Neel for all your guidance, continuous support and encouragement during my Ph.D study. You have been a tremendous mentor for me with your immense knowledge, inspiration, enthusiasm, and criticism. I also thank you for the critical reading of this thesis.

I am very grateful to Dr. Toshiyuki Araki as a co-supervisor for my research project. Thank you for sharing your knowledge, good discussions and friendship.

I thank the Department of Medical Biophysics at University of Toronto for giving me the opportunity to study here. I greatly appreciate the insightful discussions and comments from all my supervisory committee members, Dr. Peter Backx, Dr. Vuk Stambolic and Dr. Benjamin Alman. Your advice on both research, as well as on my career, have been invaluable.

This research project would not have been possible without the help of many people. Special thanks to Dr. Peter Backx and Dr. Jeremy Simpson as important collaborators on the cardiology studies of my mouse model. Dr. Jeremy Simpson performed all the echocardiography, invasive hemodynamic analysis and transverse aortic constriction experiments. Our collaboration has continued after he established his own lab at the University of Guelph. Thanks to Dr. Kyoung-Han Kim for preparing the neonatal cardiomyocytes for me, and for your patience in teaching me this technique. I also thank Dr. Tara Paton at The Centre for Applied Genomics (TCAG) at SickKids for helping me with the mouse genotyping array and Dr. Pingzhao Hu in the same facility for providing statistical analysis support. I would also like to thank Dr. Jason Moffat (University of Toronto) for providing lentiviral shRNA vectors and Dr. Bruce Gelb (Mt Sinai Hospital, NY) for providing the human RAF1 cDNA construct used in my research.

I am thankful to all of the past and present members in the Neel lab, Jocelyn Stewart, Rob Karish, Shengqing Gu, Xiannan Wang, Peter Bayliss, Angel Sing, Cathy Iorio, Gordon Chan, Ziqiang Yang, Richard Marcotte, Dong Hu, Robert Banh, Yang Xu, Lingyan Jiang, Anderson Chang, Erica Tiberia, Kwan Ho Tang, Paulina Cybulaska, Richard Chan and many others. The group has been a source of friendships, as well as good advice and collaboration. All of them made my study in this lab a cherished memory. Thank you Shengqing Gu for your help of
analyzing the hematological defects in my mice, and for your biostatistics support. I am honored
to have had the opportunity to supervise two talented and hard-working summer students, Jenny
Hong and Connie Yin. I am grateful for all their help in many aspects of my project. I also would
like to thank our previous lab member, Dr. Nirusha Thavarajah, for synthesizing the MEK
inhibitor. I thank Peter Bayliss, and Angel Sing for their technical support, and our lab manager,
Cathy Iorio, for making the lab organized and functional.

The Frederick Banting and Charles Best Canada Graduate Scholarship from Canadian
Institutes of Health Research (CIHR) is greatly appreciated. This research also was supported by
grants from the CIHR, the National Institutes of Health (NIH) and the Heart and Stroke
Foundation of Ontario (H&SFO).

Finally, I dedicate this thesis to my parents, Feng Yu and Shouzhi Wu, my husband,
Kevin Hu, and my beloved daughter, Claire Hu, for their constant support and unconditional love.
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<tbody>
<tr>
<td>ADHD</td>
<td>attention deficit-hyperactive disorder</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate-activated protein kinase</td>
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<tr>
<td>AN</td>
<td>anal-nasal</td>
</tr>
<tr>
<td>Ang-II</td>
<td>angiotensin-II</td>
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<td>ANP</td>
<td>atrial natriuretic peptide</td>
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<td>AP1</td>
<td>activating protein-1</td>
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<td>AS</td>
<td>aortic stenosis</td>
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<td>ASK1</td>
<td>apoptosis signal-regulating kinase 1</td>
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<td>ATF1</td>
<td>activating transcription factor 1</td>
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<td>BNP</td>
<td>B-type natriuretic peptide</td>
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<tr>
<td>CaMK</td>
<td>calmodulin-dependent kinase</td>
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<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
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<tr>
<td>CFCS</td>
<td>cardio-facio-cutaneous syndrome</td>
</tr>
<tr>
<td>CHD</td>
<td>congenital heart defects</td>
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<tr>
<td>CK2</td>
<td>casein kinase 2</td>
</tr>
<tr>
<td>CNK</td>
<td>connector enhancer of KSR</td>
</tr>
<tr>
<td>CO</td>
<td>cardiac output</td>
</tr>
<tr>
<td>COT</td>
<td>Cancer Osaka Thyroid oncogene</td>
</tr>
<tr>
<td>CR</td>
<td>conserved region</td>
</tr>
<tr>
<td>CRD</td>
<td>cysteine-rich domain</td>
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<td>CREB</td>
<td>cAMP response element binding protein</td>
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<td>CS</td>
<td>Costello syndrome</td>
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<tr>
<td>CSK</td>
<td>C-terminal SRC kinase</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DUSP</td>
<td>dual specificity phosphatase</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>Definition</td>
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<tr>
<td>EDC</td>
<td>epidermal differentiation cluster</td>
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<tr>
<td>EDV</td>
<td>end-diastolic volume</td>
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<tr>
<td>EF</td>
<td>ejection fraction</td>
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<td>eIF-4E</td>
<td>eukaryotic initiation factor-4E</td>
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<td>ELK-1</td>
<td>Ets-like gene 1</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>ESV</td>
<td>end-systolic volume</td>
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<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FIAU</td>
<td>1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5 iodouracil</td>
</tr>
<tr>
<td>FS</td>
<td>fractional shortening</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<td>GH</td>
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<tr>
<td>GHD</td>
<td>growth hormone deficiency</td>
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<td>glycine-rich loop</td>
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<td>glycoprotein-130</td>
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<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>GRB2</td>
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<td>glycogen synthase kinase-3β</td>
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<tr>
<td>HCM</td>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>IEG</td>
<td>immediate-early gene</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor I</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of NF-κB kinase</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>IQGAP</td>
<td>IQ motif-containing GTPase-activating protein</td>
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</table>
IkB  inhibitor of NF-κB
JMML  juvenile myelomonocytic leukemia
JNK  c-Jun N-terminal kinase
KSR1  kinase suppressor of Ras 1
LAH  loose anagen hair
LAMP2  lysosome-associated membrane protein-2-α-galactosidase
LD  linkage disequilibrium
LEOPARD  Lentigines, ECG conduction abnormalities, Ocular hypertelorism, Pulmonic stenosis, Abnormal genitalia, Retardation of growth, and sensorineural Deafness
LPA  lysophosphatidic acid
LS  LEOPARD syndrome
LV  left ventricle
LVH  left ventricular hypertrophy
LVIDd  left ventricular internal end-diastolic dimension
LVIDs  left ventricular internal end-systolic dimension
LVPWd  left ventricular diastolic posterior wall thickness
MAP  mean arterial pressure
MAPK  mitogen-activated protein kinase
MAPKK  mitogen-activated protein kinase kinase
MAPKKK  mitogen-activated protein kinase kinase kinase
MEF  mouse embryonic fibroblast
MEF2  myocyte enhancer factor 2
MEK1  MAPK/ERK kinase 1
MI  myocardial infarction
MKP  MAP kinase phosphatase
MLP  muscle LIM-domain protein
MNK  MAPK-interacting kinase
MORG1  MAPK organizer 1
MHC  myosin heavy chain
MP1  MEK partner-1
MPNST  malignant peripheral nerve sheath tumors
<table>
<thead>
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<tr>
<td>MSK</td>
<td>mitogen- and stress-activated protein kinase</td>
</tr>
<tr>
<td>MST2</td>
<td>mammalian STE20-like protein kinase 2</td>
</tr>
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<td>MSV</td>
<td>murine sarcoma virus</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>NEAA</td>
<td>non-essential amino acid</td>
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<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<td>NS</td>
<td>Noonan syndrome</td>
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<td>NS/LAH</td>
<td>Noonan-like syndrome with loose anagen hair</td>
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<td>optic pathway gliomas</td>
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<td>PDK</td>
<td>phosphoinositide-dependent kinase</td>
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<td>plekstrin homology</td>
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<tr>
<td>PHB</td>
<td>prohibitin</td>
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<td>RAF</td>
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<td>Raf kinase inhibitor protein</td>
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</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RSK</td>
<td>ribosomal S6 kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricle</td>
</tr>
<tr>
<td>SAP1</td>
<td>SRF accessory protein 1</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
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<tr>
<td>SEF</td>
<td>similar expression to FGF</td>
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<tr>
<td>SERCA2a</td>
<td>sarcoplasmic reticulum Ca$^{2+}$-ATPase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
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<td>Src homology 3</td>
</tr>
<tr>
<td>SHPS-1</td>
<td>Shp Substrate-1</td>
</tr>
<tr>
<td>SOS</td>
<td>son of sevenless</td>
</tr>
<tr>
<td>SPRED1</td>
<td>Sprouty-related EVH1 domain-containing protein 1</td>
</tr>
<tr>
<td>SRE</td>
<td>serum response element</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>SPRY</td>
<td>SPROUTY</td>
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<tr>
<td>SV</td>
<td>stroke volume</td>
</tr>
<tr>
<td>TAC</td>
<td>transverse aortic constriction</td>
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<td>TAK</td>
<td>TGFβ-activated kinase</td>
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<tr>
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<td>ternary complex factor</td>
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<tr>
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<td>tumor progression locus 2</td>
</tr>
<tr>
<td>UBF</td>
<td>upstream binding factor</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage-dependent anion channel</td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
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Chapter 1

Introduction
1.1 The RAS-RAF-MEK-ERK MAPK pathway

1.1.1 MAPK pathways

Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved kinase modules that link extracellular signals to the machinery that controls fundamental cellular processes, such as proliferation, differentiation, migration and apoptosis (1). MAPK pathways are comprised of a three-tier kinase module in which a MAPK is activated upon phosphorylation by a mitogen-activated protein kinase kinase (MAPKK) which, in turn, is activated when phosphorylated by a mitogen-activated protein kinase kinase kinase (MAPKKK) (Figure 1-1).

To date, three main families of MAPKs have been characterized in mammals, which are grouped by their structures and functions: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK, also known as stress-activated protein kinase or SAPK)1/2/3 and the p38 isoforms α/β/γ (ERK6)/δ (2-5). Members of the ERK family can be divided further into four subgroups: the ERK1/2 group, comprising classic MAPKs that consist primarily of a kinase domain, and the three large MAPK groups, ERK3/4, ERK5 and ERK7/8, containing enzymes that consist of both a kinase domain and a C-terminal domain ranging in size from 60 kDa to greater than 100 kDa (6-9). The C-terminal regions function as protein interaction domains that regulate kinase localization (10), activation (9, 11), and transcriptional activity (7).

All MAPKs contain a TXY (Thr-X-Tyr) motif within their activation loops. The phosphorylation of both the threonine and the tyrosine within the activation loop is necessary and sufficient for their activation. The majority of ERK family members contain a TEY (Thr-Glu-Tyr) activation motif (12), except for ERK3/4, which possesses an SEG (Ser-Glu-Gly) sequence (6). The p38 family has a TGY (Thr-Gly-Tyr) activation motif (13), whereas JNK family members contain TPY (Thr-Pro-Tyr) in their activation loop (14).

The ERK pathway is the best studied mammalian MAPK pathway and is activated by numerous extracellular signals (Figure 1-1). In the ERK1/2 MAPK module, ERK1/2 are activated upon phosphorylation by MAPK/ERK kinase1/2 (MEK1/2), which is itself activated when phosphorylated by Rapid Accelerated Fibrosarcoma (RAF) family proteins. The details of this signaling cascade will be discussed later. Although RAF isoforms are the primary MAPKKKs in the ERK1/2 module, MAP3K8, also called TPL2 (tumor progression locus 2) or
Figure 1-1. Schematic overview of MAPK pathways.
COT (Cancer Osaka Thyroid oncogene) also can act as a MAPKKK that regulates the ERK1/2, ERK5, JNK and p38 pathways in a cell type- and stimulus-specific manner (15). MEKK1 (16) and MOS (17) are two additional ERK1/2 MAPKKKs utilized in more restricted cell type- and stimulation-specific situations. The MAPKK for ERK5 is MEK5, which is activated upon phosphorylation by MEKK2/3 or TPL2 (15, 18). The MAPKKs and MAPKKKs for ERK3/4 and ERK7/8 remain unknown.

Many MAPK pathways participate in stress signaling. Stress-activated MAPK cascades contain a large number of MAPKKKs, probably because stresses come in many forms (Figure 1-1). JNK family kinases can be activated by cytokines, UV radiation, oxidative stress, growth factor deprivation and DNA-damaging agents (19). JNK activation requires dual phosphorylation on the TPY motif, which is catalyzed by MEK4 or MEK7. MEK4/7 are themselves phosphorylated and activated by a wide range of MAPKKKs, including MEKK1–4, DLK, MLK2/3, TPL-2, TAO1/2, TAK1 and ASK1/2. Like ERKs, JNKs translocate from the cytoplasm to the nucleus following activation, and phosphorylate transcription factors (e.g., c-JUN, ATF-2 and STAT3) (20). Active JNK also functions in the cytoplasm, although relatively little is known about cytoplasmic JNK substrates.

p38 MAPKs are strongly activated by physical and chemical stresses, such as high osmolarity, oxidative stress, hypoxia and UV irradiation, and pro-inflammatory cytokines, such as endotoxin, TNF-α, and IL-1 (21, 22). In addition to its role in stress responses, the p38 pathway also helps regulate apoptosis, cell cycle progression, growth and differentiation, which reflects its activation by a broad range of extracellular stimuli, including growth factors (e.g., FGF, IGF-I, PDGF and nerve growth factor) and hormones. The conserved TGY phosphorylation motif of p38 isoforms is phosphorylated by MEK3 or MEK6, which themselves are activated by various MAPKKKs shared with JNK, including TAK1, ASK1/2, DLK, MEKK4, TAO1/2/3 and MLK2/3. In some instances, p38 can be activated by MEK4 (23). The downstream targets of the active p38 MAPKs include a wide array of cytoplasmic (e.g., Caspase-3/6, Caspase 8, and MNK1/2) and nuclear (e.g., ATF2, p53, ELK1 and STAT1) targets (22).
1.1.2 The RAS-RAF-MEK-ERK MAPK pathway

The RAS-RAF-MEK-ERK MAPK signal transduction cascade (hereafter, the RAS/ERK pathway) functions in many cellular processes, including proliferation, differentiation, survival, cell adhesion, migration and metabolism (Figure 1-2) (24). The pathway is initiated by the activation of one of three small guanosine triphosphatases (GTPases) KRAS, NRAS or HRAS, which are stimulated by guanine-nucleotide exchange factors (GEFs), such as son of sevenless (SOS) (25). Active RAS proteins interact with a wide range of effectors and stimulate downstream signaling components, including RAF kinases, phosphoinositol-3 kinase (PI3K), RAL guanine nucleotide dissociation stimulator (RALGDS), MEKK1, RAS interaction/interference 1 (RIN1), ALL-1 fusion partner in chromosome 6 (AF-6), phospholipase C epsilon, and novel RAS effector (NORE)/mammalian STE20-like protein kinase (MST) (26, 27). In the RAS/ERK pathway, RAS recruits RAF proteins to the cell membrane, where they are activated and subsequently form complexes with MEK1/2 and ERK1/2, aided by scaffolds, such as KSR. Activated RAF proteins phosphorylate MEK1/2, which in turn phosphorylate ERK1/2. ERKs phosphorylate cytosolic substrates and also translocate to the nucleus to stimulate diverse gene expression programs by phosphorylating several transcription factors (26, 28).

Deregulation of the RAS/ERK pathway is associated with various pathologies, most notably cancer (29, 30), but also developmental abnormalities (31, 32). Oncogenic mutations in human KRAS occur in about 90% of pancreatic, 40% of colorectal, and 30% of biliary cancers, whereas NRAS mutations occur in approximately 15-20% of melanomas. Overall, RAS genes are activated in about 30% of all human cancers (26, 33, 34). Activating mutations in BRAF occur in approximately 7% of all human cancers, including 27-70% of malignant melanomas, 36-53% of papillary thyroid cancers, 30% of ovarian cancers, and 5-22% of colorectal cancers (35, 36). The RASopathies are a group of developmental syndromes caused by germline mutations in genes that alter the RAS/ERK pathway (31, 32), and will be discussed later in detail.

The conversion of inactive guanine diphosphate (GDP)-bound form to active guanine triphosphate (GTP)-bound form of RAS is promoted by the action of several receptor tyrosine kinases (RTKs), including those of the epidermal growth factor receptor (EGFR) family, the insulin-like growth factor receptor (IGFR), the vascular endothelial growth factor receptor (VEGFR) family, and many others (37). Membrane-spanning cell surface RTKs are endowed
Figure 1-2. Schematic representation of the structure of RAS-RAF-MEK-ERK MAPK pathway.
with intrinsic tyrosine kinase activity. All RTKs contain an extracellular ligand-binding domain, a single hydrophobic transmembrane helix, and a cytoplasmic region that contains a conserved protein-tyrosine-kinase domain (TKD) plus additional C-terminal and juxamembrane regulatory regions that are subject to autophosphorylation and phosphorylation by other kinases. Most RTKs are monomers at the cell membrane, with ligand binding or ectopic over-expression resulting in receptor dimerization and tyrosine “autophosphorylation” in trans, although some RTKs exist as oligomers; e.g., the insulin receptor and IGFR, which form disulfide-linked dimers in the absence of activating ligand. Whether the “inactive” state is monomeric or oligomeric, activation of the RTKs still requires the bound ligand to stabilize the individual receptor molecules in an “active” dimer or oligomer. In some RTKs, such as the insulin receptor, KIT, and TIE2, trans-phosphorylation of tyrosines within the activation loop, the juxtamembrane segment, and/or the C-terminal region disrupts their cis-autoinhibitory interaction with TKD, and promotes receptor activation (38-40). However, the EGFR family and RET are exceptions. Their activation does not require trans-phosphorylation of their activation loop or elsewhere. Instead, dimerization of these receptors promotes conformational changes and thereby allosteric activation of their TKDs (41, 42).

Following RTK activation, additional tyrosines are autophosphorylated in other parts of the receptor cytoplasmic region. The resulting phospho-tyrosines provide a mechanism for the recognition and assembly of signaling complexes, functioning as binding sites for a variety of cytoplasmic signaling molecules containing Src homology 2 (SH2) domain (43) and/or phosphotyrosine binding (PTB) (44) domains. One such signaling protein is growth factor receptor-bound protein 2 (GRB2). GRB2, a cytosolic adaptor, contains a central SH2 domain, flanked by two Src homology 3 (SH3) domains, which allow constitutive association with the proline-rich regions of SOS (45). For example, phospho-tyrosine 1068 of the activated EGFR is a binding site for the SH2 domain of GRB2 (46), either directly or through the assistance of another SH2 adaptor, SHC (47). Activated EGFR phosphorylates SHC on Tyr317, which promotes the interaction between SHC and GRB2 (48). The recruitment of GRB2 from the cytoplasm to the plasma membrane brings SOS near membrane-bound RAS, where SOS enhances GDP release and GTP binding to RAS, converting this GTPase into its active conformation. It is well accepted that the protein-tyrosine phosphatase SHP2 (encoded by PTPN11) has a positive effect on RAS/ERK activation, but the mechanisms involved have
remained elusive (49). Several mechanisms have been proposed, one of which is that SHP2 may act as an adaptor protein leading to the recruitment of the GRB2/SOS complex to the cell membrane (50-53). Alternatively, SHP2 can dephosphorylate several targets, which, when dephosphorylated, will promote ERK activation. One possible target is SPROUTY (SPRY), which, when phosphorylated, purportedly sequesters GRB2/SOS in the cytoplasm (54-56). SHP2 also can dephosphorylate RAS-GAP binding site borne by RTKs or GAB1, and thereby exclude GAPs from signaling complexes and promote RAS activation (57, 58). Several studies suggest that SHP2 could act upstream of the SRC family kinases through dephosphorylation of SRC-regulatory proteins (59-61).

In addition to RTKs, signals that activate G-protein coupled receptors (GPCRs), such as lysophosphatidic acid (LPA), angiotensin II and beta-adrenergic agonists, can activate the RAS/ERK pathway (62, 63). Integrins, which are integral membrane proteins that mediate cellular adhesion to the extracellular matrix (ECM) and to other cells, also can lead to the activation of the RAS/ERK cascade (64).

RAS family members belong to a large family of small GTPases that bind and hydrolyze GTP. First discovered as transforming oncogenes of murine sarcoma viruses, three highly related 21 kDa mammalian proteins, Harvey-RAS (HRAS), Kirsten-RAS (KRAS), and Neuroblastoma-RAS (NRAS) have been identified (65). RAS family members are anchored to the cytoplasmic face of the plasma membrane, and such anchoring is essential for their biological function. Membrane-targeting of RAS is achieved through lipid-anchors by post-translational lipid modification of its C-terminal Cys residues (66). Unlike KRAS, NRAS and HRAS also signal from endomembranes and cycle between the plasma membrane and Golgi apparatus depending on their palmitoylation status (67). The localization to the inner leaflet brings RAS into close proximity with RAS-GEFs, catalyzing the exchange of GDP for the more abundant GTP. GTP loading alters RAS conformation, allowing it to interact with a number of downstream effectors (68). Within the ERK signaling cascade, active RAS functions as an adaptor that binds to effector RAF kinases with high affinity, causing their translocation to the cell membrane, where RAF activation takes place. The detailed mechanism of RAF activation will be discussed later. Active RAS-GTP is converted to the inactive RAS-GDP when its intrinsic RAS-GTPase activity is stimulated by GTPase activating proteins (GAPs) (69). The balance between GEF and GAP activity determines the guanine nucleotide status of RAS, thereby regulating RAS activity.
The RAF family kinases, including ARAF, BRAF and RAF1/CRAF in vertebrates, catalyze the phosphorylation and activation of the dual-specificity protein kinases, MEK1 and MEK2 (also known as M KK1 and M KK2) (70). RAF family activation of MEK1/2 occurs through phosphorylation of two serine residues, at positions 218 and 222 in the activation loop of MEK1 (S222 and S226 on MEK2). Different RAF isoforms are not equal in their ability to activate MEK: ARAF appears to be a poor MEK activator (71); BRAF displays a higher affinity for MEK1 and MEK2 than RAF1, and is more efficient in phosphorylating MEKs (71-74).

MEK1/2 catalyze the phosphorylation of threonine and tyrosine residues in the activation segment of ERK1/2, their only known physiological substrates (75). Several regulatory phosphorylation sites on MEK outside the activation loop either positively or negatively regulate the MAPKK. Phosphorylation of Ser298 by p21-activated kinase-1 (PAK1), downstream of the small G-protein RAC, enhances the association of MEK with ERK, and results in RAF-independent activation of MEK1 (76, 77). Conversely, in vivo phosphorylation by an unknown kinase at Ser212, a site conserved in all MAPKKs, sharply decreases MEK1 activity (78). MEK1 and MEK2 can form heterodimers subject to negative feedback by ERK-catalyzed phosphorylation of MEK1 on T292, which is absent on MEK2, facilitating the dephosphorylation of S218/222 in the MEK1 activation loop (79).

The MAP kinases ERK1 and ERK2, also known as MAPK3 and MAPK1, are 44- and 42-kDa protein serine/threonine kinases, respectively (80). Initially isolated and cloned as kinases activated in response to insulin and nerve growth factor (NGF) (81, 82), ERK1 and ERK2 are expressed ubiquitously, with ERK2 levels generally higher than ERK1 (83). All known cellular stimuli of the ERK1/2 pathway lead to the parallel activation of ERK1 and ERK2 (83, 84). Knocking-down ERK1 and/or ERK2 by RNA interference indicates that both ERK1 and ERK2 are positive regulators of cell proliferation and immediate-early gene (IEG) transcription, and the positive role of ERK1 on cell proliferation is uncovered only when the ERK2 level is markedly reduced and becomes limiting (83). Gene ablation studies in mice demonstrate that either ERK may at least partially compensate for the other's loss, although some evidence has been provided for differential functions of ERK1 and ERK2. The Erk1 gene is dispensable for the development of mice, but ablation of the Erk2 gene is embryonic lethal (85, 86). Erk2-null mice fail to form mesoderm, although embryonic stem (ES) cell proliferation is unaffected (86). The development of embryonic trophoblast and placental vasculature is severely
impaired in Erk2-deficient mice, which leads to embryonic lethality (87, 88). Taken together, these studies suggest that Erk1 is unable to compensate for Erk2 deficiency in vivo. Erk1-deficient mice are viable, fertile, and of normal size with minor defects, such as impaired terminal differentiation of T lymphocytes (85), and decreased adiposity with fewer adipocytes (89), suggesting a specific role of Erk1 in thymocyte development and adipogenesis.

Dual threonine and tyrosine phosphorylations activate both ERKs, at Thr202/Tyr204 for human ERK1 and Thr185/Tyr187 for human ERK2. Unlike MEK, significant ERK activation requires phosphorylation at both sites, with tyrosine phosphorylation preceding that of threonine (90). The ERKs are proline-directed protein kinases, phosphorylating Pro-neighboring Ser or Thr residues. Docking sites present on physiological substrates confer additional specificity (91). These docking interactions, through non-catalytic regions on ERK, team with scaffold proteins to ensure signaling fidelity and enzymatic efficiency both to and from the MAPK. Unlike the RAF kinases and MEK1/2, which have narrow substrate specificity, ERK1 and ERK2 have more than 175 documented cytoplasmic and nuclear substrates (92) (Figure 1-3).

Cytosolic substrates for ERK include several pathway components involved in negative feedback regulation (Figure 1-3). Negative feedback by ERK has been proposed to occur through direct phosphorylation of the EGFR at Thr669, which inhibits EGFR kinase activity (93, 94). Multiple residues on SOS are phosphorylated by ERK following growth factor stimulation (95, 96). SOS phosphorylation destabilizes the SOS-GRB2 complex, eliminating SOS recruitment to the plasma membrane and interfering with RAS activation of the ERK pathway. The RAF family kinases also are substrates of activated ERK (97, 98). Six phosphorylation sites on RAF1 and four phosphorylation sites on BRAF have been identified, which contribute to the down-regulation of RAS/ERK pathway by inhibiting binding to activated RAS or disrupting BRAF/RAF1 heterodimers (98). Finally, ERKs have also been demonstrated to negatively regulate themselves by phosphorylating MAP kinase phosphatases (MKPs), which reduces the degradation of these phosphatases through the ubiquitin-directed proteasome complex (99, 100).

MAPK-interacting kinase 1 (MNK1) and MNK2 are cytosolic serine/threonine protein kinases initially discovered as ERK-interacting proteins (101). Both ERK and p38, but not JNK, activate MNK by phosphorylation at Thr197 and Thr202. Activated MNK1, and possibly MNK2, upregulates eukaryotic initiation factor-4E (eIF-4E) in vitro through phosphorylation at
Figure 1-3. Downstream targets of ERK signaling.

ERK1 and ERK2 positively regulate transcription directly and indirectly via phosphorylation of p90 ribosomal protein S6 kinases (RSKs), mitogen- and stress-activated protein kinases (MSKs), and ternary complex factors (TCFs). Additionally, ERK1/2 indirectly regulate translation. ERK1/2 also provide negative feedback loops for the RAS/ERK signaling pathway.
Ser209, which is believed to enhance translation efficiency (102). The 90 kDa ribosomal S6 kinases (RSK) family of proteins are directly activated by ERK1/2 in response to growth factors, many polypeptide hormones, chemokines and other stimuli (103, 104). RSKs are characterized by the presence of two functional domains, the N-terminal kinase domain and the C-terminal kinase domain, connected by a linker region, which are activated in a sequential manner by a series of phosphorylation events following the binding of active ERK1 or ERK2 to an ERK docking site (D domain) located at the extreme carboxyl terminus of cytoplasmic RSK (105). RSKs phosphorylate many cytosolic and nuclear targets, regulating diverse cellular processes, including cell proliferation, survival, growth and motility (106). For example, RSKs regulate translation by modulating the PI3K-mTOR pathway at various steps (107-110). RSKs also play a direct role in cell-cycle regulation. For example, RSKs have been shown to phosphorylate the cyclin-dependent kinase (CDK) inhibitor p27^KIP1, which promotes its association with 14-3-3, prevents its translocation to the nucleus, and thereby promotes G1-phase progression (111). Activated RSKs also translocate to nucleus and regulate transcription by direct phosphorylation of transcription factors involved in IEG expression or by post-translational modification of IEG products (104, 112) (Figure 1-3).

Upon phosphorylation, ERK1 and ERK2 translocate into the nucleus (113), where they phosphorylate a wide range of targets, including transcription factors and a family of RSK-related kinases, the mitogen- and stress-activated protein kinases (MSKs) (114). MSK1 and MSK2 share the same tandem kinase structure as the RSKs, and also appear to be activated by sequential phosphorylation following ERK docking. MSKs phosphorylate and activate the activating transcription factor 1 (ATF1) at Ser63 (115), and the cAMP response element binding protein (CREB) at Ser133 (116). MSKs also are the major kinases for Histone H3 and high-mobility-group protein HMG-14, facilitating rapid induction of IEGs in response to mitogenic and stress stimuli in fibroblasts (117). The best-characterized transcription factor substrates of ERKs are ternary complex factors (TCFs), including Ets-like gene 1 (ELK-1) and SRF Accessory Protein 1 (SAP1) and SAP2, which are directly phosphorylated by ERKs at multiple sites (118, 119). Phosphorylated TCFs form complexes with the serum response factor (SRF) and activate the transcription of numerous mitogen-inducible genes regulated by serum response elements (SREs) (120). Another direct target of ERK is the proto-oncogene protein MYC, which is a transcription factor that regulates cell-fate decisions, including proliferation, growth and
apoptosis (121). Phosphorylation on Ser62 by ERK stabilizes MYC, and allows it to activate transcription as a heterodimeric partner with MAX (122). ERK can also directly phosphorylate components of activating protein-1 (AP1) family of transcription factors, c-JUN and c-FOS (123).

Finally, the ERK pathway has been demonstrated to directly link growth factor signaling to ribosome biogenesis. Following serum stimulation, ERK directly binds to and phosphorylates the BRF1 subunit of the RNA polymerase (pol) III-specific transcription factor TFIIB, which enhances translational efficiency by affecting tRNA and 5S rRNA synthesis (124). ERK also activates ribosomal RNA transcription following EGF stimulation by POL I through phosphorylation of upstream binding factor (UBF), preventing its interaction with DNA (125).

### 1.2 Noonan syndrome and the RASopathies

#### 1.2.1 Noonan syndrome

Noonan syndrome (NS; OMIM 163950) is the eponymous name for the genetic disorder described by the pediatric cardiologist Jacqueline Noonan (126). NS is thought to be relatively common, although its prevalence has not been determined accurately to date. Most authors cite the figure of 1 in 1,000–2,500 live births first reported by Nora and colleagues (127). NS is characterized by short stature, distinctive facial dysmorphic features, a wide spectrum of congenital heart defects (CHD), and an increased risk of hematopoietic malignancy. Other relatively common features are bleeding diathesis, ectodermal anomalies, lymphatic dysplasias, cryptorchidism, and cognitive deficits (128-130) (Table 1-1).

Facial features of NS include high forehead, hypertelorism, downslanting palpebral fissures, epicantal folds, ptosis, and low-set and/or posteriorly rotated ears (Figure 1-4). Besides the short and/or webbed neck, a low posterior hairline is common. Cardiac defects and short stature are the major reasons that patients with NS seek medical attention. While birth length is typically normal, growth parameters usually drop below the 3rd centile during the first years of life. Because there is often some attenuation and/or delay of the pubertal growth spurt, the prevalence of short stature in NS is highest during the age of normal puberty. This is accompanied by a delay in bone age. Although there have been reports of growth hormone (GH) deficiency, neurosecretory dysfunction, or GH resistance in NS (131-133), a consistent pattern of
Table 1-1. Clinical features of Noonan syndrome.

<table>
<thead>
<tr>
<th>Short stature</th>
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<tbody>
<tr>
<td>Facial dysmorphism</td>
</tr>
<tr>
<td>Triangular face shape</td>
</tr>
<tr>
<td>Broad forehead</td>
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<tr>
<td>Hypertelorism (widely set eyes)</td>
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<tr>
<td>Epicanthal folds (extra fold of skin at the inner corner of the eye)</td>
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<tr>
<td>Ptosis (drooping of the eyelids)</td>
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<tr>
<td>Down-slanting palpebral fissures</td>
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<tr>
<td>Low set and/or backward rotated ears</td>
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<tr>
<td>Cardiovascular defects</td>
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<tr>
<td>Pulmonic stenosis</td>
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<tr>
<td>Atrial septal defects</td>
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<tr>
<td>Ventricular septal defects</td>
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<tr>
<td>Mitral valve defects</td>
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<tr>
<td>Hypertrophic cardiomyopathy</td>
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<td>Hematological disorders</td>
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<tr>
<td>Leukemia</td>
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<td>Lymphedema</td>
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<tr>
<td>Developmental</td>
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<tr>
<td>Delay</td>
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<tr>
<td>Attention deficit/hyperactivity disorder</td>
</tr>
<tr>
<td>Skeletal</td>
</tr>
<tr>
<td>Pectus excavatum and/or carinatum</td>
</tr>
<tr>
<td>Vertebral anomalies</td>
</tr>
<tr>
<td>Webbed neck with low posterior hairline</td>
</tr>
<tr>
<td>Cryptorchidism (undescended testicles)</td>
</tr>
<tr>
<td>Dental/Oral problems</td>
</tr>
<tr>
<td>Feeding difficulties</td>
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</tbody>
</table>
Figure 1-4. Facial dysmorphism in Noonan syndrome.

abnormal GH secretion or action has not been shown so far, and it seems unlikely that there is a simple link between GH and the growth deficits in NS. GH treatment in NS is still a matter of debate. Recent research using our NS mouse model expressing the D61G mutant of Shp2, showed that NS-causing Shp2 mutants inhibit insulin-like growth factor 1 (IGF-I) release via GH-induced Erk hyperactivation, contributing to early postnatal growth delay (134).

NS patients exhibit a wide spectrum of cardiac disease (135, 136). Pulmonic stenosis (PS), septal defects, and HCM occur most commonly, but other lesions also are observed. Feeding problems are noted in the majority of affected infants and can cause failure to thrive (137). Developmental delay and learning problems are quite common. Some motor delay can be attributed to the hypotonia often observed in affected infants. An increased incidence of attention deficit/hyperactivity disorder and frank mental retardation also are observed. Available data indicate that the heterogeneity in the cognitive abilities observed in NS is at least partially attributed to the specific causative gene mutation (138, 139). Skeletal anomalies most frequently consist of pectus deformities, cubitus valgus, vertebral defects, and scoliosis. Children with NS also are at increased risk of developing myeloproliferative disorder (MPD) (140). Juvenile myelomonocytic leukemia (JMML; OMIM 607785), a rare MPD of childhood (141), arises at increased prevalence in NS, although it affects only a small percentage of patients (142). The MPD in most children with NS usually regresses without treatment, but occasionally follows an aggressive clinical course.

The diagnosis of NS depends primarily on clinical features, although the prevalence of the characteristic features among affected individuals has not been assessed rigorously and depends on the patient’s age (143). In newborns, the facial features can be less apparent and length is typically normal, but lymphedema and excess nuchal folds may be present. With time, several features become more obvious, including facial dysmorphism, pectus deformities, and reduced growth. Hypertrophic cardiomyopathy (HCM) also may develop during the first few years of life. The facial features also can become more difficult to detect in later adolescence and adulthood.

NS is a Mendelian trait transmitted in an autosomal dominant manner, and as observed for other dominant disorders, a significant percentage of cases is due to de novo mutations. Although NS is genetically heterogeneous (31, 32, 144), all known cases are caused by germ-line
mutations in conserved components of the canonical RAS/ERK pathway. Mutations in PTPN11, which encodes the protein tyrosine phosphatase SHP2, account for approximately half of NS cases (145). Other known NS genes include SOS1 (~10%) (146, 147), RAF1 (3-5%) (148, 149), KRAS (<2%) (150, 151), NRAS (152) and BRAF (153) (<1%). The causative genes responsible for the remaining 30% of NS cases remain to be identified. Mutations in some of these genes, as well as in genes encoding other RAS/ERK pathway components, also cause phenotypically related disorders, such as neurofibromatosis type 1 (NF1), Costello syndrome, cardio-facio-cutaneous syndrome (CFCS), and LEOPARD syndrome; together with NS, these syndromes are now termed “RASopathies” (32) (Figure 1-5).

The most common gene associated with NS is PTPN11, which accounts for approximately 50% of cases. SHP2, the protein product of PTPN11, is a non-receptor protein-tyrosine-phosphatase (PTP) that positively modulates RAS/ERK signaling (154). SHP2 is composed of two tandem N-terminal SH2 domains (N-SH2 and C-SH2), which function as phospho-tyrosine binding domains and mediate the interaction of this PTP with its substrates, followed by a catalytic PTP domain and a C-terminal tail with tyrosyl phosphorylation sites and a proline-rich motif (155). In the basal state, the catalytic function of the protein is auto-inhibited by interaction between the N-SH2 domain and the PTP domain, which blocks substrate access. Binding of an appropriate phosphotyrosyl peptide alters the conformation of the N-SH2 domain, prevents its binding to the PTP domain and causes catalytic activation of the protein (156). Most NS-causing missense mutations in PTPN11 affect residues involved in the N-SH2/PTP auto-inhibitory binding and up-regulate SHP2 function by impairing the switch from the active to inactive conformations. There also are mutations affecting residues located in the phosphopeptide-binding pocket of the N-SH2 or C-SH2 domains or in the linker stretch connecting these domains, which promote SHP2 gain of function by increasing the binding affinity, altering the binding specificity, or changing the flexibility of the N-SH2 domain in a manner that inhibits the N-SH2/PTP interaction (157-159).

SOS1 is the second most frequently mutated NS disease gene, accounting for approximately 10% of cases (146, 147, 160). The majority of NS-associated SOS1 mutations affect multiple domains that are responsible for stabilizing the protein in a catalytically autoinhibited conformation. Approximately half affect residues located in the short helical linker connecting the plekstrin homology (PH) and the RAS exchanger motif (REM) domains. A
Figure 1-5. RASopathies and the RAS-RAF-MEK-ERK MAPK pathway.

Schematic diagram showing the RAS/ERK pathway and affected disease genes in RASopathies. NS, Noonan syndrome; NS/LAH, Noonan-like syndrome with loose anagen hair; LS, LEOPARD syndrome; CS, Costello syndrome; CFCS, cardiofaciocutaneous syndrome; NF1, neurofibromatosis type 1; NFLS, neurofibromatosis type 1-like syndrome (also known as Legius syndrome).
second mutation cluster is located within the PH domain, while a third functional cluster resides at the interacting regions of the Dbl homology and REM domains. A single amino acid change (E846K) within the Cdc25 domain accounts for more than 10% of NS cases with SOSI mutations. Biochemical data demonstrate that NS-causing SOSI mutations disrupt the auto-inhibition of SOS1 RAS-GEF activity resulting in gain-of-function of SOS1 and a subsequently enhanced RAS/ERK activation (146, 147).

Germline KRAS mutations account for a small percentage (< 2%) of affected subjects in NS (150, 151, 161). NS-causing KRAS mutations up-regulate protein function and increase signaling down the RAS/ERK pathway by either reducing the intrinsic or GAP-stimulated GTPase activity and, consequently, impairing the switch between the active and inactive conformation, or by interfering with the binding of KRAS to guanine nucleotide (150, 162). More recently, two germline missense mutations of NRAS (T50I and G60E) conferring enhanced stimulus-dependent MAPK activation, were reported to account for a few NS cases (152).

Two groups (148, 149) identified multiple missense mutations of RAF1 in NS, which cluster in three regions (Figure 1-6). Approximately 70% of NS-associated RAF1 alleles alter the motif flanking S259 within the so-called CR2 domain, which binds to 14-3-3 proteins and is critical for auto-inhibition (163, 164). The second group of mutations (~15%) affects residues within the activation segment of the kinase domain (D486 and T491). The remaining alleles (~15%) involve two adjacent residues (S612 and L613) located C-terminal to the kinase domain. Transient transfection studies indicate that mutations affecting the 14-3-3 binding motif or the C-terminus of the protein enhance RAF1 kinase activity and increase MEK/ERK activation in cells. By contrast, mutations that cluster in the activation segment are kinase-impaired and reportedly act as dominant negative or null alleles (148, 149). Previous work suggested that the increased kinase activity of NS-associated CR2 domain mutants results from decreased S259 phosphorylation and consequent dissociation from 14-3-3 (149, 165, 166), but the mechanism underlying increased kinase activity of the RAF1 C-terminal mutants remained unclear. Likewise, how kinase-defective RAF1 alleles cause NS had remained obscure, if not paradoxical. Studies of kinase-defective BRAF alleles strongly implicate enhanced MEK/ERK activation and heterodimerization with RAF1 in human melanoma pathogenesis (167, 168). The paradoxical activation of the MEK/ERK pathway in wild type cells treated with selective small molecule
Figure 1-6. RAF1 domain structure and location of residues altered in NS.

RAF1 protein domains are indicated (CR, conserved region) along with two serine residues (below the cartoon), phosphorylation of which serve as 14-3-3 binding sites. The mutations observed in NS are shown above the cartoon.
BRAF inhibitors also has been attributed to the ability of these inhibitors to induce BRAF/RAF1 heterodimer formation (169, 170). The relevance of these observations for *RAF1* alleles expressed at physiological expression levels remained to be determined.

Germline *BRAF* mutations have also been documented in a small percentage of subjects with phenotypes fitting or suggestive of NS (< 1% of cases) (153). Of note, *BRAF* has previously been identified as a major disease gene underlying CFCS (50-75% of cases) (171, 172). NS-associated mutations largely do not overlap with those occurring in CFCS, suggesting a genotype-phenotype correlation.

NS is characterized by marked phenotypic variability, which can be explained, in part, by the underlying molecular lesions. *PTPN11* mutations are more prevalent among subjects with PS and short stature, and less common in individuals with HCM and/or severe cognitive deficits (173, 174). JMML is associated with a narrow spectrum of mutations affecting the *PTPN11* gene (142, 175), but also can be associated with certain germline *KRAS* mutations (150). Germline *KRAS* mutations are generally associated with a highly variable but generally severe phenotype (150, 151, 161). Subjects with a mutated *SOS1* allele tend to exhibit a distinctive phenotype characterized by ectodermal abnormalities and generally associated with a lower prevalence of cognitive deficits and short stature (146, 160). NS patients with *RAF1* mutations have a much higher incidence (~75%) of hypertrophic cardiomyopathy (HCM) than is found in the overall NS population (~18%). Notably, kinase-activating and kinase-impaired *RAF1* alleles are associated with different syndromic phenotypes. Only *RAF1* alleles encoding kinase-activated mutants are highly associated (~95%) with HCM (148, 149).

### 1.2.2 RASopathies: disorders clinically related to Noonan syndrome

#### 1.2.2.1 LEOPARD syndrome

LEOPARD syndrome (LS; OMIM 151100) is a rare autosomal dominant disorder that overlaps phenotypically with NS, including a ‘Noonan-like’ appearance as well as Lentigines, ECG conduction abnormalities, Ocular hypertelorism, Pulmonic stenosis, Abnormal genitalia, Retardation of growth, and sensorineural Deafness (acronym LEOPARD). Similar to NS, there are age-dependent aspects of the phenotype. Craniofacial dysmorphism is similar to that of NS
but is usually milder (176). Multiple lentigines, which are flat, black-brown macules, are dispersed primarily on the face, neck, and upper part of the trunk, sparing the mucosae. Growth retardation is observed in 25% of affected individuals. Approximately half of affected individuals have heart defects, which are similar to those in NS but occur with different frequencies. ECG anomalies, progressive conduction defects and HCM are the most frequent features. Of note, HCM is detected in up to 80% of LS patients with heart defects, most commonly appearing during infancy. LS is allelic with NS with a restricted spectrum of mutations in \textit{PTPN11} that cause decreased phosphatase activity and account for the vast majority of affected individuals (~90%) (Figure 1-5) (177-180). In a small proportion of cases, LS has been linked to mutations in \textit{RAFI} or \textit{BRAF} (149, 153, 181).

1.2.2.2  Noonan-like syndrome with loose anagen hair

Patients with Noonan-like syndrome with loose anagen hair (NS/LAH; OMIM 607721) show facial features reminiscent of NS, including macrocephaly, high forehead, hypertelorism, and low-set and posteriorly rotated ears, in addition to short and wedded neck and pectus abnormalities (182-184). Ectodermal involvement, severe short stature associated with proven GH deficiency (GHD), significant cognitive deficits and distinctive hyperactive behavior are other common features associated with these subjects. The hair anomalies include easily pluckable, sparse, thin, and slow-growing hair in the anagen phase, which fit a well-known condition termed loose anagen hair (LAH) syndrome (185), and suggest that this is a disorder distinct from NS. Most affected individuals also have darkly pigmented skin with eczema or ichthyosis. Cardiac anomalies are observed in the majority of cases, with mitral valve and septal defects significantly overrepresented compared with the general NS population. The voice of affected individuals is characteristically hypernasal. To date, NS/LAH appears to be genetically homogeneous, as all affected individuals share the same 4A>G missense mutation (an S2G amino acid substitution) in \textit{SHOC2} (Figure 1-5) (183), which encodes a leucine-rich repeat-containing scaffold protein required for the efficient transmission of information from RAS to the MAPK cascade (186). Functional studies of the SHOC2-S2G mutant demonstrated aberrant protein N-myristoylation, which resulted in aberrant targeting of SHOC2 to the plasma membrane and impaired translocation to the nucleus upon growth factor stimulation.
1.2.2.3  **CBL-mutation associated Noonan-like syndrome**

Three independent studies recently reported that heterozygous germline mutations in the *CBL* tumor suppressor gene, which is mutated in myeloid malignancies and encodes a multivalent adaptor protein with E3 ubiquitin ligase activity, underlie a previously unrecognized condition with clinical features fitting or partially overlapping NS in some individuals (Figure 1-5) (187-189). Missense mutations alter the evolutionarily conserved residues located in the RING finger domain or the linker connecting this domain to the N-terminal tyrosine kinase binding domain, a known mutational hot spot in myeloid malignancies that affects CBL-mediated receptor ubiquitylation and dysregulates signal flow through RAS (187). Common features occurring in *CBL* mutation-positive subjects include variable developmental delay, reduced growth, facial dysmorphism, café-au-lait spots, and predisposition to JMML during childhood (188, 189).

1.2.2.4  **Neurofibromatosis type 1 and related phenotypes**

Neurofibromatosis type 1 (NF1; OMIM 162200) is one of the most common autosomal dominant disorders (1 in 3000–4000 live births) caused by a single gene (190, 191). NF1 is characterized by multiple café-au-lait spots, axillary and inguinal freckling, Lisch nodules of the iris, cutaneous, subcutaneous and/or plexiform neurofibromas, skeletal dysplasia including short stature, dystrophic scoliosis and sphenoid wing dysplasia, learning deficits and behavioral abnormalities, and a predisposition for developing benign and malignant neoplasms, such as malignant peripheral nerve sheath tumors (MPNST) and optic pathway gliomas (OPG). Cardiovascular abnormalities in NF1 include congenital heart defects such as pulmonary artery stenosis, vasculopathy and hypertension. Heterozygous mutations of the *NF1* gene, which encodes Neurofibromin, a RAS-GTPase activating protein (RASGAP) that activates the intrinsic GTPase activity of RAS and negatively regulates its role in signal transduction, are observed in the vast majority of affected individuals (>90%) (Figure 1-5) (192). Loss-of-function mutations or deletions of the *NF1* gene lead to increased RAS/ERK signalling.

A disorder related to NF1 is NF1-like syndrome (NFLS; OMIM 611431), also known as Legius syndrome (193, 194). The most common clinical features of NFLS are multiple café-au-lait spots, axillary freckling, macrocephaly, NS-like facial dysmorphism, learning disabilities,
and attention deficit-hyperactive disorder (ADHD). Despite the clinical overlap with NF1, some typical features of NF1, such as Lisch nodules, neurofibromas and central nervous system tumors are absent in NFLS patients. NFLS is caused by loss-of-function mutations of the \textit{SPRED1} gene (193, 195), which encodes Sprouty-related EVH1 domain-containing protein 1 (SPRED1), a negative modulator of RAS/ERK pathway that suppresses phosphorylation and activation of RAF (Figure 1-5) (196).

1.2.2.5 Cardiofaciocutaneous syndrome

Cardiofaciocutaneous syndrome (CFCS; OMIM 115150) is an extremely rare and severe genetic disorder characterized by cardiac abnormalities, distinctive craniofacial dysmorphism, cutaneous abnormalities, failure to thrive, severe feeding problems, developmental delay, reduced growth, and abnormalities of the gastrointestinal tract and central nervous system (197, 198). Recurrent craniofacial features include macrocephaly, which is usually associated with prominent forehead, bitemporal constriction, and facial dysmorphia that is coarser compared with NS. The skin abnormalities include dryness and hyperkeratosis, ichthyosis, eczema, unusually sparse, brittle and curly scalp hair, and absent/sparse eyebrows and eyelashes. Pigmentary changes (such as café-au-lait spots, nevi or lentigines) and hemangiomas are observed. Cardiac defects occur in the majority of affected individuals, and consist of pulmonic stenosis and other valve dysplasias, septal defects, HCM and rhythm disturbances. Some form of neurologic and/or cognitive delay (ranging from mild to severe) is seen in all affected individuals; seizures are also frequent. CFCS is genetically heterogeneous, and is usually the result of \textit{de novo} dominant mutations in the \textit{KRAS}, \textit{BRAF}, \textit{MAP2K1} or \textit{MAP2K2} genes, which occur in approximately 60-90% of affected individuals (Figure 1-5) (171, 172).

1.2.2.6 Costello syndrome

Costello syndrome (CS; OMIM 218040) is the eponymous name for a rare genetic disorder described by pediatrician Dr. Jack Costello in 1970, as a condition characterized by prenatal overgrowth followed by severe failure to thrive, delayed development and mental retardation, distinctive coarse facial features, short stature, and cardiac defects (most commonly HCM, septal defects, valve thickening and/or dysplasia, and tachycardia), and musculoskeletal and skin abnormalities (unusually flexible joints and loose folds of extra skin on hands and feet)
Beginning in early childhood, affected individuals are predisposed to certain malignancies (most commonly rhabdomyosarcoma, neuroblastoma and transitional cell carcinoma) (201). Cutaneous papillomas in the perinasal and perianal regions are the most common benign tumors seen with this condition. CS is caused by germline missense mutations in HRAS (Figure 1-5) (202), but not in other RAS family genes. CS-associated HRAS mutations at codons 12 and 13 promote the active, GTP-bound conformation and constitutively activate downstream effectors such as MAP kinase, PI-3 kinase and RALGDS (203).

**1.2.3 Noonan syndrome mouse models**

In order to study the molecular pathogenesis of NS, several mouse models carrying NS-associated mutations have been generated and characterized in our and other laboratories. Araki *et al.* generated the first knock-in mouse model expressing the NS-associated gain-of-function mutant Shp2D61G (204). When homozygous, the D61G mutant causes embryonic lethality, whereas heterozygotes have decreased viability. Surviving *Ptpn11*<sup>D61G/+</sup> embryos (approximately 50%) have short stature, craniofacial abnormalities similar to those in NS patients, and MPD. Severely affected *Ptpn11*<sup>D61G/+</sup> embryos (approximately 50%) have multiple cardiac defects, including atrial, atrioventricular or ventricular septal defects, double-outlet right ventricle (DORV), and markedly enlarged outflow tract and atrioventricular valve primordia, similar to those in mice lacking Nf1 (205, 206). Their endocardial cushions have increased Erk activation, but Erk hyperactivation is cell- and pathway-specific. These data show that a single *Ptpn11* gain-of-function mutation can evoke all major features of Noonan syndrome by acting on multiple developmental lineages in a gene dosage-dependent and pathway-selective manner. Transgenic mice expressing a different NS-associated *Ptpn11* mutant (Q79R) also show valvulospetal defects and facial abnormalities seen in NS patients, which are prevented by the genetic ablation of *Erk1/2* or pre-natal pharmacological inhibition of Mek (207-209).

Later, using an inducible gene knock-in approach, our laboratory further elucidated the mechanism underlining the cardiac defects in NS caused by *Ptpn11* mutations, and showed that all cardiac defects in NS result from mutant Shp2 expression in the endocardium, not in the myocardium or neural crest (210). Moreover, the penetrance of NS defects is affected by genetic background and the specific *Ptpn11* allele. Finally, *ex vivo* assays and pharmacological approaches showed that NS mutants cause cardiac valve defects by increasing Erk MAPK
activation. However, we did not observe HCM in these NS mouse models caused by \textit{Ptpn11} mutations.

Chen \textit{et al.}, in collaboration with our laboratory, observed that activation of multiple signaling pathways causes developmental defects in mice with a Noonan syndrome–associated \textit{Sos1}^{E846K} gain-of-function mutation (211). Both heterozygous and homozygous mutant mice showed many NS-associated phenotypes, including growth defects, distinctive facial dysmorphism, hematologic abnormalities, and cardiac defects. The phenotypes in \textit{Sos1}^{E846K} mice and our NS-associated \textit{Ptpn11} mutation mouse models (204, 210) overlap to some degree, but are distinguishable. \textit{Sos1}^{E846K/+} mice develop left ventricular hypertrophy (LVH) and fibrosis, with or without aortic stenosis (AS). \textit{Sos1}^{E846K} mutation appears to selectively affect semilunar valves, whereas \textit{Ptpn11}^{D61G} has a more profound effect on atrioventricular valves. Although NS patients with \textit{SOS1} mutations develop PS, PS was not observed in the \textit{Sos1}^{E846K/+} hearts. The Ras/Erk pathway, as well as Rac and Stat3, are activated in \textit{Sos1}^{E846K} mutant hearts, suggesting that Rac and Stat3 activation might also contribute to NS phenotypes. Furthermore, prenatal administration of a MEK inhibitor ameliorated the embryonic lethality, cardiac defects, and NS features of the homozygous mutant mice.

1.3 Function and regulation of RAF1

The first \textit{raf} gene was described in 1983 as a retroviral oncogene, \textit{v-raf}, transduced by the murine sarcoma virus (MSV-3611) (212). Later, an avian homolog, \textit{v-mil}, was found in the acutely transforming avian retrovirus MH2 (213). These two retroviruses encoded the first oncogene with serine/threonine kinase activity to be discovered (214). After the cellular proto-oncogenes \textit{c-raf} (215) and \textit{c-mil} (216) were cloned, RAF proteins have been studied intensely. Initial studies demonstrated that CRAF (also known as RAF1) plays a critical role in mediating the cellular effects of growth factor signals (217-219). Later, RAF proteins were identified as the direct activators of MEK (220, 221) and as downstream effectors of RAS (222-226), acting as essential connectors between RAS and the MEK-ERK pathway. Most subsequent work focused on understanding this role and the regulation of RAF proteins in detail, until new kinase-independent functions of RAF1 in the regulation of apoptosis (227-229) and cell migration (230) emerged in the last decade.
Three different RAF isoforms, originating from three independent genes, are present in mammals: RAF1/CRAF, BRAF, and ARAF. All Raf isoforms are expressed ubiquitously in embryonic and adult mouse tissues. However, the Araf and Braf genes are more restricted in their expression, with Araf mRNA expressed particularly highly in urogenital organs and Braf mRNA abundant in neuronal tissues (231-233). Genetic studies in mice have shown that the Raf proteins have non-redundant functions in development. On a predominantly C57BL/6 genetic background, Araf-deficient mice survive to birth, but die between 7 and 21 days post-partum, displaying neurological and gastrointestinal defects (234). By contrast, Araf\(^{-/-}\) animals on a 129/OLA background, survive to adulthood, are fertile and do not display obvious intestinal abnormalities, although they do have a subset of the neurological defects seen on the C57BL/6 background. Mice with a targeted disruption in Braf die of vascular defects during mid-gestation. Braf\(^{-/-}\) embryos show growth retardation, an increased number of endothelial precursor cells, dramatically enlarged blood vessels and apoptotic death of differentiated endothelial cells (235). Raf1-deficient embryos also are growth retarded and die at mid-gestation with vascular defects in the yolk sac and placenta, abnormalities in the fetal liver, as well as increased apoptosis of embryonic tissues (236-238). The lack of compensation between the Raf proteins in mice does not seem to be the result of differences in expression patterns, which implies that the different isoforms have distinct functions.

1.3.1 Structure of RAF family kinases

All RAF isoforms share a common structure, consisting of three conserved regions (CR) with distinct functions (Figure 1-7). The CR1 region contains elements required for membrane recruitment, including a RAS binding domain (RBD), which binds to active RAS-GTP, and a cysteine-rich domain (CRD), which has a zinc finger structure homologous to those of protein kinase C (PKC). The CRD stabilizes the association with RAS through interaction with the lipid moiety present on processed RAS, and also is necessary for the interaction of CR1 with the kinase domain for RAF autoinhibition (222, 239-242). CR2 contains important inhibitory phosphorylation sites participating in negative regulation of RAS binding and RAF activation (243). Finally, CR3 comprises the kinase domain, including the activation segment, whose phosphorylation is crucial for kinase activation (244). In addition, phosphorylation of residues in the negatively-charged (N) region upstream of the CR3 is necessary for RAF activation (245). Functionally, the RAF structure can be split into a regulatory N-terminal region, containing CR1
Figure 1-7. Common structure of RAF proteins.

Schematic structure of RAF proteins depicting the three conserved region (CR1-3), the RAS-binding domain (RBD) and cysteine-rich domain (CRD), as well as the negative-charged regulatory region (N-region), glycine-rich loop (G-loop) and activation segment in which the phosphorylation sites crucial for activation are located.
and CR2, which is critical for activation as well as inhibitory phosphorylation, and a catalytic C-terminal region, which includes the phosphorylation sites necessary for kinase activation. The regulatory domain restrains the activity of the kinase domain (240, 246, 247) and its removal results in constitutive oncogenic activation (248). However, the activity of the isolated RAF1 kinase domain is subjected to further regulation and can be stimulated by phorbol esters, v-Src, and phosphorylation (247, 249). This observation is in keeping with the finding that the most common oncogenic mutation in $BRAF^{V600E}$ activates BRAF kinase activity by mimicking phosphorylation of the activation loop that releases its inhibitory interaction with the ATP-binding domain (167).

1.3.2 Regulation of RAF1

RAF regulation is highly complex and involves many steps, including membrane recruitment, dimerization, protein-protein interaction, conformational changes and phosphorylation (250-252).

1.3.2.1 The RAF1 activation/deactivation cycle

In the quiescent state, RAF1 is thought to exist in a closed, inactive conformation wherein the N-terminal regulatory region folds over and blocks the catalytic region (Figure 1-8) (253). This conformation is stabilized by 14-3-3 dimer binding to an N-terminal site, phospho-S259 (pS259), and a C-terminal site, pS621 (Figure 1-9). Although RAF1 activation is incompletely understood, numerous studies have suggested the following sequence of events (Figure 1-8).

Dephosphorylation of pS259 at the cell membrane by specific phosphatases, including protein phosphatase 2 (PP2A) and PP1, releases 14-3-3 from its N-terminal binding site on RAF1, allowing conformational changes to occur that unmask the RBD and CRD in the CR1 region to enable RAS binding and membrane recruitment (164, 254-258). RAS activation is under negative feedback regulation mediated by ERK and its downstream substrate RSK, which phosphorylate and inhibit SOS1 (95, 259). On the other hand, binding of RAF1 to RAS can be facilitated by the scaffolding protein SUR-8/SHOC2 (186). The CRD is necessary, but not sufficient, for stable membrane recruitment and activation of RAF1 (260, 261). RAF translocation to the membrane also is aided by the ability of RAF to interact with lipids (262,
These data suggest that the CRD may stabilize the primary recruitment of RAF1 exerted by the RBD through forging interactions with the lipid tails of RAS proteins (264). Furthermore, RAS isoforms reside in different subcellular compartments, which can influence interactions with RAF family members, and can profoundly influence the mechanism and kinetics of RAF activation (265).

Phosphorylation of multiple residues in the N-region upstream of CR3 and in the activation segment in CR3 is required for full RAF1 activation (Figure 1-8 and Figure 1-9). The N-region in RAF1 contains the S338/9 and Y340/1 phosphorylation sites, which are not only essential for full kinase activation but also for interaction with the substrate MEK (266-268). PAK (269, 270) and SRC family tyrosine kinases (271, 272), respectively, reportedly phosphorylate these sites. Other studies suggest that Ser338 is autophosphorylated upon RAF dimerization induced by mitogens (273), whereas others demonstrate that casein kinase 2 (CK2) phosphorylates Ser338 as a component of the KSR1 scaffold complex recruited to RAF (274). S338/9 phosphorylation itself only slightly elevates RAF1 kinase activity and mainly seems to serve as a priming event that initiates further activating modifications (266). S471 has been identified as a growth factor-induced RAF1 phosphorylation site, which is critical for RAF1 kinase activity and MEK binding (275). Finally, the phosphorylation of T491/S494 in the activation loop is RAS-dependent and required for full activation (244, 276), but the identity of the respective kinase(s) is unknown. Beside these major sites, phosphorylation at several minor sites also has been reported, including T268/269 (277, 278), T481 (275) and S497/499 (279). The role of these phosphorylations in RAF1 regulation remains unresolved, but they do not notably affect RAF1 activation. RAF homodimerization and heterodimerization have recently emerged as important regulatory mechanisms that drastically enhance kinase activity and downstream signaling, and are discussed in detail later.

Deactivation of RAF1 is initiated by specific binding of protein phosphatase 5 (PP5) to activated RAF1, which results in the dephosphorylation of pS338 (280). The phosphorylated N-region also serves as a binding site for the RAF kinase inhibitor protein (RKIP) (281, 282), which dissociates RAF1 from its substrate MEK (283, 284). In addition, RAF1 is subjected to direct feedback phosphorylation on multiple sites by ERK (Figure 1-9), which inhibit the activation of RAF1 by RAS and promote the subsequent dephosphorylation and resensitization of RAF1 by PP2A (97). Negative feedback from ERK to RAF1 also is suggested by a systematic
In quiescent cells, intramolecular autoinhibition of RAF1 is stabilized by the binding of 14-3-3 to the pS259 and pS621 residues. Upon membrane recruitment by activated RAS, pS259 is dephosphorylated by PP1 or PP2A. Subsequently, phosphorylation of the N-region and activation loop by as yet incompletely defined upstream kinases, and homodimerization or heterodimerization with other RAF isoforms, cause full activation of RAF1. Following MEK/ERK activation, RAF1 is deactivated through PP5-mediated dephosphorylation of pS338, and ERK-mediated feedback phosphorylation, which desensitize the kinase. PP2A, through the prolyl isomerase PIN1, and maybe other unknown phosphatases, dephosphorylate the remaining activating sites and the ERK feedback sites. RAF1 ultimately reverts to its closed, inactive conformation upon rephosphorylation of S259 by AKT, PKA or unknown kinases, allowing intramolecular bidentate 14-3-3 rebinding. Activating events are colored red and deactivating processes are in black.
Figure 1-9. Regulatory phosphorylation sites of RAF proteins.

The structure and phosphorylation residues of the three RAF isoforms. Red residues indicate activating phosphorylation sites, black are ERK feedback phosphorylation sites, and blue are 14-3-3 binding sites.
analysis of feedback regulation of the RAS/ERK pathway based on mathematical modeling (285), although several ERK feedback phosphorylation sites also were described as stimulating RAF1 activity (286).

RAF1 also is negatively regulated by cAMP–activated kinase (PKA)-catalyzed phosphorylation of several sites, including S43, S233, S259 and S621. The phosphorylation of S43 interferes with RAS binding (287, 288), whereas phosphorylation of S233 and S259 enhances the binding of 14-3-3 and suppresses RAF1 kinase activity (289, 290). Studies also show that S259 could be phosphorylated by AKT directly or indirectly (291-293). The phosphorylation of S621 and binding of 14-3-3 to pS621 appears to have multiple roles in RAF1 regulation. On the one hand, it serves as a negative regulatory phosphorylation site to stabilize the closed, inactive conformation in resting cells (294, 295); on the other hand, its inhibitory function is converted into an essential component of RAF1 activation following stimulation (296). For example, pS621 increases the stability of RAF1 by preventing proteasome-mediated degradation (297). Binding of 14-3-3 to pS621 also enhances RAF dimerization (98, 168, 298, 299), and is required for the kinase domain to bind ATP (296). However, several studies demonstrate that S621 phosphorylation is largely mediated by autophosphorylation (296, 297). The phosphatase(s) responsible for dephosphorylating S621 is(are) currently unknown.

1.3.2.2 RAF homodimers and heterodimers

Dimerization is a frequent mechanism for the activation of kinases. Homodimerization was initially highlighted as a potentially important step of RAF1 activation by two studies showing that forced interaction of RAF1 monomers tagged with inducible dimerizing tags robustly induced kinase activity (300, 301). Both studies proposed that active RAS would promote the formation of dimers. This hypothesis was later extended to heterodimerization between RAF1 and BRAF, which was found to be inducible by active RAS (302). These initial studies showed that homodimerization and heterodimerization can hyperactivate RAF kinases. Later, Rushworth et al. demonstrated that endogenous BRAF and RAF1 heterodimerize in multiple cell lines in response to mitogens (298). Biochemical fractionation of RAF heterodimers from homodimers and monomers showed that RAF1/BRAF heterodimers accounted for the majority of the mitogen-induced RAF kinase activity, suggesting that RAF1 and BRAF cooperate to drastically elevate their kinase activity when forming heterodimers. Given that
heterodimers between wild-type RAF1 and kinase-defective BRAF still display elevated kinase activity, as do heterodimers between wild-type BRAF and kinase-impaired RAF1, either kinase-competent RAF isoform is sufficient to confer high catalytic activity to the heterodimers.

Heterodimerization plays a pathological role in certain cancers. When BRAF mutations were discovered in cancer (35), a puzzling observation was that while the most frequent mutation, V600E, massively enhanced BRAF kinase activity, several less frequent BRAF mutations only mildly increased, or even impaired, kinase activity (167). Nevertheless, even kinase-impaired BRAF mutants could hyperactivate the MEK/ERK pathway. Intriguingly, this activation was dependent on the presence of RAF1, and a subsequent study demonstrated that the kinase-impaired BRAF mutants found in human cancers indeed could promote RAF1 heterodimer formation (168). Although physiological RAF1/BRAF heterodimerization is induced by RAS activation, oncogenic BRAF mutants constitutively dimerize with RAF1 (168, 298). More recently, an important role for RAF heterodimers in response to ATP-competitive RAF kinase inhibitors was described. The original observation that RAF inhibitors paradoxically induce ERK cascade signaling can now be explained by the ability of RAF inhibitors to promote RAF heterodimerization and activation in the presence of oncogenic or normally activated RAS (169, 170, 303).

Mutation of S621 abrogates RAF heterodimerization, suggesting that 14-3-3 binding to pS621 is essential for RAF heterodimerization (302). Indeed, heterodimerization is enhanced by wild type 14-3-3, but not a dimerization-negative 14-3-3 mutant, suggesting that the 14-3-3 dimer crosslinks RAF1 and BRAF by binding to the C-terminal sites on each kinase (298). This observation suggests a mechanism for how 14-3-3 can stabilize both inactive and active RAF1 conformations. In the inactive conformation, 14-3-3 clasps the RAF1 regulatory domain to the kinase domain via intramolecular binding to pS259 in the N-terminus and p621 in the C-terminus. Dephosphorylation of pS259 and binding to activated RAS displaces 14-3-3 from S259, leaving one 14-3-3 arm free to contact with the 14-3-3 binding site on BRAF in order to facilitate heterodimerization. RAF heterodimerization also is regulated by ERK-mediated feedback phosphorylation on RAF (97, 298). The feedback phosphorylation mainly serves to limit the lifetime of RAF1/BRAF heterodimers, and mutation of the relevant sites enhances ERK signaling and the associated biological activities. Other regulators include KSR1 (304) and MLK3 (305), which both enhance heterodimerization.
The precise mechanism by which the juxtaposition of RAF molecules stimulates kinase activity was elucidated recently by Rajakulendran et al. (299). They demonstrated that activation of the kinase domain of RAF is controlled by an allosteric interaction between two kinase domains in a specific side-to-side dimer configuration, which is thought to position a critical helix (helix αC) in the kinase domain in a productive conformation necessary for catalytic activity (Figure 1-10). The dimer interface region in the RAF kinase domain is conserved in the KSR kinase domain, which also can serve as an allosteric activator of RAF by forming KSR/RAF side-to-side heterodimers. Given that all isoforms of RAF and KSR share a nearly identical side-to-side dimer interface, the question still remains as to how the cell regulates specific dimer formation between the multiple isoforms of RAF and KSR proteins found in higher-order metazoans. Presumably, cells must have a separate mechanism for selectively driving dimer formation between any two specific isoforms. For example, a recent study showed that most RAF inhibitors induce KSR1/BRAF binding, but promote little complex formation between KSR1 and RAF1 (306). These works also found that KSR1 competes with RAF1 for inhibitor-induced binding to BRAF.

1.3.2.3 Scaffolds and modulators of RAF signaling

Multiple studies have revealed scaffolding as a mechanism that helps the ERK cascade to transduce signals with high efficiency and specificity. Scaffolds also appear to be involved in the spatial restriction of ERK activity and signaling to distinct subcellular compartments (Figure 1-11) (307). Therefore, scaffolds can have a huge impact on the biochemical and biological behavior of the ERK pathway (308, 309). However, our knowledge of their role in the functional modulation of the pathway and their exact mechanism of action is still limited.

The best-characterized scaffold of the ERK pathway is kinase suppressor of Ras (KSR) (310). KSR1 has a kinase domain with high homology with RAF1. But this kinase domain contains mutations in residues critical for catalytic activity. Whether KSR1 has kinase activity or is a pseudokinase have been debated. As a scaffold for the ERK pathway, KSR1 can interact with all kinases of the ERK pathway. MEK is bound constitutively, while RAF and ERK are recruited to KSR1 upon mitogen stimulation (311, 312). However, KSR1 binds less than 5% of endogenous RAF1 (313), indicating that it might affect only a subset of RAF functions. Experiments with KSR-deficient mice indicate that KSR is not absolutely required, but enhances
Figure 1-10. Allosteric mechanism for activation of the kinase domain of RAF1.

RAF1 exists in an inactive conformation characterized by the monomeric state of its kinase domain. The N- and C-terminal lobes of RAF or KSR kinase domains are indicated. Following RAS activation, the kinase domain of RAF1 transitions from an inactive monomeric state to an active side-to-side dimer, characterized by the juxtaposition of a critical helix (helix $\alpha$C) in the N-lobe. The cellular pool of RAF/KSR proteins contains multiple isoforms, and the mechanism by which the cell selectively drives dimer formation between two given isoforms is currently unknown.
Figure 1-11. Scaffolding proteins in RAF-MEK-ERK signaling.

Scaffolding proteins form RAF-MEK-ERK signaling platforms at different subcellular localizations. See text for details.
signaling from RAS (314). Recent studies have shown that KSR is a functional protein kinase, presumably stimulated allosterically by forming side-to-side dimers with RAF, and catalyzes phosphorylation of MEK1 on non-activation segment residues to facilitate RAF-mediated phosphorylation of MEK1 activation segment serines (315, 316).

Another group of RAF scaffolds is the connector enhancer of KSR (CNK) family of proteins, which lack kinase activity but contain different protein-protein interaction domains that can bind a variety of proteins, including RAF (309, 317). CNK1 can augment RAF1 activation by increasing tyrosine phosphorylation of the N-region through the recruitment of c-SRC (318). Interestingly, CNK1 also can bind Ras association domain-containing protein 1A (RASSF1A) and enhance apoptosis in a MST2-dependent manner, which may play a role in balancing apoptosis and proliferation by coordinating MST2 binding to RASSF1A or RAF1 (319). Another family of multi-domain scaffolding proteins is IQ motif containing GTPase-activating proteins (IQGAPs), which directly interact with, and modulate the functions of, BRAF, MEK, and ERK (320, 321). Prohibitin (PHB) facilitates the displacement of 14-3-3 from RAF1 by activated RAS, thereby promoting plasma membrane localization and phosphorylation of RAF1 at the activating S338 (322, 323).

Scaffolding proteins also are crucial for the localization of members of the ERK pathway to different subcellular signaling platforms. Similar expression to FGF (SEF1), which is located at the Golgi apparatus, is a transmembrane scaffold for MEK and ERK (324). Importantly, SEF1 only binds activated MEK and inhibits the dissociation of the MEK-ERK complex, which blocks nuclear translocation of ERK, allowing the activation only of cytoplasmic ERK targets (324). The β-arrestins are proposed to augment ERK activation in clathrin-coated pits by scaffolding RAF1, MEK, and ERK (325). Similar to SEF1, β-arrestins also prevent ERK nuclear translocation and therefore restrict RAS signaling to cytoplasmic effectors of the pathway. The small scaffold MEK partner-1(MP1) is an obligatory heterodimer with p14, and this complex interacts with MEK and ERK, targeting them to late endosomes (326, 327). In addition, MP1 may target MEK-ERK to high molecular weight protein complexes (328), organized by MAPK organizer 1 (MORG1), which was identified as an interaction partner of MP1 as well as RAF1, BRAF, MEK, and ERK (329). MP1 also forms the Ragulator complex with p14 and p18, which is required for the recruitment and activation of mTORC1 at the lysosomal surface (330). The
multidomain protein paxillin is a component of focal adhesions, providing a structural and signaling link between the actin cytoskeleton and the extracellular matrix (ECM) (331). Paxillin constitutively interacts with MEK, but also binds to activated RAF and ERK in response to growth factors, directing activated ERK to focal adhesions (332).

1.3.3 MEK-independent functions of RAF1

Studies using conventional and conditional Raf1 knockout mice (237, 238, 333) led to the discovery of new RAF1 effector pathways in which regulation occurs through protein-protein interactions. For some of these pathways, RAF1 kinase activity is dispensable (Figure 1-12).

1.3.3.1 RAF1 and apoptosis

RAF1 regulates apoptosis through multiple targets. A mitochondrial pool of RAF1 has been shown to protect cells from apoptosis (Figure 1-12). RAF1 can be targeted to the mitochondria via its interaction with BCL2 (334). In addition, RAF1 serves as a scaffold to recruit protein kinase C theta (PKCθ) to phosphorylate and inactivate the pro-apoptotic BCL-2 family member BAD (335, 336). Another possible mechanism is the direct interaction between RAF1 and mitochondrial voltage-dependent anion channels (VDACs), which may be responsible for the RAF-induced inhibition of cytochrome c release from mitochondria (337).

\textit{Raf1} ablation in mice demonstrated that Raf1 is required for survival and protection against apoptosis (237, 238). Surprisingly, reconstituting \textit{Raf1}–/– mice with a kinase-impaired Raf1 mutant (Raf-1^{YY340/1FF}) fully rescues the apoptotic phenotype and produces viable mice (238). Studies have shown that several mechanisms account for the anti-apoptotic function of Raf1 (Figure 1-12). These may operate in a tissue-specific manner, but none requires Raf1 kinase activity. One mechanism is through the deregulation of the Rho effector kinase Rok-α, which is up-regulated and mislocalized to the membrane in \textit{Raf1}-deficient cells (229). This results in a defect in the internalization of the Fas death receptor, which increases Fas clustering and membrane expression, enhancing Fas sensitivity.

The other targets of RAF1 in apoptosis suppression include two pro-apoptotic kinases, apoptosis signal-regulating kinase 1 (ASK1) (227, 338) and MST2 (228, 339) (Figure 1-12). ASK1 is a member of the MAP kinase kinase kinase family that selectively activates JNK and p38 to promote apoptosis induced by various cytotoxic stresses, such as reactive oxygen species
RAF1 can suppress apoptosis in a MEK-independent fashion in several ways: 1) by binding to and inhibiting ASK1; 2) by suppressing cytochrome C release from mitochondria through voltage-dependent anion channels (VDACs); 3) by acting as scaffold to recruit PKCθ to phosphorylate and inactivate BAD; 4) by binding to and inhibiting the mammalian MST2 pathway; and 5) by inhibiting ROK-α-induced FAS maintenance and clustering at the cell membrane. Inhibition of ROK-α by RAF1 binding also is required for regulating motility through the actin cytoskeleton and for skin tumorigenesis by preventing keratinocyte differentiation and sustaining MYC expression.
(ROS), or by death receptors, such as the TNF-α receptor or FAS (340). RAF1 interacts with the N-terminal regulatory fragment of ASK1 and inhibits its proapoptotic function (227). Cardiac-specific disruption of Raf1 results in heart dilation and dysfunction with a significant increase in cardiomyocyte apoptosis, all of which are rescued by the ablation of Ask1 (338). MST2 was identified in a proteomics screen for RAF1-associated proteins (228). RAF1 binds to the SARAH domain of MST2, thereby preventing the dimerization and phosphorylation of the activation loop of MST2, independent of RAF1 kinase activity. RASSF1A, a tumor suppressor, can disrupt the RAF1–MST2 complex and promote the assembly of a proapoptotic signaling complex consisting of RASSF1A, MST2, LATS1, and YAP1 (339, 341). Activated YAP1 translocates into the nucleus and interacts with p73, promoting the expression of proapoptotic BH3-only protein p53-upregulated modulator of apoptosis (PUMA) to induce apoptosis (341). Interestingly, RAS binding to RAF1 enables RAF1 to activate the MEK-ERK pathway and promote proliferation, but also induces dissociation of the RAF1-MST2 complex and promotes apoptosis (342).

1.3.3.2 RAF1 regulates cell motility and differentiation through ROK-α

Conditional gene ablation of Raf1 in keratinocytes delays migration and wound healing (230). The target of Raf1 in motility is Rok-α (Figure 1-12). Raf1 knockout fibroblasts and keratinocytes show a contracted appearance and fail to migrate due to the hyperactivity and incorrect localization of Rok-α to the plasma membrane. Similar to Raf1, Rok-α is regulated by auto-inhibition, and its C-terminal regulatory region features a domain highly homologous to the CRD in Raf1. The Raf1 regulatory domain can cross-regulate Rok-α by binding to the Rok-α kinase domain and repressing its function in trans (343). The biological relevance of the Raf1: Rok-α complex was observed in a Ras-induced skin tumor mouse model (344). In this model, the inhibition of Rok-α by Raf1 was required for Ras transformation by decreasing the expression of epidermal differentiation cluster (EDC) and activating the Stat3/Myc pathway, thereby promoting dedifferentiation of tumor cells.

1.4 Hypertrophic cardiomyopathy and the RAS/ERK pathway

1.4.1 Cardiac hypertrophy and hypertrophic cardiomyopathy

The mammalian heart is a dynamic organ, which is composed of cardiomyocytes, non-myocytes (e.g., fibroblasts, endothelial cells, mast cells and vascular smooth muscle cells) and
surrounding extracellular matrix (345, 346). Cardiomyocytes are specialized muscle cells composed of bundles of myofibrils, which contain repeating contractile units known as sarcomeres (347). It is generally believed that most cardiomyocytes lose their ability to proliferate at or soon after birth, and that the subsequent growth of heart occurs primarily as a result of an increase in myocyte size (348). In adults, the growth of heart is closely matched to its functional load. In response to elevated workload, the heart undergoes a hypertrophic response, which is characterized by an increase in the size of individual cardiomyocytes, to compensate for the increase in wall stress (345). This hypertrophic response can be classified as one of two general types, physiological or pathological, which are caused by different stimuli, are functionally distinguishable, and are associated with distinct structural and molecular phenotypes (Figure 1-13) (349, 350).

Physiological growth of the heart includes the embryonic and fetal stages of development and the rapid postnatal growth stage (345). In the adult, physiological hypertrophy usually occurs in response to chronic exercise training (351) or pregnancy (352). Pathological cardiac hypertrophy is caused by genetic mutations (primary) (353, 354) or in response to diverse stimuli, such as hypertension, valvular stenosis or myocardial infarction (secondary) (345, 355). With increased cardiac stress, pathological cardiac hypertrophy might initially represent a compensatory mechanism for increasing cardiac function and decreasing ventricular wall tension. However, chronic pathological hypertrophy eventually leads to functional decompensation, and predisposes individuals to ventricular dilatation, heart failure, arrhythmia and/or sudden death (356, 357). By contrast, physiological hypertrophy does not decompensate into dilated cardiomyopathy or heart failure (351, 358).

Physiological and pathological hypertrophy can be further sub-classified as concentric (hearts with thick walls and relatively small cavities) or eccentric (hearts with chamber enlargement and a proportional change in wall thickness) (359, 360). Isotonic exercise (e.g., running, walking or swimming) causes volume overload and produces eccentric physiological hypertrophy, whereas isometric or static exercise (e.g., weight lifting) causes pressure overload and results in concentric physiological hypertrophy (345, 358). Pathological stimuli causing pressure overload, such as hypertension or aortic stenosis, produce concentric hypertrophy. By contrast, volume overload (e.g., mitral or aortic regurgitation) that causes increased diastolic wall stress or myocardial infarction (MI), can result in eccentric hypertrophy (359, 360). Concentric
Figure 1-13. Types of cardiac hypertrophy.

RV, right ventricle; LV, left ventricle.
hypertrophy is characterized by assembly of sarcomeres in parallel, resulting in a relative increase in the width of individual cardiomyocytes. By contrast, eccentric hypertrophy is characterized by assembly of sarcomeres in series, leading to a relatively greater increase in the length than in the width of cardiomyocytes. As mentioned above, concentric pathological hypertrophy may progress to eccentric hypertrophy, and then eventually to cardiac dilation with associated systolic heart failure (361).

Animal studies have demonstrated that physiological and pathological hypertrophy are associated with distinct histological and molecular characteristics (362-364). In mice, a surgical model of pathological hypertrophy can be established by transverse aortic constriction (TAC), which causes pressure overload on the left ventricle (365, 366). TAC initially leads to compensated hypertrophy, which often is associated with a temporary increase of cardiac contractility. However, over time, the response to the chronic hemodynamic overload becomes maladaptive, resulting in cardiac dilatation and heart failure. Pathological hypertrophy usually is associated with increased apoptosis, necrosis and interstitial fibrosis (367). In response to pathological stimuli, cardiac fibroblasts and extracellular matrix proteins accumulate disproportionately and excessively surrounding the cardiomyocytes, which can lead to mechanical stiffness, and contribute to cardiac dysfunction (368). Pathological cardiac hypertrophy typically is associated with reactivation of fetal genes, including atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and genes for the fetal isoforms of contractile proteins, such as skeletal α-actin and β-myosin heavy chain (β-MHC). These changes can be accompanied by down-regulation of genes normally expressed at higher levels in the adult, such as α-MHC and sarcoplasmic reticulum Ca2+-ATPase (SERCA2a) (369). This altered gene expression program affects cardiac function, leading to altered contractility, elevated end-diastolic pressure and arrhythmias. By contrast, these histological and molecular changes are not observed in models of physiological cardiac hypertrophy induced by exercise training (364, 370, 371).

Primary hypertrophic cardiomyopathy (HCM) is the prototypical genetic form of pathological hypertrophy. The hallmark of HCM is cardiac hypertrophy in the absence of an obvious inciting hypertrophic stimulus (372). Complications of HCM include left ventricular outflow tract obstruction, arrhythmias, diastolic dysfunction, MI, stroke, infective endocarditis.
and sudden cardiac death (373). Hearts from HCM patients commonly show cardiomegaly and left ventricular hypertrophy (LVH). In end-stage disease, cardiac remodeling occurs, leading to dilatation of the ventricles and atria and thinning of the ventricular wall due to myocardial scarring (374). Myocyte disarray, the loss of the normal parallel alignment of cardiomyocytes, is the most characteristic and common histological finding in HCM (373). Also, HCM usually is associated with interstitial myocardial fibrosis and variation in the size of myocyte nuclei.

Autosomal dominant mutations in several genes encoding proteins of the cardiac sarcomeric apparatus (e.g., β-MHC, cardiac troponin T, and myosin-binding protein C) account for 60-70% of all cases of primary HCM (373). Such mutations usually alter sarcomere structure and function and result in mechanical, biochemical and/or bioenergetic stresses that activate cardiomyocyte signaling pathways to mediate the hypertrophic phenotype (375-378). Non-sarcomeric genes responsible for myocardial metabolism also have been identified in HCM, such as lysosome-associated membrane protein-2-α-galactosidase (LAMP2) (379). Aberrant activation of hypertrophic signaling pathways can themselves result in hypertrophy. For example, germ line mutations in adenosine monophosphate-activated protein kinase (AMPK) are a rare cause of HCM (380-382).

Moreover, the discovery of mutated RAS/ERK pathway-related genes in RASopathies has underlined the relevance of this signaling pathway to HCM (32). HCM occurs with a high incidence in CS (200, 383) and LS (176, 384), whereas it is less common in CFCS (171, 385) and NS (146, 173) patients. However, NS-associated kinase-activating RAF1 mutants are highly associated with HCM (148, 149).

1.4.2 Signaling pathways involved in cardiac hypertrophy

Studies of transgenic and knockout mice, in combination with surgical and exercise models, have revealed signaling cascades that play important roles in regulating cardiac hypertrophy, such as the GPCR signaling, the calcineurin-nuclear factor of activated T cells (NFAT) pathway, the insulin-like growth factor-I (IGF-I)-phosphoinositide-3 kinase (PI3K) cascade, and the MAPK pathway (349, 350, 386) (Figure 1-14). To date, the best characterized examples of pathways that play distinct roles in pathological and physiological hypertrophy are \( G_{aq/11} \)-dependent and IGF-I signaling, respectively.
The secretion of cardiac paracrine and/or autocrine factors, including angiotensin-II (Ang-II), endothelin-1 (ET-1) and catecholamines (α-adrenergic), in response to a pathological stimulus plays an important role in the development of pathological cardiac hypertrophy (387-391) (Figure 1-14). These ligands bind to specific GPCRs that are coupled to heterotrimeric G proteins of the $G_{aq/11}$ subclass, which leads to the activation of downstream signaling molecules, such as protein kinase C (PKC), and the mobilization of internal Ca$^{2+}$. Ca$^{2+}$ stores have been shown to regulate cardiac hypertrophy by activating the calcineurin-NFAT pathway (392, 393) and by promoting the calmodulin-dependent kinase (CaMK)-mediated nuclear export of histone deacetylases (HDACs) (394). Calcineurin, which is a Ca$^{2+}$-dependent serine/threonine protein phosphatase, binds to and dephosphorylates transcription factors of the NFAT family, resulting in their nuclear translocation and the activation of pro-hypertrophic gene expression (392). The class II HDACs are implicated in cardiac hypertrophy by means of chromatin remodelling and altered gene expression. Nuclear export of class II HDACs triggered by CaMKII-mediated phosphorylation releases the suppression of hypertrophic gene transcription and induces cardiac hypertrophy (394). Activation of $G_{aq/11}$ also can induce MAPK pathways in cardiomyocytes, although the exact mechanism remains unknown (395).

IGF-I has been implicated in the regulation of developmental and physiological growth of the heart, and is released in response to exercise training (396-400) (Figure 1-14). IGF-I acts via the IGF-I receptor, a receptor tyrosine kinase that activates class IA PI3K ($PI3K_\alpha$). PI3K activation results in the sarcolemmal recruitment and activation of the kinase AKT (also known as PKB) (401). Glycogen synthase kinase-3β (GSK-3β), which is inhibited by AKT-mediated phosphorylation, is an important downstream target of AKT in the heart. GSK-3β negatively regulates hypertrophic transcriptional effectors (e.g., GATA4, β-catenin, c-Myc and NFAT), and inhibits the translation initiation factor eIF2B. AKT also enhances protein synthesis and cell growth by activating the mTOR pathway (402). However, the role of the PI3K-AKT signaling pathway in promoting cardiac hypertrophy is complex, as each of the effectors in this pathway can have adaptive or maladaptive influences on the myocardium, depending on the type and duration of the stimuli (350, 354). For example, short-term activation of AKT in cardiomyocytes results in physiological adaptive hypertrophy, whereas chronic activation produces pathological hypertrophy (403). Excessive PI3K-AKT-mTOR signaling also has been recently associated with pathological cardiac hypertrophy in LS, wherein rapamycin treatment reverses LS cardiac
Figure 1-14. Signaling pathways involved in cardiac hypertrophy.

ANP, atrial natriuretic peptide; Ang II, angiotensin II; BNP, B-type natriuretic peptide; CaMK, calmodulin-dependent kinase; CDK, cyclin dependent kinase; DAG, diacylglycerol; Endo-1, endothelin-1; GC-A, guanylyl cyclase-A; GPCR, G-protein coupled receptors; GSK3β, glycogen synthase kinase-3β; HDAC, histone deacetylases; IκB, inhibitor of NF-κB; IGF-I, insulin-like growth factor-I; IKK, inhibitor of NF-κB kinase; MEF, myocyte enhancer factor; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PKD, protein kinase D; PLA2, phospholipase A2; PLC, phospholipase C; Pol II, RNA polymerase II; TAK, TGFβ-activated kinase; TGFβ, transforming growth factor-β; TNFα, tumour necrosis factor-α. Adopted from Heineke and Molkentin, Nature Reviews Molecular Cell Biology 7, 589-600 (350).
defects (404).

MAPK signaling is initiated in cardiomyocytes by GPCRs (395), RTKs (e.g. IGF-I, EGF and FGF receptors) (405), receptor serine/threonine kinases (e.g. transforming growth factor-β1 (TGF-β1) receptor) (406), glycoprotein-130 (gp130), the receptor for the interleukin-6 (IL-6)-related cytokines (407), and by stress stimuli, such as mechanical stretch (408) (Figure 1-14). Activated MAPKs each phosphorylate numerous downstream targets, including transcription factors that induce the reprogramming of cardiac gene expression.

The role of the RAS/ERK pathway in cardiac hypertrophy has been controversial. Some data argue that excessive activity of this pathway causes HCM, whereas other evidence suggests its involvement in physiological, but not pathological, cardiac hypertrophy (409, 410). Transgenic mice with cardiac-specific expression of oncogenic HRas (G12V) display significant cardiac hypertrophy, decreased contractility, diastolic dysfunction associated with interstitial fibrosis, induction of cardiac fetal genes and sudden death (411-413), all of which are consistent with HCM. However, besides RAF-MEK-ERK activation, RAS also can activate the JNK branch of the MAPK cascade, PI3K and other signaling pathways. Therefore, the cardiac defects observed with constitutive RAS activation could be independent of MEK-ERK signaling. In cultured cardiomyocytes, depletion of Erk1/2 with antisense oligonucleotides, or pharmacological inhibition of Mek1/2, attenuates the hypertrophic response to agonist stimulation (414, 415). Mice with cardiac-specific over-expression of “dominant-negative” Raf1 show reduced phosphorylation of the Erk target Elk1 and have no overt phenotype, but they are resistant to the development of cardiac hypertrophy in response to pressure overload (416). These data suggest that signals from Raf1 are necessary for promoting the pathological hypertrophic response. The RAS/ERK pathway also has been implicated in inducing the re-expression of fetal cardiac genes by altering the levels and activities of cardiac transcription factors, such as GATA4, MEF2 and NFAT (417).

On the other hand, transgenic mice expressing an activated Mek1 allele under the control of the α-MHC promoter have concentric hypertrophy with enhanced contractility, show no signs of decompensation over time and reportedly do not progress to pathological hypertrophy up to 6 months of age (418). This study also found that the Mek1-Erk1/2 pathway induces cardiac hypertrophy partially by enhancing the transcriptional activity of Nfat, indicating crosstalk
between the Ras/Erk and the calcineurin-Nfat pathways (419). Another study argued against any role for Erk1/2 in cardiac hypertrophy, as Erk1<sup>−/−</sup>Erk2<sup>+/−</sup> mice, as well as transgenic mice with cardiac-specific expression of dual specificity phosphatase 6 (Dusp6), an Erk1/2-specific phosphatase, showed a normal hypertrophic response to pressure overload and exercise (420). A more recent study from the same group using mice lacking all Erk1/2 proteins in the heart demonstrated that Erk1 and Erk2 regulate the balance between eccentric and concentric cardiac growth (421). Although loss of Erk1/2 in the heart (Nkx2.5-Cre) or specifically in the cardiomyocytes (α-MHC-Cre) does not block the cardiac hypertrophic response to either aging or pathological stimuli, it does dramatically alter how the heart grows: adult cardiomyocytes from the Erk1<sup>−/−</sup>Erk2<sup>2fl/fl:Cre</sup> heart show eccentric growth with a significant increase in length, whereas myocytes from activated Mek1 transgenic hearts show concentric growth with increased width.

The MEK5-ERK5 branch of MAPKs pathways also has been identified as a regulator of cardiac growth. Transgenic mice with cardiac-specific expression of activated Mek5 show eccentric cardiac hypertrophy, and progress to dilated cardiomyopathy and sudden death (422). Consistent with this, targeted deletion of Erk5 in the heart attenuates the hypertrophic response following pressure overload, and induces apoptosis in the heart (423). By contrast, activation of p38 (transgenic mice expressing activated Mek3 or Mek6) (424) or JNK (transgenic mice expressing activated Mek7) (425, 426) in the heart does not induce cardiac hypertrophy. Such mice develop lethal cardiomyopathy as juveniles, characterized by reduced functional performance, fibrosis and dilated ventricular walls.

Finally, in addition to signaling events that are initiated by ligand-receptor interactions, cardiomyocytes can directly sense biomechanical deformation or stretch through an internal sensory apparatus (427, 428). The integrin-interacting molecule melusin has been implicated as a mechanical stress sensor in cardiomyocytes, essential for the inactivation of GSK-3β (428). The muscle LIM-domain protein (MLP) might be a second sensory apparatus at the level of the Z-disc within sarcomeres (427). MLP anchors to specific proteins at the Z-disc and functions as an internal stretch sensor through a complex of transducing proteins that regulate calcineurin-NFAT signaling (429).
1.5 Rationale and hypothesis

The RAS/ERK pathway controls fundamental cellular processes, such as proliferation, differentiation and apoptosis, so it is not surprising that malfunction of this pathway results in the multiple clinical manifestations seen in RASopathies. Yet, how mutations in the same signaling pathway cause similar, yet clearly distinct, phenotypes remains unclear. Consequently, detailed understanding of RASopathy pathogenesis could yield new insights into RAS/ERK pathway regulation.

Primary HCM is the most common inherited cardiovascular disorder (1 in 500 individuals in the general population) (373, 430) and a leading cause of sudden death in the young (431). Genetic and cellular models have identified multiple signaling pathways that can cause or contribute to pathological hypertrophy, but the detailed mechanism by which aberrant activation of these pathways evokes HCM remains incompletely understood. Delineating the molecular pathways that distinguish physiological and pathological cardiac hypertrophy, and identifying ways to reverse the latter, are of obvious medical importance. The role of the RAS/ERK pathway in cardiac hypertrophy has been controversial, and remains to be resolved. Given the fact that HCM is found in nearly all (~95%) NS patients bearing RAF1 mutations that cause increased kinase activity (148, 149), this disorder provides a good opportunity to study the role of RAS/ERK pathway in the pathogenesis of HCM.

The hypotheses of my project were: (1) Specific RAF1 mutations identified in NS patients cause the common features of NS; (2) Kinase-activating RAF1 mutations cause NS with HCM, whereas kinase-impaired RAF1 mutations are not associated with HCM; (3) Different RAF1 mutations cause NS through distinct molecular mechanism(s), and/or by acting in different cell types. To test my hypotheses, I generated and analyzed lines of knock-in mice with HCM-associated kinase-activating (L613V) or non-HCM-associated kinase-impaired (D486N) Raf1 mutations, respectively. I also investigated the effects and biochemical properties of various other NS-associated RAF1 mutants expressed at more physiological levels than in previous transient transfection studies. Together, my project aimed to advance the field in understanding the molecular pathogenesis of NS as well as HCM, which could provide us with potentially important prognostic, diagnostic and even therapeutic implications for this disease. My findings
could also increase our understanding of other forms of cardiac disease, as well as normal cardiac development.
Chapter 2

Materials and methods
2.1 Mice

All animal studies were approved by the University Health Network Animal Care Committee (Toronto, ON, Canada) and performed in accordance with the standards of the Canadian Council on Animal Care.

2.1.1 Generation of $Raf^1_{L613V}$ knock-in mice

To construct the targeting vector for inducible $Raf^1_{L613V}$ knock-in mice, a “short arm”, containing $Raf^1$ exon 12 (SacII-NotI genomic fragment) and a “long arm”, which includes exons 13-16 (BamHI-ClaI genomic fragment) were ligated into the vector pGK Neo-HSV-1 TK (432). The L613V (exon 16) mutation, marked by a unique DraIII site, was introduced by site-directed mutagenesis. A splice acceptor sequence, a $Raf^1$ cDNA fragment encoding wild-type exons 13-16 and a pGK-Neo (Neo) gene were positioned after the first loxP site as a SalI-XbaI fragment. The targeting vector was linearized with SacII and electroporated into G4 ES cells (129S6 x C57BL/6 F1 background). Genomic DNA, isolated from doubly G418/1-(2-deoxy-2-fluoro-$\beta$-D-arabinofuranosyl)-5 iodouracil (FIAU)-resistant (positive and negative selection, respectively) ES clones, was screened by PCR using primers outside and inside the targeting vector (Table 2-1), followed by NotI digestion, which marks the targeting vector. Homologous recombinants were confirmed by Southern blotting using Neo and external (5’ and 3’) probes (Table 2-1). For these experiments, genomic DNA was digested with XbaI (5’ and Neo probes) or BamHI (3’ probe).

To validate the desired properties of the targeted locus, correctly targeted ES cells were transfected with a Cre-expressing plasmid (MSCV-GFP-Cre) to excise the cDNA-Neo cassette (see below for detailed methods). Expression of $Raf^1_{L613V}$ mRNA was confirmed by RT-PCR (Table 2-1), followed by digestion with DraIII, which marks the L613V allele. Chimeras were generated by outbred morula aggregation (Toronto Centre of Phenogenomics), and germ line transmission was obtained (L613Vfl/+ mice). L613Vfl/+ mice (129Sv X C57BL/B6) were crossed to CMV-Cre (C57BL/B6) mice, which express Cre ubiquitously, and then to WT (129S6) mice to generate mice with global Raf1$^{L613V}$ expression (L613V/+, 129Sv X C57BL/B6). Mice on a 129Sv X C57BL/B6 mixed background were used for all experiments.
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<th>PCR Screening</th>
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<td>Neo probe</td>
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<td>5'-GAACGGGTTGTATCCTGATCCGATTACTTCTC-3'</td>
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<td>antisense 5'-CGCCGGCAAGCTCTTCAGCAA-3'</td>
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<tr>
<td>5’ probe</td>
<td>sense 5'-TGCTCTGGAGCTCAAACCCTCAGTGTAG-3'</td>
<td>antisense 5'-CATGGCTGAGTGAGGACGTCAGGTG-3'</td>
</tr>
<tr>
<td>3’ probe</td>
<td>sense 5'-GAGACGCGCAGATCCTCAGTAGTACTTG-3’</td>
<td>antisense 5'-ACGGTGTTAGTTGTGTCTTTGGCCATG-3’</td>
</tr>
<tr>
<td>RT-PCR for Raf1 mRNA</td>
<td>sense 5'-TCTCCATGAAGGCCTCACGGTG-3’</td>
<td>antisense 5'-AGACTGTTAGCCTTGGGGATGTAG-3’</td>
</tr>
<tr>
<td>Genotyping for Raf1L613V</td>
<td>sense 5'-ATCCCTCTGATCTCAGCAGGCTCTAC-3’</td>
<td>antisense 5'-AGTAGTCTAGGTCCTTAGGCAGGC-3’</td>
</tr>
</tbody>
</table>

Table 2-1. PCR primers for generating the Raf1L613V mice.
For genotyping, genomic DNA was prepared from tails, and then subjected to PCR (Table 2-1) and digestion with DraIII.

2.1.2 Generation of $\text{RafI}^{D486N}$ knock-in mice

To construct the targeting vector for our inducible $\text{RafI}^{D486N}$ knock-in mice, a “short arm”, containing $\text{RafI}$ exon 12 (SacII-NotI genomic fragment), and a “long arm,” which includes exons 13-16 (BamHI-ClaI genomic fragment), were ligated into the vector pGK Neo-HSV-1 TK (432). The D486N (exon 13) mutation, marked by a unique ApoI site, was introduced by site-directed mutagenesis. A splice acceptor sequence, a $\text{RafI}$ cDNA fragment encoding wild-type exons 13-16, and a pGK-Neo (Neo) gene were positioned after the first loxP site as a Sall-Xbal fragment. The targeting vector was linearized with SacII and electroporated into G4 ES cells (129Sv x C57BL/6 F1 background). Genomic DNA, isolated from doubly G418/FIAU-resistant ES clones, was screened by PCR using primers outside and inside the targeting vector (Table 2-2). Homologous recombinants were confirmed by Southern blotting, using Neo and external (5’ and 3’) probes (Table 2-2). For these experiments, genomic DNA was digested with XbaI (5’ and Neo probes) or BamHI (3’ probe).

To validate the desired properties of the targeted locus, correctly targeted ES cells were transfected with a Cre-expressing plasmid (pMSCV-GFP-Cre) to excise the cDNA-Neo cassette (see below for detailed methods). Expression of $\text{RafI}^{D486N}$ mRNA was confirmed by RT-PCR (Table 2-2) followed by digestion with ApoI, which marks the D486N allele. Chimeras were generated by outbred morula aggregation (Toronto Centre of Phenogenomics), and germ line transmission was obtained (D486Nfl/+ mice). D486Nfl/+ mice (129Sv X C57BL/6 background) were crossed to EIIa-Cre (129Sv) mice, which express Cre ubiquitously, and then to WT (C57BL/6) mice to generate mice with global $\text{RafI}^{D486N}$ expression (D486N/+, 129Sv X C57BL/6). Mice on a 129Sv X C57BL/6 mixed background were used for all experiments. For genotyping, genomic DNA was prepared from tails, and then subjected to PCR (Table 2-2) and digestion with ApoI.
<table>
<thead>
<tr>
<th>PCR screening for 5’loxP site</th>
<th>sense</th>
<th>5’-TCCAGCTAATTGACATTGCCCCGACAGACAGCTCAG-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>antisense</td>
<td>5’-GAACGGGTTGTCATCCTGCATCCGGATTACTTTCTG-3’</td>
</tr>
<tr>
<td>PCR screening and probe for</td>
<td>sense</td>
<td>5’-GGA TTG CAC GCA GGT TCT CCG-3’</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5’-CGC CGC CAA GCT CTT CAG CAA-3’</td>
</tr>
<tr>
<td>5’ probe</td>
<td>sense</td>
<td>5’-TGC TCT GGA GCT CAA ACC CTC AGT GTA G -3’</td>
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<tr>
<td></td>
<td>antisense</td>
<td>5’-CAT GGC TGA GTG GAC GGT CAG GCT G-3’</td>
</tr>
<tr>
<td>3’ probe</td>
<td>sense</td>
<td>5’-GAG ACG GCA GAT CCT CAG TAG TAC TTG-3’</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5’-ACG GTG GTA GTT GTG TCT TTG GCC ATG-3’</td>
</tr>
<tr>
<td>RT-PCR for Raf1 mRNA</td>
<td>sense</td>
<td>5’-TCT CCA TGA AGG CCT CAC GGTG-3’</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>5’-AGA CTG GTA GCC TTG GGG ATG TAG-3’</td>
</tr>
<tr>
<td>PCR screening &amp; genotyping</td>
<td>sense</td>
<td>5’-TGTGCACATGCTCCGTCTCGTTCCCTGTC -3’</td>
</tr>
<tr>
<td>for Raf1D486N</td>
<td>antisense</td>
<td>5’-GCACCCTACTCTGGCCCCAGTAATTCC -3’</td>
</tr>
</tbody>
</table>

Table 2-2. PCR primers for generating the Raf1D486N mice.
2.2 Cell culture

2.2.1 Mouse embryonic stem (ES) cells

ES cells (G4) were cultured on γ-irradiated mouse embryonic fibroblast (MEF) feeders in knockout Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), containing 15% ES-tested (HyClone, Thermo Scientific) fetal bovine serum (FBS), 2mM L-glutamine (Invitrogen), 0.1mM Non-Essential Amino Acids (NEAA) (Invitrogen), 0.1mM β-mercaptoethanol (Sigma), 100 U/ml penicillin/streptomycin (Invitrogen), and 500U/ml LIF (ESGRO, Chemicon). ES cells were transfected with MSCV-GFP-Cre plasmid using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s protocol. GFP-positive cells were purified by Fluorescence Activated Cell Sorting (FACS) at 48 hours post-transfection, and then used for RNA isolation.

For biochemical studies, ES cells were cultured under feeder-free condition, starved in knockout DMEM containing 1% FBS for 6 hr and then stimulated with 10^3U/ml LIF before harvesting.

2.2.2 Mouse embryonic fibroblasts (MEFs)

Primary MEFs were prepared from E13.5 embryos as described (433). In brief, embryos were incubated in 0.25% trypsin/EDTA (Invitrogen) for 30 min at 37°C, and dissociated cells were collected by centrifugation and cultured in DMEM (WISENT INC.) containing 10% FBS and 100 units/ml penicillin/streptomycin (Invitrogen). Different independent MEF strains were used for experiments, with similar results.

Primary MEFs were immortalized by the 3T3 protocol (434). Immortalized MEFs were cultured in DMEM containing 10% FBS and 100units/ml penicillin/streptomycin.

For biochemical studies, MEFs were starved in serum-free DMEM for 16 hr before stimulation with 10ng/ml EGF or 50ng/ml PDGF (both from PeproTech) before harvesting.
2.2.3 Neonatal cardiomyocytes and cardiac fibroblasts

Neonatal mouse ventricular myocytes (neonatal cardiomyocytes) were isolated using methods adapted from a previous study (435). In brief, 1-day-old mouse hearts were harvested and pre-digested with 0.15 mg/mL trypsin (Invitrogen) at 4°C for 12-16 hours, followed by 50U/ml Type II Collagenase (Worthington Biochemical) and 0.2 mg/mL trypsin in calcium- and bicarbonate-free Hank’s buffer with HEPES (pH 7.5) (137mM NaCl, 5.36mM KCl, 0.81mM MgSO₄, 5.55 mM Dextrose, 0.44mM KH₂PO₄, 0.34mM NaH₂PO₄·H₂O and 20mM HEPES) for 1-2 hr at 37°C. Non-cardiomyocytes were depleted by differential plating for 1 hour. Cardiomyocytes were counted, seeded at 2.5 × 10⁵ cells/ml on Falcon Primaria™ tissue-culture plates (BD Biosciences), and cultured at 37°C in DMEM/Ham's F-12 [1:1 (v/v); Invitrogen], 10% FBS, and 100 U/ml penicillin/streptomycin (Invitrogen), supplemented with 0.1 mM bromodeoxyuridine (Sigma-Aldrich) and 20 μM arabinosylcytosine (Sigma-Aldrich) to inhibit rapidly proliferating cells. For biochemical studies, the medium was replaced with serum-free DMEM/Ham’s F12 (1:1) medium supplemented with 1% insulin-transferrin-selenium supplements-X (Invitrogen) after 24 hr. After an additional 24 hr, cardiomyocytes were stimulated with 10ng/ml IL-6 (PreproTech), 1μg/ml Angiotensin II (Sigma-Aldrich), 100ng/ml IGF-I (PreproTech), 50ng/ml EGF (PreproTech) or 100ng/ml NRG (heregulin β1, PeproTech) before harvesting.

Non-cardiomyocytes from the above preparation, mainly comprising cardiac fibroblasts, were cultured in DMEM containing 10% FBS and 100 units/ml penicillin/streptomycin. For biochemical studies, cardiac fibroblasts were starved in serum-free DMEM for 16 hr, and then stimulated with EGF (50 ng/ml), IGF-I (100ng/ml), PDGF (100ng/ml), or FGF2 (100ng/ml), all from PeproTech, before harvesting.

2.2.4 Flp-In T-REx 293 cell lines

Flp-In T-REx 293 cell lines were cultured in DMEM (WISENT INC.) containing 10% tetracycline-tested GIBCO® FBS (Invitrogen) and 100 units/ml penicillin/streptomycin (Invitrogen). To induce expression of the indicated gene, a final concentration of 1μg/ml tetracycline (Sigma-Aldrich) was added to the cells, followed by incubation for 24 hours before harvesting for analysis.
For biochemical studies, Flp-In T-REx 293 cell lines were starved in serum-free DMEM for 16 hr before stimulation with 50ng/ml EGF before harvesting.

2.3 Generation of Flp-In T-REx 293 expression cell lines

RAF1 mutations were introduced into a Flag-tagged human RAF1 construct (a gift from Dr. Bruce Gelb, Mt Sinai Hospital, NY, NY) by site-directed mutagenesis. Flag-tagged human WT or mutant RAF1 coding sequences were cloned into the KpnI and XhoI restriction sites of the vector pcDNA5/FRT/TO (Invitrogen). Flp-In T-Rex 293 host cells (Invitrogen) were co-transfected with pOG44 (Invitrogen) and pcDNA5/FRT/TO expression plasmid DNAs, using FuGENE HD Transfection Reagent (Promega), according to the manufacturer’s protocol. Hygromycin-resistant colonies were picked and expanded to assay for tetracycline-regulated expression of Flag-RAF1.

2.4 Inducible RAF1/BRAF heterodimerization

Inducible RAF1/BRAF heterodimerization was achieved by using the ARGENT regulated heterodimerization kit (ARIAD). A Flag-tagged human RAFl\textsuperscript{R401H/D486N} cDNA was cloned into the EcoRI and XbaI restriction sites of the vector pC\textsubscript{4}EN-F1. A human BRAF cDNA was cloned into the XbaI restriction site of the vector pC\textsubscript{4}-R\textsubscript{H}E. The resultant FKBP and FRB fusion protein constructs were co-transfected into Flp-In T-REx 293 host cells, using FuGENE HD Transfection Reagent (Promega), as above.

2.5 Lentivirus production and transduction

Lentiviral shRNA expression plasmids (hairpin-pLKO.1) were obtained from Dr. Jason Moffat (University of Toronto). shRNA oligonucleotide sequences against murine Braf (5’ CCACATCATTGAGACAAATT 3’ or 5’ CGAGGATACCTATCTCCAGAT 3’), murine Araf (5’ CAGGCTCATCAAAGGAAGAAA 3’) and a control shRNA against Luciferase (5’ CAAAATCACAGAATCGTCGTAT 3’) were used. To generate lentiviruses, 2X10\textsuperscript{6} 293T packaging cells in growth medium (DMEM+10%FBS) were transfected with 3µg lentiviral shRNA construct, 2.7 µg packaging plasmid (pCMV-dR8.91) and 0.3 µg envelope plasmid (VSV-G) in 10cm cell culture plates, using FuGENE HD Transfection Reagent (Promega) according to the manufacturer’s protocol. Transfection medium was changed the following
morning and replaced with 10ml high serum growth medium (DMEM+30%FBS) for viral harvest. Virus-containing supernatants were collected at 48h post-transfection, and subsequently passed through a 0.45µm filter to remove cell debris, aliquotted, and stored at -80°C.

For knock-down experiments, cells were transduced in 10cm cell culture plates with 1ml virus in the presence of 6µg/ml polybrene for 24 hours, followed by selection with 2.5µg/ml puromycin for 48 hours. After puromycin selection, transduced cells were re-plated for biochemical analysis.

2.6 Retrovirus production and transduction

A Myc epitope tag was added to the N-terminus of human BRAF cDNA (The Centre for Applied Genomics) by using PCR. The BRAF<sup>R509H</sup> mutation was introduced by site-directed mutagenesis. RNAi-insensitive BRAF<sup>WT</sup> and BRAF<sup>R509H</sup> mutants were generated by introducing three silent mutations into the shRNA target sequence in BRAF by site-directed mutagenesis. Blunt-ended Myc-BRAF<sup>WT</sup> or Myc-BRAF<sup>R509H</sup> coding sequence was cloned into the blunt-ended XhoI site of the pMSCV-IRES-EGFP vector upstream of the IRES sequence.

To generate retroviruses, 1X10<sup>6</sup> 293T packaging cells in growth medium (DMEM+10%FBS) in 6cm cell culture plates were transfected with 3µg pMSCV retroviral and 3 µg EcoPac packaging plasmid using FuGENE HD Transfection Reagent (Promega), according to the manufacturer’s protocol. Transfection media was changed the following morning and replaced with 5ml fresh growth medium for viral harvest. Virus-containing supernatants were collected at 48h post-transfection, and passed through a 0.45µm filter to remove cell debris. MEFs were transduced in 15cm cell culture plates with 5ml virus in the presence of 6µg/ml polybrene for 24 hours, followed by fluorescence-activated cell sorting (FACS) to select for GFP positive cells.

2.7 Biochemical analysis

Total protein extracts from cells or tissues were prepared by homogenization in RIPA buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 2mM EDTA, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS) containing a protease and phosphatase inhibitor cocktail (40 µg/ml PMSF, 20mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub>, 10mM β-glycerophosphate, 10mM sodium pyrophosphate, 2µg/ml antipain,
2μg/ml pepstatin A, 20μg/ml leupeptin, and 20μg/ml aprotinin). Homogenates were centrifuged at 16,100 x g for 15 min at 4 °C, and the supernatants were collected. Lysates (10-25μg protein) were resolved by SDS-PAGE and analyzed by immunoblotting.

For immunoprecipitations, total cell extracts were prepared in NP40 buffer (20mM Tris-HCl, pH8.0, 137mM NaCl, 2mM EDTA, 1% NP40 and 10% glycerol) containing the protease and phosphatase inhibitor cocktail described above. Homogenates were centrifuged at 16,100 x g for 15 min at 4 °C, and the supernatants were collected. Lysates were incubated with anti-Raf1 antibody (BD Biosciences) and Protein-G Sepharose 4 Fast Flow (GE Healthcare), or anti-FLAG M2 affinity agarose gel (Sigma-Aldrich) for 3 hours at 4 °C with rotation. Beads were washed four times with NP40 buffer, and immunoprecipitates were analyzed by immunoblotting.

Antibodies for immunoblots included: Raf1 (BD Biosciences), SH-PTP2 (C-18) and ERK2 (D2) (Santa Cruz Biotechnology Inc.), and phospho-MEK1/2, MEK1/2, phospho-p44/42 MAPK, phospho-S6 (Ser235/236), p38, phospho-p38, phospho-JNK1/2, Akt1, phospho-Akt (Ser473), phospho-GSK3β (Ser9), phospho-P70S6K (Cell Signaling Technology). Primary antibody binding was visualized by IRDye infrared secondary antibodies using the Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification of immunoblots was performed by using Odyssey V3.0 software.

2.8 Body size analysis and morphometry

For growth curves, body length [anal-nasal (AN) length] and weight were measured weekly. For skeletal morphometry, mice were anesthetized with 2% isoflurane and scanned by using a Locus Ultra microCT scanner (GE Healthcare). Three-dimensional images of the skeleton were generated and analyzed with GEHC MicroView software (GE Healthcare). Skull measurements were made according to the Standard Protocol and Procedures from the Jackson Laboratory (http://craniofacial.jax.org/standard_protocols.html).

2.9 Histology and immunohistochemistry

Hearts for morphometry and histochemistry were fixed in the relaxed state by infusion of 1% KCl in PBS, followed by 10% buffered formalin. Hearts were then incubated in 10% buffered formalin overnight and embedded in paraffin. Sections (5μm) were prepared and
stained with H&E, Picro sirius red (PSR) or Masson-Trichrome. Cell membranes were stained with TRITC-conjugated wheat germ agglutinin (WGA) (Sigma-Aldrich), according to the manufacturer’s protocol. Nuclei were stained with DAPI. Cross-sectional area of cardiomyocytes with centrally located nuclei (to ensure the same plane of sectioning) was measured by using ImageJ. Five individual samples were analyzed for each genotype, with 200 cells measured in each.

2.10 BrdU incorporation assays

For proliferation assays, pregnant mice (E16.5) were injected intraperitoneally (IP) with BrdU (100 μg/g body weight) 1 h before sacrifice. Embryos were fixed in 10% buffered formalin overnight and embedded in paraffin. BrdU incorporation was detected by using rat anti-BrdU primary antibody (1:50; Abcam). Immune complexes were visualized using F(ab)₂ biotin-conjugated donkey anti-rat IgG (1:500; Research Diagnostics Inc.) and the VECTASTAIN Elite ABC Kit (Vector Laboratories). Sections were counterstained with hematoxylin. For each sample, BrdU+ cells were counted in 10 randomly selected fields.

2.11 Hematopoietic analysis

Myeloid colony assays (in the absence of added cytokines) were performed as described previously (436). In brief, bone marrow (BM) cells were suspended in MethoCult® M3234 without cytokines (Stem Cell Technologies) at 10^5 cells/ml, and colonies were scored after 7-9 days. Complete blood counts were determined by using a Hemavet 850FS (Drew Scientific).

2.12 Echocardiographic and hemodynamic measurements

For echocardiography and cardiac catheterization, mice were anesthetized with isoflurane/oxygen (2%/100%), and body temperature was maintained at ∼37.5°C. Transthoracic 2D and M-mode echocardiography was performed from the long axis view of the heart at the level of the papillary muscle with a Visualsonics Vevo 770 imaging system (Visualsonics) equipped with a 30-MHz linear transducer (RMV707B). Measurements of the left ventricular (LV) end-systolic diameter (LVIDs) and end-diastolic diameter (LVIDd) internal dimensions and of the LV diastolic posterior wall thickness (LVPWd) were made under Time Motion-mode. The papillary muscles were excluded from all measurements. Measurements were averaged from at
least 3 separate cardiac cycles. From TM-mode measurements, end-diastolic volume (EDV) was calculated as \((4.5 \times \text{normalized LVIDd}^2)\), end-systolic volume (ESV) was calculated as \((3.72 \times \text{normalized LVIDs}^2)\), and stroke volume (SV) was calculated as EDV-ESV. Cardiac output (CO) was calculated as SV X Heart rate. Fractional shortening (FS) was calculated as \((\text{LVIDd} - \text{LVIDs})/\text{LVIDd} \times 100\%\), and ejection fraction (EF) was calculated as \((\text{EDV} - \text{ESV})/\text{EDV} \times 100\%\).

For invasive hemodynamic assessments, a 1.2F catheter (model # FTS-1211B-0018, Scisense Inc.) was inserted via the right carotid artery into the left ventricle. Hemodynamic signals were digitized at a sampling rate of 1kHz and acquired to a computer using the MP100 imaging system and Acqknowledge software (BIOPAC Systems, Inc). Following recording of left ventricular pressure, the catheter was relocated to the ascending aortic for measurement of aortic blood pressure. Mean arterial pressure (MAP) was calculated as \((\text{Systolic pressure} + \text{Diastolic pressure} \times 2)/3\).

2.13 Transverse aortic constriction (TAC)

Eight to nine week-old male mice (25-30g body weight) were anesthetized with 2% isoflurane, intubated, connected to a ventilator (Harvard Apparatus) and ventilated at a tidal volume of \(~230\mu\text{l} and 135 \text{breaths/min}. A para-sternal thoracotomy was performed to expose the transverse aorta, which was then constricted with a 7/0 silk suture tied around a 27G needle. Pressure overload was maintained for various times as indicated in Results and Figure Legends.

2.14 MEK inhibitor and Rapamycin treatment

\(\text{N-[(R)-2,3-dihydroxy-propoxy]-3,4-difluoro-2-(2-fluoro-4-iodo-phenylamino)-benzamide (PD0325901) was synthesized according to the disclosure in document WO2007042885(A2). All chemicals necessary for the synthesis were purchased from Sigma Aldrich.}\)

\(\text{PD0325901 was dissolved in DMSO at a concentration of 50mg/ml, then resuspended in vehicle (0.5% hydroxypropylmethylcellulose with 0.2% Tween 80) at a concentration of 0.5mg/ml, and injected intraperitoneally (5mg/kg body weight) daily for the indicated times.}\)
Control mice were injected with vehicle. The same protocol was used to inject lactating females for early post-natal treatment.

Rapamycin (LC Laboratories) was dissolved in DMSO at a concentration of 20mg/ml, then resuspended in vehicle (0.5% hydroxypropylmethylcellulose with 0.2% Tween 80) at a concentration of 0.2mg/ml, and injected intraperitoneally (2mg/kg body weight) daily for the indicated times. Control mice were injected with vehicle.

2.15 Quantitative real time RT-PCR

Total RNA from the left ventricle was prepared using the RNeasy mini-kit (QIAGEN). RNA (2 µg) was reverse transcribed using SuperScriptIII (Invitrogen). TaqMan probe-based gene expression analyses (Applied Biosystems) for Myh7 (Mm00600555_m1), Myh6 (Mm00440359_m1), Nppa (Mm01255748_g1) and Nppb (Mm00435304_g1) were conducted according to the manufacturer’s instructions. Each sample was measured in triplicate, and their relative expression was normalized to Gapdh (4352932E).

2.16 Statistics

All data are presented as mean±SEM. Statistical significance was determined using Student’s t-test, one-way ANOVA or two-way repeated measure ANOVA, as appropriate. If ANOVA was significant, individual differences were evaluated using Bonferroni post-test. Deviation of progeny from Mendelian frequency was assessed by χ² test. Kaplan–Meier survival curves were analyzed using the Log-rank test. For experiments in Figure 3-19, significant outliers were identified using Grubbs’s test. All statistical analyses were performed with GraphPad Prism 5. For all studies, p<0.05 was considered significant.
Chapter 3

MEK-ERK Pathway Modulation Ameliorates Disease Phenotypes in a Mouse Model of Noonan Syndrome Associated with the $Raf^{1L613V}$ Mutation

This Chapter is a modified version of a paper published in the Journal of Clinical Investigation (2011 Mar;121(3):1009-25).
3.1 Abstract

Hypertrophic cardiomyopathy (HCM) is a leading cause of sudden death in children and young adults. Abnormalities in several signaling pathways are implicated in the pathogenesis of HCM, but the role of the RAS-RAF-MEK-ERK MAPK pathway has been controversial. Noonan syndrome (NS) is one of several autosomal-dominant conditions known as “RASopathies”, which are caused by mutations in different components of this pathway. Germ-line mutations in \textit{RAF1} (which encodes the serine-threonine kinase RAF1) account for approximately 3–5% of cases of NS. Unlike other NS alleles, \textit{RAF1} mutations that confer increased kinase activity are highly associated with HCM. To explore the pathogenesis of such mutations, we generated “knock-in” mice expressing the NS-associated \textit{Raf1}^{L613V} mutation. Like NS patients, mice heterozygous for this mutation (referred to herein as L613V/+ mice) had short stature, craniofacial dysmorphia, and hematologic abnormalities. Valvuloseptal development was normal, but L613V/+ mice exhibited eccentric cardiac hypertrophy and aberrant cardiac fetal gene expression, and decompensated following pressure overload. Agonist-evoked MEK/ERK activation was enhanced in multiple cell types, and post-natal MEK inhibition normalized the growth, facial, and cardiac defects in L613V/+ mice. These data show that different NS genes have intrinsically distinct pathological effects, demonstrate that enhanced MEK-ERK activity is critical for causing HCM and other RAF1-mutant NS phenotypes, and suggest a mutation-specific approach to the treatment of RASopathies.

3.2 Background

Cardiac hypertrophy is a major way by which cardiomyocytes respond to various stresses, including abnormal neuro-hormonal stimuli, hemodynamic overload and injury. There are two general types of cardiac hypertrophy (349, 350): physiological, which is associated with exercise or pregnancy, and pathological, caused by genetic defects (primary), or excessive afterload, resulting from conditions such as hypertension or valvular stenosis (secondary). With increased cardiac stress, cardiac hypertrophy may initially represent a compensatory response of the myocardium. However, chronic pathological hypertrophy predisposes to ventricular dilatation, heart failure, arrhythmia and/or sudden death (356, 357). Physiological hypertrophy is typically concentric, with preservation of chamber shape, the absence of inflammation or fibrosis and normal cardiac gene expression. By contrast, pathological hypertrophy eventually progresses
to chamber dilatation (eccentric hypertrophy), is often associated with fibrosis, and typically leads to the reactivation of a fetal gene expression program, characterized by increased levels of (among others) atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and beta-myosin heavy chain (β-MHC) (437). Delineating the molecular pathways that distinguish physiological and pathological hypertrophy, and identifying ways to reverse the latter, are of obvious medical importance.

Primary hypertrophic cardiomyopathy (HCM), the prototypic genetic form of pathologic hypertrophy, is a leading cause of sudden death in the young (431). The hallmark of HCM is cardiac hypertrophy in the absence of an obvious inciting hypertrophic stimulus (372). Mutations in genes encoding sarcomeric proteins (e.g., β-MHC, cardiac troponin T, and myosin-binding protein C) account for most (~75%) cases of primary HCM. Such mutations usually alter sarcomere structure and function and result in mechanical, biochemical and/or bioenergetic stresses that activate cardiomyocyte signaling pathways to mediate the hypertrophic phenotype (375-378). Aberrant activation of hypertrophic signaling pathways can themselves result in hypertrophy. For example, germ line mutations in adenosine monophosphate-activated protein kinase (AMPK) are a rare cause of HCM (380-382). Moreover, genetic and cellular models have identified multiple signaling systems that can cause or contribute to pathological hypertrophy, including the calcineurin/NFAT, PI3K/Akt/mTOR, GSK3β and JNK pathways (349, 350, 386). The detailed mechanism by which aberrant activation of these pathways evokes pathological hypertrophy remains incompletely understood.

The RAS-RAF-MEK-ERK MAPK pathway (hereafter, the RAS/ERK pathway) is a central signaling cascade evoked by multiple agonists, including growth factors (e.g., Heregulin, IGF-1, EGF, PDGF), cytokines (e.g., IL6, cardiotrophin, LIF), G-protein coupled receptor agonists (angiotensin-II, beta-adrenergic agonists), and physical stimuli (e.g., mechanical stretch), in cardiomyocytes as well as other cell types (26, 349, 350). The pathway is initiated by the activation of RAS, which requires RAS-guanine nucleotide exchange factors (RAS-GEFs) such as SOS1 and, in most cell types, the protein-tyrosine phosphatase SHP2 (encoded by PTPN11 gene). RAS recruits RAF proteins (RAF1, BRAF, ARAF) to the cell membrane, where they are activated and subsequently form complexes with MEK1/2 and ERK1/2, aided by scaffolds, such as KSR. Activated RAF proteins phosphorylate MEK1,2 which, in turn, phosphorylate ERK1,2. ERKs phosphorylate cytosolic substrates and also translocate to the
nucleus to stimulate diverse gene expression programs by phosphorylating several transcription factors (26, 28).

The role of the RAS/ERK pathway in cardiac hypertrophy has been controversial. Some data argue that excessive activity of this pathway causes HCM, whereas other evidence suggests involvement in physiological, but not pathological, hypertrophy (409, 410). Transgenic mice with cardiac-specific expression of oncogenic HRas (G12V) display significant cardiac hypertrophy, decreased contractility, diastolic dysfunction associated with interstitial fibrosis, induction of cardiac fetal genes and sudden death (411-413), all of which are consistent with HCM. In cultured cardiomyocytes, depletion of Erk1/2 with antisense oligonucleotides or pharmacological inhibition of Mek1/2 attenuates the hypertrophic response to agonist stimulation (414, 415). Mice with cardiac-specific over-expression of “dominant-negative” Raf1 have no overt phenotype, but they are resistant to the development of cardiac hypertrophy in response to pressure overload (416), suggesting that signals from Raf1 are necessary for the hypertrophic response. On the other hand, transgenic mice expressing an activated Mek1 allele under the control of the alpha-MHC promoter have concentric hypertrophy with enhanced contractile performance, show no signs of decompensation over time and reportedly do not progress to pathological hypertrophy (418). A recent study even argued against any role for ERK1/2 in cardiac hypertrophy, as Erk1−/−Erk2+/− mice, as well as transgenic mice with cardiac-specific expression of dual specificity phosphatase 6 (Dusp6), an ERK1/2-specific phosphatase, showed a normal hypertrophic response to pressure overload and exercise (420).

Over the past ten years, germ line mutations in genes encoding several members of the RAS/ERK pathway have been identified in a set of related, yet distinct, human developmental syndromes (31, 32, 144, 438, 439), now collectively termed the RASopathies (32, 439). These disorders, some (but not all) of which include HCM as a syndromic phenotype, present an opportunity to clarify the role of the RAS/ERK pathway in cardiac hypertrophy. Noonan syndrome (NS), a relatively common autosomal dominant disorder (~1/1,000–2500 live births), typically presents with proportional short stature, facial dysmorphism, and cardiovascular abnormalities. Many (25-50%) NS patients exhibit some form of myeloproliferative disorder (MPD), which is usually transient and resolves spontaneously; rarely, NS patients develop the severe childhood MPD, juvenile myelomonocytic leukemia (JMML) or other forms of leukemia (440). Mutations in PTPN11 that increase SHP2 phosphatase activity account for ~50% of NS
cases (145); other known NS genes include \textit{SOS1} (~10\%) (146, 147), \textit{RAF1} (3-5\%) (148, 149), \textit{KRAS} (1-2\%) (150, 151), \textit{NRAS} (<1\%) (152) and \textit{SHOC2} (<1\%) (183).

Although NS patients typically have valvuloseptal defects, ~20\% have HCM (441). Moreover, different NS genes are differentially associated with HCM. Only ~10\% of NS patients with \textit{PTPN11} mutations (442) and ~20\% of those with mutations in \textit{SOS1} (146) develop HCM. By contrast, HCM is found in nearly all (~95\%) patients bearing \textit{RAF1} mutations that cause increased kinase activity (148, 149). The frequency of HCM also varies in other RASopathies. HCM is the most frequent (~80\%) cardiovascular manifestation of LEOPARD syndrome (LS), caused by phosphatase-inactivating mutations of \textit{PTPN11} (158, 179, 180, 384), but also is common (~50\% in each) in Costello Syndrome (CS), caused by gain-of-function mutations in \textit{HRAS} (202, 383), and Cardio-facio-cutaneous (CFC) syndrome, caused by \textit{BRAF}, \textit{MEK1} or \textit{MEK2} mutations (171, 172, 385). Whether these differences represent differential effects of specific RAS/ERK pathway mutations, the effects of modifiers in the outbred human population, or both, remains unclear.

Mouse models have begun to address such issues and to provide insight into the detailed pathogenesis and potential therapeutic approaches to these disorders. For example, we previously generated a knock-in mouse model of the NS-associated \textit{Ptpn11}^{D61G} mutation, which recapitulates the major features of NS, including short stature, facial dysmorphia, mild MPD and valvuloseptal defects. These mice, like most \textit{PTPN11} mutant NS patients, do not have HCM (204). Transgenic mice expressing a different NS-associated \textit{Ptpn11} mutant (Q79R) also show valvuloseptal defects and facial abnormalities seen in NS patients, which are prevented by the genetic ablation of \textit{Erk1/2} and or pre-natal pharmacological inhibition of Mek, respectively (207-209). Genetic ablation of \textit{Erk1} also prevents the development of valvuloseptal defects in mice expressing a highly activated \textit{Ptpn11} mutant in endocardial cells (210). A knock-in mouse model of CS, caused by \textit{HRas}^{G12V} mutation shows HCM, but these mice also have aortic stenosis, making it unclear whether hypertrophy is primary or secondary (443).

Here, we have generated “knock-in” mice expressing the kinase-activating NS mutant \textit{Raf1}^{L613V} (L613V). We find that, whereas similar to \textit{Ptpn11} mutant mice, mice expressing this \textit{Raf1} allele have short stature, facial dysmorphia, and hematological abnormalities, they do not have valvuloseptal abnormalities but instead develop HCM. Remarkably, nearly all phenotypic
abnormalities in Raf1 mutant mice are reversed by post-natal MEK inhibitor treatment. Our results show that different NS genes have intrinsically distinct pathological effects, and demonstrate that enhanced MEK/ERK activity is critical for causing HCM and other RAF1-mutant NS phenotypes. Along with the companion study on LS-associated HCM by Marin et al. (444), these findings suggest a mutation-specific approach to the treatment of RASopathies.

3.3 Results

3.3.1 Generation of L613V/+ mice

Expression of an activated Raf1 mutant during development might cause embryonic lethality. Therefore, to investigate the effects of the NS-associated, kinase-activating RAF1L613V mutant, we designed an “inducible knock-in” Raf1L613V allele (L613Vfl) (Figure 3-1A). The targeting vector included a cassette containing a splice acceptor sequence, a Raf1 cDNA fragment encoding wild type (WT) exons 13-16 and a pGK-Neo (Neo) gene. The fusion cDNA/Neo cassette was flanked by loxP sites and was positioned upstream of exons 13-16 of the Raf1 gene itself, with an L613V mutation introduced into exon 16 and an HSV-TK cassette for negative selection. In the absence of Cre recombinase (445), Raf1 exon 12 should be spliced to the cDNA (exon 13-16), leading to the production of WT Raf1. When Cre is present, the floxed cassette should be excised, evoking transcription of the mutant Raf1 allele.

The targeting construct was electroporated into G4 embryonic stem (ES) cells, and correctly targeted clones (L613Vfl/) were identified by PCR and confirmed by Southern blotting (Figure 3-1B). We also validated the desired properties of the targeted locus in L613Vfl/+ ES cells (Figure 3-1C). As expected, expression of the mutant allele was undetectable (by RT-PCR) in the absence of Cre, but it was induced effectively upon introduction of a Cre-expression vector (MSCV-GFP-Cre). Mutant Raf1 (protein) also was expressed at levels comparable to WT Raf1. Chimeras were then generated by outbred morula aggregation, and germ line transmission was obtained. L613Vfl/+ progeny were crossed to CMV-Cre mice, which express Cre ubiquitously, and then to WT mice, thereby generating mice with global Raf1L613V expression (L613V/+ mice) on a 129S6 x C57BL/6 mixed background.

L613V/+ mice were obtained at the expected Mendelian ratio at weaning, indicating that on this mixed background, Raf1L613V expression during development is compatible with
Figure 3-1. Generation of inducible Raf1\textsuperscript{L613V} knock-in mice.

(A) Targeting strategy. Structures of the Raf1 locus, targeting vector, mutant allele and location of probes for Southern blotting are shown. (B) Correct targeting of ES cells. Genomic DNA from WT ES cells and PCR-positive L613Vfl/+ ES clones was digested with XbaI (5' and Neo probe) or BamHI (3' probe) and subjected to Southern blotting with 5', 3' or Neo probes, respectively. Blots with 5' and 3' probes represent non-adjacent lanes on the same gel. (C) Expression of Raf1\textsuperscript{L613V} allele is inducible. RNA was isolated from WT and L613Vfl/+ ES cells with or without prior transfection of MSCV-Cre-GFP plasmid and reverse transcribed into cDNA. A PCR product, obtained by using primers within exon 11 and at the end of exon 16 of the Raf1 cDNA, was digested with DralIII. Note that the mutant allele is silent until Cre is introduced, and then is expressed efficiently.
embryonic viability. However, similar to mice expressing NS-associated Ptpn11 mutant alleles (204), L613V/+ mice could not be obtained after (>three generations) backcrossing to C57BL/6 mice. Consequently, all experiments herein were performed on the 129S6 x C57BL/6 mixed background.

3.3.2 L613V/+ mice show multiple NS phenotypes

L613V/+ newborns showed normal size at birth (Figure 3-2A). At weaning, though, L613V/+ mice were significantly smaller than their WT littermates, and they remained shorter throughout their lives (Figure 3-2B and Figure 3-2C). Although their overall body proportions were normal, L613V/+ mice exhibited facial dysmorphia (Figure 3-3). Consistent with their decreased body size, the skulls of L613V/+ mice were significantly shorter than WT. Their skull width was increased, however, resulting in a significantly greater width/length ratio. As a result, L613V/+ mice had a ‘triangular’ facial appearance with a blunter snout and widely-set eyes (increased inner canthal distance). These features are reminiscent of the facial phenotype of mice expressing NS-associated Ptpn11 mutations (204, 209, 210) and represent the mouse equivalent of the facial abnormalities seen in NS patients (446).

Like mouse models of Ptpn11 mutation-associated NS (204, 210) and many NS patients (447), L613V/+ mice had hematological defects. There was abnormal expansion of myeloid progenitors, and bone marrow (BM) from L613V/+ mice yielded factor-independent myeloid colonies (Figure 3-4A). L613V/+ mice also developed splenomegaly, which became more severe as they aged (Figure 3-4B). Peripheral blood counts were normal at 4 months, but by 1 year, L613V/+ mice had developed subtle but statistically significant leukocytosis, neutrophilia and monocytosis (Figure 3-4C) with normal hematocrit and platelet counts.

3.3.3 L613V/+ mice show cardiac hypertrophy with chamber dilatation

Unlike PTPN11 alleles, which are negatively associated with hypertrophic cardiomyopathy (HCM) in NS patients (442) and in mouse models (204, 208, 210), RAF1 mutations that encode proteins with increased kinase activity are strongly associated with HCM (148, 149). Remarkably, L613V/+ mice showed evidence of cardiac hypertrophy as early as 2 weeks after birth, as indicated by an increased heart weight to body weight ratio (Figure 3-5A)
Figure 3-2. Short stature in L613V/+ mice.

(A) Body weight of neonatal WT (n=30) and L613V/+ (n=35) mice. ns, not significant. (B) Gross appearance of 2-month-old WT and L613V/+ male mice. (C) Growth curves of WT (n=45) and L613V/+ (n=45) male and female mice. Differences were significant at all time points (p<0.0001, two-way repeated measure ANOVA; *** p<0.0001, Bonferroni post-test).
Morphometry of skulls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT (n=13)</th>
<th>L613V/+ (n=11)</th>
</tr>
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<tbody>
<tr>
<td>Length (mm)</td>
<td>22.9±0.1</td>
<td>21.4±0.3***</td>
</tr>
<tr>
<td>Width (mm)</td>
<td>10.4±0.1</td>
<td>10.9±0.1***</td>
</tr>
<tr>
<td>Width/Length</td>
<td>0.46±0.01</td>
<td>0.51±0.01***</td>
</tr>
<tr>
<td>Inner canthal distance (mm)</td>
<td>6.1±0.1</td>
<td>6.5±0.1***</td>
</tr>
</tbody>
</table>

Figure 3-3. L613V/+ mice have facial dysmorphia.

(A) Gross facial appearance of WT and L613V/+ mice. (B) Representative microCT scans of skulls from WT and L613V/+ mice. Double-headed arrows indicate inner canthal distance. (C) Morphometric measurements from microCT scans of a cohort of 2-month-old WT and L613V/+ male mice. *** p<0.0001, 2-tailed Student’s t-test.
Figure 3-4. L613V/+ mice have hematological defects.

(A) Cytokine-independent myeloid colonies from bone marrow of 2 month-old mice (n=6 for each genotype). *** p<0.0001, 2-tailed Student’s t-test. (B) Splenomegaly in L613V/+ mice. Representative gross appearance (left) and spleen weight/body weight (mg/g) ratio (448) in WT (n=25) and L613V/+ (n=25) mice at 4 months. *** p<0.0001, 2-tailed Student’s t-test. (C) Increased total white blood cells (WBC), neutrophils (NE) and monocytes (MO) in 1 year-old L613V/+ mice (n=8 for each genotype). * p<0.05; *** p<0.0001, 2-tailed Student’s t-test.
Cardiac enlargement became even more obvious in adult L613V/+ mice, with histological analysis revealing substantial thickening of the ventricular wall and septum (Figure 3-5B). Increased heart size can reflect a larger number of cardiomyocytes (e.g., as a consequence of excess proliferation during development) and/or cardiomyocyte hypertrophy. Cardiomyocyte proliferation, as measured by BrdU incorporation assays, was comparable in E16.5 L613V/+ and WT embryos (Figure 3-5C). By contrast, cross-sectional area was markedly (~35%) increased in cardiomyocytes from 8-week-old L613V/+, compared with WT, mice (Figure 3-5D), indicative of cardiac hypertrophy.

Cardiac hypertrophy can be secondary to pressure overload caused by stenotic valves or hypertension. Notably, mice expressing the NS-associated Ptpn11D61G mutation have severe valvuloseptal abnormalities, including atrial, atrioventricular or ventricular septal defects and double-outlet right ventricle (204). By contrast, valvuloseptal development, as assessed by histology, appeared normal in 1 week-old L613V/+ mice (Figure 3-5E). Invasive hemodynamic studies established that ventricular pressure was actually lower in L613V/+ mice compared with WT controls (see below).

To assess cardiac morphology and function, we performed echocardiography on L613V/+ mice and littermate controls at 2 and 4 months of age. As expected, left ventricular diastolic posterior wall thickness (LVPWd) was increased in L613V/+ mice (Figure 3-6A and Figure 3-6B). Although chamber size was normal in 2 month-old mice, by 4 months, L613V/+ hearts showed an increase in left ventricular internal end-diastolic dimension (LVIDd). Left ventricular end-systolic dimension (LVIDs) remained within normal limits (Figure 3-6A and Figure 3-6C), indicating preserved or enhanced function. Consistent with the latter interpretation, stroke volume (SV), ejection fraction (EF), fractional shortening (FS), and cardiac output (CO) were increased in L613V/+ mice (Table 3-1).

Invasive hemodynamic studies confirmed and extended these conclusions (Figure 3-6D and Table 3-2). L613V/+ mice showed increased dP/dt Max, consistent with enhanced contractility, but no change in cardiac relaxation (-dP/dt). Afterload (systolic pressure) was slightly lower in L613V/+ mice. Although this finding rules out hypertension as a cause of hypertrophy in L613V/+ mice, it complicates comparison of dP/dt Max values. For this reason,
Figure 3-5. L613V/+ mice show cardiac hypertrophy with normal cardiomyocyte proliferation and valve development.

(A) L613V/+ mice show cardiac hypertrophy, as indicated by heart weight/body weight ratio (mg/g) as early as 2 weeks after birth (n=14 for each genotype). (B) Representative gross appearance (left top) and H&E-stained cross-sections (left bottom; original magnification, 4X; black bar, 2mm) of WT (n=25) and L613V/+ (n=25) hearts (8 weeks); heart weight/body weight (mg/g) ratio of 4 month-old WT and L613V/+ mice is shown at right. (C) No difference in BrdU incorporation in E16.5 WT (n=4) and L613V/+ (n=3) hearts. (D) Cross-sectional area of cardiomyocytes (original magnification, 400X; white bar, 100µm), measured in WGA-strained sections from 8 week-old mice (n=5 samples for each genotype, with 200 cells counted for each sample using ImageJ). (E) Representative H&E-stained cross-sections of aortic valves in 1 week-old mice (original magnification, 40X; two individual samples are shown for each genotype). No obvious abnormalities were noted in other cardiac valves either. *** p<0.0001, 2-tailed Student’s t-test.
Figure 3-6. L613V/+ mice show cardiac hypertrophy with chamber dilatation.

(A) Representative echocardiograms of hearts from 4 month-old mice. Arrows indicate left ventricular diastolic dimension. (B) Left ventricular diastolic posterior wall thickness (LVPWd) at 2 and 4 months, measured by echocardiography (n=13 for WT; n=11 for L613V/+). (C) Left ventricular chamber dimensions of 2 and 4 month-old WT (n=13) and L613V/+ hearts (n=11). LVIDd, left ventricular internal end-diastolic dimension; LVIDs, left ventricular internal endsystolic dimension. (D) Cardiac contractility of 4-month-old WT (n=13) and L613V/+ hearts (n=11) hearts, as measured by invasive hemodynamic analysis. * p<0.05; ** p<0.005, 2-tailed Student’s t-test.
Table 3-1. Echocardiographic parameters in WT and L613V/+ mice.

Shown are data from 2 and 4 month-old mice. SV, stroke volume; EF, ejection fraction; FS, fractional shortening; CO, cardiac output. * p<0.05; ** p<0.005; *** p<0.0001, 2-tailed Student’s t-test.

<table>
<thead>
<tr>
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<th>2 month</th>
<th>4 month</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT (n=13)</td>
<td>L613V/+ (n=11)</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>456±19</td>
<td>454±17</td>
</tr>
<tr>
<td>SV (µl)</td>
<td>38±1</td>
<td>49±3**</td>
</tr>
<tr>
<td>EF%</td>
<td>56±1</td>
<td>63±2*</td>
</tr>
<tr>
<td>FS%</td>
<td>29±1</td>
<td>34±2**</td>
</tr>
<tr>
<td>CO (ml/min)</td>
<td>18±1</td>
<td>22±2*</td>
</tr>
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we also compared dP/dt estimated at left ventricular pressure (LVP) of 40mm Hg (dP/dt@LVP40), therefore reducing or eliminating the influence of afterload (449). Importantly, dP/dt@LVP40 was increased in L613V/+ animals, providing conclusive evidence of increased contractility (Figure 3-6D). Moreover, there was no pressure gradient across the aortic valves of L613V/+ mice (Table 3-2), ruling out aortic valve stenosis as a cause of their cardiac hypertrophy. Taken together, our finding of eccentric cardiac hypertrophy in the absence of pressure overload is consistent with the conclusion that L613V/+ mice have pathological hypertrophy.

Mice (and humans) with pathological hypertrophy often reactivate specific fetal genes (431, 437). There are two isoforms of cardiac myosin: alpha-myosin heavy chain (alpha-MHC, faster kinetics) and beta-myosin heavy chain (beta-MHC, slower kinetics). In rodents, the \textit{Myh7} (beta-Mhc) gene is expressed mainly in late fetal life, whereas \textit{Myh6} (alpha-Mhc) is expressed predominantly in the adult. Re-expression of \textit{Myh7} and a shift from alpha-Mhc to beta-Mhc is a marker for phenotypic reprogramming and HCM (437). Indeed, \textit{Myh6} mRNA levels were decreased significantly in L613V/+ hearts, and there was a trend (p=0.09, 1-tailed Student’s t-test) towards increased \textit{Myh7} expression (Figure 3-7A). Consequently, the \textit{Myh7}/\textit{Myh6} ratio increased significantly. Expression of \textit{Nppa} (atrial natriuretic peptide, Anp) and \textit{Nppb} (brain natriuretic peptide, Bnp), two other fetal genes often associated with cardiac hypertrophy (437, 450), was unaffected in L613V/+ hearts (Figure 3-7B).

3.3.4 Enhanced hypertrophic response and functional decompensation in L613V/+ hearts following pressure overload

Although L613V/+ mice show cardiac hypertrophy, they displayed enhanced cardiac function without signs of heart failure for at least a year of life. To gain further insight into the nature of the hypertrophy in L613V/+ mice, we assessed their response (compared with controls) to biomechanical stress by transverse aortic constriction (TAC). L613V/+ mice had an unusually high acute death rate after this procedure (Figure 3-8A). Furthermore, the hearts of surviving L613V/+ mice showed dramatic ventricular, as well as left atrial, enlargement compared with WT mice (Figure 3-8B). Although WT mice had an ~45% increase in heart weight to body weight ratio following TAC, L613V/+ mice had an ~ 72% increase. L613V/+ mice also
Table 3-2. Additional hemodynamic parameters of hearts from 4 month-old mice.

Cardiac catheterizations were performed and analyzed as described in Methods. LVP, left ventricular systolic pressure; EDP, end diastolic pressure; MAP, mean arterial pressure. * p<0.05, 2-tailed Student’s t-test.

<table>
<thead>
<tr>
<th></th>
<th>WT (n=12)</th>
<th>L613V/+ (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>516±17</td>
<td>521±17</td>
</tr>
<tr>
<td>LVP (mmHg)</td>
<td>121±3</td>
<td>113±2*</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>4.1±0.7</td>
<td>3.9±0.5</td>
</tr>
<tr>
<td>Systolic P (mmHg)</td>
<td>117±3</td>
<td>109±2</td>
</tr>
<tr>
<td>Diastolic P (mmHg)</td>
<td>83±3</td>
<td>77±2</td>
</tr>
<tr>
<td>-dP/dt (mmHg/s)</td>
<td>-11,010±332</td>
<td>-11,190±327</td>
</tr>
<tr>
<td>-dP/dt/MAP (1/s)</td>
<td>-118±4</td>
<td>-127±3</td>
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Figure 3-7. L613V/+ mice show a shift from alpha-Mhc to beta-Mhc expression in hearts.

Alpha- and beta-Mhc gene expression (A) and Anp and Bnp gene expression (B) in 4-month-old WT (n=6) and L613V/+ (n=9) hearts, assessed by quantitative real-time PCR. * p<0.05; ** p<0.005, 2-tailed Student’s t-test. WT heart sample after 8-week transverse aortic constriction (TAC) (WT-TAC) (n=1) was served as positive control.
Figure 3-8. Abnormal response of L613V/+ mice to pressure overload.

(A) Survival curves of WT (n=25) and L613V/+ (n=24) mice following transverse aortic constriction (TAC). ** p<0.005, Log-rank test. (B) Gross appearance of hearts (left) and heart weight/body weight (mg/g) ratio (right) at 8 weeks post-TAC. Black dashed lines show markedly enlarged left atrium in L613V/+ , compared with WT, mice. ** p<0.005; *** p<0.0001 (Bonferroni post-test when ANOVA is significant); ## p<0.005 (1-tailed Student’s t-test). (C) Severe interstitial fibrosis in L613V/+ hearts (Pico Sirius Red-PSR staining; original magnification, 100X) at 8 weeks post-TAC. Percentage of pixels staining positive with PSR for interstitial fibrosis was quantified by using ImageJ (n=14 for WT; n=13 for L613V/+). *** p<0.0001, 2-tailed Student’s t-test. (D) Perivascular fibrosis in hearts (PSR staining; original magnification, 200X) at 8 weeks post-TAC. Similar results were obtained when Masson’s Trichrome stain was used to assess fibrosis.
developed more severe interstitial fibrosis (Figure 3-8C) and perivascular fibrosis (Figure 3-8D and Figure 3-9A) post-TAC. Two L613V/+ mice were excluded from analysis as by 8 weeks of TAC these mice had sustained a large (~30% of free ventricular wall) spontaneous transmural infarct; extensive fibrosis with impaired systolic and diastolic function was evident (Figure 3-9B).

These morphologic and histological findings established that L613V/+ mice have an altered response to pressure overload. Consistent with this, TAC provoked increases in left ventricular diastolic posterior wall thickness (LVPWd) in WT and L613V/+ mice, which was more pronounced in the L613V/+ mice (Figure 3-10A). Left ventricular internal end-diastolic dimension (LVIDd) did not change after TAC in WT or L613V/+ mice, but remained elevated in the latter (Figure 3-10B). Most importantly, several parameters of cardiac function, including SV and FS, deteriorated in L613V/+ mice, whereas these were unaffected in WT mice (Figure 3-10C). There also was a trend towards decreased cardiac output in L613V/+ mice subjected to TAC, although this did not reach statistical significance because these mice increased their heart rate sufficiently to compensate for decreased ventricular function (Figure 3-10D). In addition, cardiac contractility (measured as either dP/dt Max or dP/dt@LVP40) decreased in L613V/+, but not in WT mice (Figure 3-11A). Cardiac relaxation assessed by −dP/dt, normalized to mean arterial pressure (afterload), was reduced comparably, while end-diastolic pressure was increased to similar extents in WT and L613V/+ mice (Figure 3-11B). Thus, while WT mice could adapt appropriately to pressure overload, L613V/+ mice exhibited substantial, occasionally fatal, functional decompensation with reductions in SV, FS and dP/dt Max and @LVP40, consistent with early stages of heart failure by 8 weeks of TAC.

3.3.5 The Raf1^{L613V} mutant increases Mek and Erk activation in response to multiple stimuli

Compared with WT RAF1, RAF1^{L613V} has increased kinase activity in vitro and an enhanced ability to activate MEK/ERK in transfection studies (148, 149). We assessed the effect of Raf1^{L613V}, expressed at endogenous levels, on the RAS-RAF-MEK-ERK MAPK pathway. Consistent with the earlier over-expression experiments, Mek and Erk activation (as inferred from immunoblots with activation-specific antibodies) was enhanced in multiple cell types
Figure 3-9. Severe perivascular fibrosis and infarct in L613V/+ mice after TAC.

(A) Severe perivascular fibrosis in L613V/+ heart after TAC (PSR staining; original magnification, 200X). (B) Gross appearance of an LV/+ heart (left) with a severe infarct after TAC, and severe fibrosis (right) in the infarcted region (PSR staining; original magnification, 100X).
Figure 3-10. Echocardiographic parameters in WT and L613V/+ mice following pressure overload.

Left ventricular diastolic posterior wall thickness (LVPWd) (A) and left ventricular internal end-diastolic dimension (LVIDd) (B) at 8 weeks after TAC. (C) Decreased stroke volume (SV) and fractional shortening (FS) in L613V/+ mice after TAC. (D) Cardiac output (CO) and heart rate at 8 weeks after TAC. *** p<0.0001 (Bonferroni post-test when ANOVA is significant); # p<0.05; ## p<0.005; ### p<0.0001 (1-tailed Student’s t-test); ns=not significant. n=12 for WT Sham; n=11 for L613V/+ Sham; n=22 for WT TAC; n=13 for L613V/+ TAC.
Figure 3-11. Hemodynamic parameters in WT and L613V/+ mice following pressure overload.

(A) Decreased cardiac contractility in L613V/+ mice after TAC. Because left ventricular pressures (LVP) are not identical in WT and L613V/+ mice, both dP/dt Max and dP/dt@LVP40 are shown. (B) Additional invasive hemodynamic parameters of hearts after TAC. EDP, end-diastolic pressure; MAP, mean arterial pressure. * p<0.05; ** p<0.005; *** p<0.0001 (Bonferroni post-test when ANOVA is significant); ## p<0.005; ### p<0.0001 (1-tailed Student’s t-test); ns=not significant.
expressing Raf1<sup>L613V</sup>, in response to a variety of stimuli, including LIF-stimulated ES cells (Figure 3-12A), and EGF or PDGF-stimulated embryonic fibroblasts (MEFs) (Figure 3-12B and Figure 3-12C). Of direct relevance to the L613V/+ cardiac phenotype, Mek and Erk activation also were higher in L613V/+ (compared with WT) neonatal cardiomyocytes stimulated with RTK (heregulin-β1), cytokine receptor (IL-6) or GPCR (AngII) agonists (Figure 3-13). Recently, cardiac fibroblasts were implicated in the genesis of cardiac hypertrophy (451, 452); notably, L613V/+ cardiac fibroblasts also showed enhanced agonist-stimulated Mek/Erk activation (Figure 3-14). The effects (quantitative and qualitative) of the mutant Raf1 allele on Mek and Erk activation differed in detail in cardiomyocytes versus cardiac fibroblasts (or MEFs) and in response to different stimuli. In some cases, mutant Raf1 affected only the peak level (magnitude) of activation, in others, solely the duration of activation, and for still others, both magnitude and duration. Such differences might reflect distinct feedback responses to the agonists in various cell types.

Although it was difficult to detect Erk activation in the adult heart under basal conditions (data not shown), basal Mek activity was significantly higher in adult L613V/+, compared with WT, hearts (Figure 3-15A). To compare Mek and Erk activation in vivo, we monitored the response of WT and L613V/+ mice to pressure overload evoked by TAC for up to 45 minutes. Mek activation remained significantly higher in L613V/+ hearts throughout the period of acute TAC (Figure 3-15A). Erk activation was significantly higher in L613V/+ hearts after 30 min TAC (compared with WT hearts), but was similar to WT at other time points (Figure 3-15B).

We also assayed several signaling pathways implicated in other models of cardiac hypertrophy/HCM by immunoblotting with appropriate p-specific antibodies. Activation of the MAPK family members c-jun NH2-terminal kinase (JNK) and p38 was comparable in AngII-stimulated neonatal cardiomyocytes (Figure 3-16A) and EGF-stimulated cardiac fibroblasts (Figure 3-16B). Likewise, Akt, GSK3β and p70S6K activation (in response to the agonists tested) were unaffected by Raf1<sup>L613V</sup> expression in either cell type. Importantly, in the same experiments, Mek and Erk activation were enhanced in L613V/+ cardiomyocytes and cardiac fibroblasts (Figure 3-16).
Figure 3-12. Raf1^{L613V} mutant causes increased Mek and Erk activation in multiple cell types.

(A) WT and L613V/+ ES cells were removed from feeders, starved for 6 hr, and then stimulated with LIF (10³ U/ml) or left unstimulated (0'). Cell lysates (20µg protein) were resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. One of two experiments with comparable results is shown. (B, C) Primary mouse embryo fibroblasts (MEFs) from WT and L613V/+ mice were starved for 16 hr, and then either stimulated with 10 ng/ml EGF (n=4; two independent experiments in two different MEF strains) or 50 ng/ml PDGF (n=2; one experiment in each of two different MEF strains). Quantification of blots from all experiments is shown at right.
Figure 3-13. Raf1$^{L613V}$ mutant increases Mek and Erk activation in cardiomyocytes.

Cardiomyocytes prepared from neonatal WT and L613V/+ mice were starved for 24 hr, and then either left unstimulated (0'), or stimulated for the indicated times with 1µg/ml Angiotensin II (AngII) (A), 10ng/ml IL-6 (B) or 100ng/ml heregulin-β1 (C). Cell lysates (15µg protein) were immunoblotted with the indicated antibodies. Quantification of blots is shown at right. One of two experiments with comparable results is shown.
Figure 3-14. Raf1<sup>L613V</sup> mutant increases Mek and Erk activation in cardiac fibroblasts.

Cardiac fibroblasts prepared from neonatal WT and L613V/+ mice were starved for 16 hr, and then either left unstimulated (0’) or stimulated for the indicated times with 50ng/ml EGF (A), 100ng/ml IGF-I (B), 100ng/ml PDGF (C) or 50ng/ml FGF2 (D). Cell lysates (20µg protein) were immunoblotted with the indicated antibodies. Quantification of blots is shown at right. One of two experiments with comparable results is shown.
Figure 3-15. Enhanced Mek and Erk activation in L613V/+ hearts after pressure overload.

Hearts from WT and L613V/+ mice were subjected to TAC for the indicated times (n=5 for each group at each time point), then lysed and analyzed by immunoblotting with the indicated antibodies. Each lane represents an individual animal. (A) Mek activation, with all samples from a single time point analyzed on the same gel. Quantification is shown at right. (B) Representative samples of Erk activation from each time point analyzed on the same gel. Quantification of all samples is shown at right. In both cases, Erk2 levels are shown as a loading control. * p<0.05, 2-tailed Student’s t-test.
Figure 3-16. Other signaling pathways are unaffected in neonatal cardiac myocytes and fibroblasts.

(A) Cardiomyocytes from neonatal WT and L613V/+ mice were starved for 24 hr, and then either left unstimulated (0’) or stimulated with AngII (1µg/ml). (B) Cardiac fibroblasts from neonatal WT and L613V/+ mice were starved for 16 hr, and then either left unstimulated (0’) or stimulated with EGF (10ng/ml). Cell lysates (15-20µg protein) were analyzed by immunoblotting with the indicated phospho-specific antibodies for other pathways implicated in cardiac hypertrophy.
MEK inhibitor treatment normalizes NS phenotypes in L613V/+ mice

The genetics of NS (and other RASopathies), and the ability of Raf\textsuperscript{L613V} to selectively enhance Mek and Erk activation by multiple agonists in cardiomyocytes and cardiac fibroblasts, strongly implicate enhanced Mek/Erk activation in the pathogenesis of NS phenotypes, including HCM. We asked whether any of these phenotypes might be reversible if Mek/Erk activation were normalized by treatment of L613V/+ mice with a MEK inhibitor. In initial experiments, the ATP-uncompetitive inhibitor PD0325901 (453) or empty vehicle was injected intraperitoneally (IP) daily to WT and L613V/+ mice (5mg/kg body weight), beginning at 4 weeks and continuing for the succeeding 6 weeks. Importantly, at the start of the treatment period, L613V/+ mice already show significant growth defects, facial dysmorphia and cardiac hypertrophy.

Remarkably, the body length of L613V/+ mice began to catch up with WT mice after 1 week of treatment, and by 2 weeks, L613V/+ mice were the same length as untreated WT mice (Figure 3-17A). MEK inhibitor-treated WT mice also increased their body length, such that by the last two weeks of treatment, they were significantly longer than control, untreated WT mice. Notably, however, MEK inhibitor-treated L613V/+ mice achieved the same final body length as treated WT mice, arguing that increased Mek/Erk activity is the primary cause of the growth defect in L613V/+ mice (see Discussion). Inhibitor treatment also increased the body weight of L613V/+ mice, but surprisingly, they, as well as inhibitor-treated WT mice, gained substantially more weight than untreated WT mice (Figure 3-17B). Increased body weight in MEK-inhibitor-treated mice was accompanied (and presumably in large part caused) by an obvious increase in body fat; thus, increased adiposity/body mass is an unanticipated, and previously unreported, side-effect of PD0325901 (and possibly, MEK inhibitor) treatment (see Discussion).

MEK inhibitor treatment also affected the L613V/+ cardiac phenotype. The heart weight to body weight ratio (Figure 3-17C) in L613V/+ mice was restored to normal range (WT control) after treatment, whereas there was no significant change in WT mice. Echocardiography (Figure 3-17D and Figure 3-17E) and invasive hemodynamic (Figure 3-19) studies showed significant improvement in multiple parameters of cardiac morphology and function. Furthermore, histological assessment of cross-sectional area of cardiomyocytes confirmed the normalization of
Figure 3-17. MEK inhibitor treatment rescues growth defect and cardiac hypertrophy in L613V/+ mice.

Mice were injected intraperitoneally (IP) daily with PD0325901 (5mg/kg body weight) or vehicle, starting at 4 weeks of age and for the succeeding 6 weeks. Body length (A) and body weight (B) were measured weekly. Note the rapid normalization of body length, as well as the increase in body weight caused by inhibitor treatment. * p<0.05; ** p<0.005; *** p<0.0001 (two-way repeated measure ANOVA); # p<0.05; ## p<0.005; ### p<0.0001 (Bonferroni post-test when ANOVA is significant; black asterisk for WT treatment vs. WT control; red asterisk for L613V/+ treatment vs. L613V/+ control). (C) Heart weight/body weight (mg/g) ratio and (D) Left ventricular diastolic posterior wall thickness (LVPWd) are restored to within normal limits in inhibitor-treated mice. ** p<0.005; *** p<0.0001 (Bonferroni post-test when ANOVA is significant); # p<0.05 (1-tailed Student’s t-test); ns=not significant. (E) Left ventricular end-diastolic dimension (LVIDd). **p<0.005 (Bonferroni post-test when ANOVA is significant); # p<0.05; ## p<0.005 (1-tailed Student’s t-test); ns=not significant. n=14 for WT control; n=10 for L613V/+ control; n=6 for WT treatment (WT PD); n=14 for L613V/+ treatment (L613V/+ PD). (F) Cross-sectional area of cardiomyocytes (original magnification, 400X; white bar, 100µm), measured in WGA-strained heart sections (n=2 samples for each group, with 200 cells counted for each sample using ImageJ). *** p<0.0001 (Bonferroni post-test when ANOVA is significant); ns=not significant.
Figure 3-18. Normalized cardiac morphology and function after MEK inhibitor treatment.

(A) Left ventricular diastolic posterior wall thickness (LVPWd) normalized by BW^{1/3}. (B) Left ventricular internal end-diastolic dimension (LVIDd) normalized by BW^{1/3}. (C) Normalized stroke volume (SV). End-diastolic volume (EDV) = (4.5 x normalized LVIDd^2); End-systolic volume (ESV) = (3.72 x normalized LVIDd^2); SV=EDV-ESV. (D) Normalized cardiac output (CO). CO=Normalized SV X Heart rate. ** p<0.005; *** p<0.0001 (Bonferroni post-test when ANOVA is significant); # p<0.05; ## p<0.005 (1-tailed Student’s t-test); ns=not significant. n=14 for WT control; n=10 for LV/+ control; n=6 for WT treatment (WT PD); n=14 for L613V/+ treatment (L613V/+ PD).
Figure 3-19. MEK inhibitor treatment normalizes cardiac function in L613V/+ mice.

(A) Echocardiographic parameters of hearts after MEK inhibitor treatment as described in Figure 3-17. Note normalization of stroke volume (SV) and fractional shortening (FS), with a trend towards normalization of cardiac output (CO). * p<0.05; ** p<0.005; *** p<0.0001 (Bonferroni post-test when ANOVA is significant); # p<0.05 (1-tailed Student’s t-test); ns=not significant.

(B) Hemodynamic parameters, assessed by cardiac catheterization, after MEK inhibitor treatment. For calculating statistical significance, significant outliers (circled data points), as accessed by Grubbs’ test, were removed. *: p<0.05 (Bonferroni post-test when ANOVA is significant); p=0.09 when outliers are not removed. #: p<0.05 (1-tailed Student’s t-test); p=0.12 when outliers are not removed. ns, not significant. n=14 for WT control; n=10 for L613V/+ control; n=6 for WT treatment (WT PD); n=14 for L613V/+ treatment (L613V/+ PD).
cardiomyocyte size in L613V/+ mice after treatment (Figure 3-17F). The significant increase in body size and body mass caused by inhibitor treatment potentially complicates echocardiography and invasive hemodynamic comparisons of pre- and post-treatment WT and L613V/+ mice, respectively. Therefore, we compared all parameters using both nominal values and values normalized by BW\(^{1/3}\) (Figure 3-18); overall, the two analysis regimes lead to similar conclusions. First, there was a significant reduction (towards normal) in left ventricular posterior wall thickness in L613V/+ mice after treatment (Figure 3-17D); this difference was even more significant when normalized by BW\(^{1/3}\) (Figure 3-18). Nominal left ventricular end-diastolic dimension was unchanged in inhibitor-treated L613V/+ mice (Figure 3-17E), although when this value is normalized, chamber dilatation was improved significantly, becoming comparable to WT controls (Figure 3-18B). Inhibitor treatment clearly reduced the abnormal SV and FS in L613V/+ mice towards normal (untreated or treated WT) values (Figure 3-19A and Figure 3-18C). There also was a strong trend towards decreased CO in L613V/+ mice after inhibitor treatment (Figure 3-19A and Figure 3-18D). Finally, the excessive cardiac contractility (dP/dt and dP/dt@LVP40) in L613V/+ mice was ameliorated by inhibitor treatment, while cardiac relaxation remained unchanged (Figure 3-19B).

MEK inhibitor treatment did not improve the facial dysmorphia in L613V/+ mice in the above study, most likely because skull development had already been completed by the onset of drug administration. We tested whether earlier, but still post-natal, MEK inhibitor treatment could prevent/ameliorate L613V/+ facial defects. Lactating female mice were injected IP with PD0325901 (5mg/kg body weight) daily, beginning at postnatal day 0 (P0) until weaning (P21). Weaned mice were then injected individually with same dose of inhibitor for another 5 weeks. As expected from our initial treatment regimen (Figure 3-17A), the growth defect in L613V/+ mice was again prevented in this new study. Remarkably, however, earlier MEK inhibitor treatment had a dramatic effect on the appearance of L613V/+ mice: they no longer had “triangular” faces and instead, appeared indistinguishable from control (treated or untreated) WT mice (Figure 3-20A). MicroCT morphometry confirmed that inner canthal distance was reduced significantly, while skull length was increased and skull width and width/length ratio were decreased in inhibitor-treated L613V/+ mice; all of these values were indistinguishable from WT (treated or untreated) mice by the end of the treatment period (Figure 3-20B).
Figure 3-20. Early post-natal MEK inhibitor treatment rescues facial dysmorphia in L613V/+ mice.

Lactating female mice were injected IP daily with PD0325901 (5mg/kg body weight) or vehicle, starting at postnatal day 0 (P0) until weaning (P21). Weaned mice were then injected IP individually with PD0325901 (5mg/kg body weight) or vehicle daily for another 5 weeks. (A) Gross facial appearances for mice treated with PD0325901 (PD) or vehicle. (B) Morphometric measurements of skulls from microCT scans. ** p<0.005; *** p<0.0001 (Bonferroni post-test when ANOVA is significant). n=11 for WT control; n=10 for L613V/+ control; n=6 for WT treatment (WT PD); n=7 for L613V/+ treatment (L613V/+ PD).
3.4 Discussion

We describe here a knock-in mouse model for Noonan syndrome (NS) caused by a Raf1 gain-of-function mutation. Similar to mouse models of Ptpn11 mutation-associated NS, Raf1^{L613V} heterozygosity causes proportional short stature, facial dysmorphia, and hematological defects. Unlike phosphatase-activating Ptpn11 alleles, which cause valvuloseptal abnormalities (204, 207, 210), L613V/+ mice have normal valvuloseptal development and instead exhibit eccentric cardiac hypertrophy that decompensates upon pressure overload. Agonist-evoked Mek/Erk activation is enhanced in multiple cell types without changes in several other signaling pathways implicated in cardiac hypertrophy/HCM. Remarkably, post-natal MEK inhibition normalizes the growth, facial and cardiac defects in L613V/+ mice, demonstrating that continued MEK/ERK activity is critical for causing HCM and other NS phenotypes and identifying MEK inhibitors as potential therapeutic agents for the treatment of NS.

RASopathies are a class of human genetic syndromes caused by germ line mutations in genes that encode components of the RAS/ERK pathway (32, 439). Not surprisingly, these disorders share several features (albeit with varying degrees of penetrance), yet each also exhibits unique and characteristic phenotypes. Conceivably, the specific mutant gene, possibly as a consequence of its position in the pathway and susceptibility to feedback regulation, could direct the phenotype. Alternatively, genetic modifiers in the highly outbred human population could be determinative.

Previous mouse models suggest that both the gene and the genetic background are important to the ultimate RASopathy phenotype. Clearly, different mutations in the same RASopathy gene can result in distinguishable phenotypes: gain-of-function Ptpn11 mutations, depending on the degree of their phosphatase activity, cause a variable spectrum of NS phenotypes (204, 207, 210). The current study, along with a parallel analysis of knock-in mice expressing a NS-associated Sos1^{E846K} mutant (211), shows that mutations in different genes that cause the same RASopathy syndrome yield different phenotypes: mice with phosphatase-activating Ptpn11 mutations have valvuloseptal defects, but not HCM (204, 207); Sos1^{E846K/+} mice develop left ventricular hypertrophy with incompletely penetrant aortic stenosis; and Raf1^{L613V/+} mice exhibit HCM with normal valvuloseptal development.
On the other hand, mutations associated with different RASopathies also have distinct effects in mice. In contrast to the NS mice discussed above, an HRas$^{G12V}$ knock-in mouse model of Costello Syndrome (CS) shows abnormal cranial dimensions, papillomas and angiosarcomas. These mice have cardiac hypertrophy, but also aortic stenosis, making it unclear whether the hypertrophy is primary or secondary (439, 443). As described in the accompanying manuscript (444), a mouse model of LEOPARD syndrome (LS) caused by Ptpn11$^{Y279C}$, indicates that, unlike Ptpn11 alleles with increased catalytic activity, catalytically impaired mutants develop HCM and skeletal abnormalities (as well as short stature and facial dysmorphia).

While the specific mutation plays a major role in determining RASopathy phenotype, modifier loci also clearly contribute: just as there is considerable phenotypic variation between family members carrying the same NS or LS allele (454), there are differences in disease spectrum and severity of mice with Ptpn11 (204, 210) and Raf1 (data not shown) mutations on different strain backgrounds. Ptpn11$^{D61G/+}$ mice show incomplete penetrance of valvuloseptal defects on mixed background and various penetrance of embryonic lethality on different strain backgrounds (204, 210). Raf1$^{L613V/+}$ mice were obtained at the expected Mendelian ratio on mixed background, whereas on the C57BL/6 background this mutant allele almost always was lethal (data not shown). All of these data suggest that incomplete penetrance reflects strain-specific modifiers. Genomic scans using SNP panels should help to determine whether cloneable modifiers exist or heterosis accounts for the variable penetrance.

The role of the RAS/ERK pathway in cardiac hypertrophy has been controversial. Overexpression of MAPK phosphatase 1 (MKP-1) blocks both agonist-induced hypertrophy in vitro and pressure overload-associated hypertrophy in vivo (455). However, MKP-1 inactivates all three major MAPKs, so this study could not address the specific effects of Ras/Erk pathway activation. Depletion of ERK1/2 with antisense oligonucleotides or pharmacological inhibition of MEK1/2 attenuates the hypertrophic response to agonist stimulation of cultured cardiomyocytes (414, 415), consistent with a requirement for MEK/ERK activation in the hypertrophic response. Transgenic mice with cardiac-specific expression of HRas$^{G12V}$ display HCM associated with interstitial fibrosis and sudden death (411-413). Cardiac-specific Nf1-deleted mice develop marked cardiac hypertrophy, progressive cardiomyopathy, and fibrosis as adults (456).
 Conversely, other studies suggest that MEK/ERK activity is dispensable for cardiomyocyte hypertrophy. Transgenic mice with cardiac-restricted expression of activated Mek1 exhibit concentric hypertrophy without signs of cardiomyopathy (418). Although hypertrophy in this model was interpreted as physiological, these mice also have impaired diastolic function and reactivated cardiac fetal gene expression, which is more consistent with pathologic hypertrophy. A recent study showed that Erk1+/Erk2+/ mice, or transgenic mice with cardiac-specific expression of dual specificity phosphatase 6 (Dusp6), an Erk1/2-specific phosphatase, showed a normal hypertrophic response to pressure overload and exercise (420). In both of these lines of mice, however, residual Erk activity cannot be excluded. Also, it is possible that Dusp6 has other targets besides Erk1/2, which could complicate interpretation of these results. Moreover, most of these earlier studies involved cardiomyocyte-specific expression or deletion of potential hypertrophy-related genes, which excludes the potential contribution of other cell types in the heart to the hypertrophic response. Recent studies show that cardiac fibroblasts play key roles in myocardial development and function (457, 458). Embryonic cardiac fibroblasts induce myocyte proliferation, whereas adult cardiac fibroblasts promote myocyte hypertrophy (457), and evoke pathological hypertrophy and fibrosis in response to disease stimuli (451, 452). Of particular note, enhanced Ras/Erk activation in cardiac fibroblasts is implicated in pathological hypertrophy and fibrosis caused by over-expression of the beta-adrenergic receptor in cardiomyocytes (452).

Our mouse model, in which a NS-associated Raf1 mutant is expressed globally under normal promoter control, supports the conclusion that excessive Ras/Erk pathway activity causes HCM. Several lines of evidence indicate that L613V/+ mice have pathologic cardiac hypertrophy. Hypertrophy is eccentric in these mice, and they show the characteristic shift from alpha-Mhc to beta-Mhc expression seen in pathological hypertrophy. In response to pressure overload (TAC), they have an unusually high death rate, presumably due to inability to adapt to this stress or arrhythmia, while surviving mice show clear evidence of functional decompensation. Importantly, in our model, unlike many previous studies (see above), the mutant allele is expressed in both cardiomyocytes and cardiac fibroblasts (as well as multiple other cell types). Moreover, Mek/Erk activation is enhanced in response to multiple agonists in these cells. It will be important to determine whether mutant expression in cardiomyocytes, cardiac fibroblasts, or both is important for HCM in L613V/+ mice; our inducible Raf1 allele should facilitate such
analyses. Most importantly, post-natal MEK inhibitor treatment substantially normalizes the cardiac defects in L613V/+ mice, providing strong evidence for the critical role of the RAS/ERK pathway in initiating and maintaining the cardiac hypertrophic response.

Post-natal MEK inhibitor treatment also normalizes the growth defects and, if administered early enough, the facial dysmophia in L613V/+ mice. Notably, MEK inhibitor treatment also increases the body length of WT mice, but there is no difference in the final body length (after treatment) between the WT and mutant-treated groups. Likewise, MEK inhibitor treatment (at doses that effectively normalized L613V/+ cardiac anatomy and function) had little effect on cardiac function in WT mice. These results strongly suggest that all of these NS phenotypes are due to excessive MEK/ERK activity (as opposed to the MEK inhibitor acting on a parallel pathway to mitigate syndromic features).

Unexpectedly, we found that PD0325901 treatment caused a significant increase in body weight with an obvious increase in body fat. Although we cannot exclude the possibility that this is an idiosyncratic (i.e., off-target) effect of this specific MEK inhibitor, other evidence points to a potential obesity-promoting effect of MEK/ERK inhibition. For example, leptin activates Erk via an Shp2-dependent pathway (459, 460) and deletion of Shp2 in post-mitotic forebrain neurons causes early-onset obesity with decreased ERK activation and evidence of leptin resistance (461). We suspect that MEK inhibition may act in analogous ways to promote obesity in our mice.

In summary, our data demonstrate a critical role of the RAS/ERK pathway in the genesis of HCM in NS, and show that NS phenotypes can be rescued by pharmacological inhibition of MEK1/2. Previous studies showed that genetic ablation of Erk1/2 (207, 210) or pre-natal treatment with a MEK inhibitor (209, 211) can prevent some NS phenotypes. While these studies provide evidence for the key role of Mek/Erk hyperactivity in NS pathogenesis, they did not resolve whether MEK inhibition can reverse these phenotypes. By contrast, our results suggest that MEK inhibition may be useful for the specific treatment of Raf1 mutant NS, and possibly for other RASopathies associated with increased MEK/ERK pathway activity. Interestingly, a parallel study show that LS-associated HCM is associated with hyper-activation of PI3K/Akt pathway, and can be rescued by Rapamycin treatment (444). Taken together, these studies argue for a mutation-specific, “personalized” approach to RASopathy therapy.
Chapter 4

Increased BRAF heterodimerization is the common pathogenic mechanism for Noonan Syndrome-associated RAF1 mutants

This Chapter is a modified version of a paper published in Molecular and Cellular Biology (2012 Oct;32(19):3872-90).
### 4.1 Abstract

Noonan syndrome (NS) is a relatively common autosomal dominant disorder, characterized by congenital heart defects, short stature and facial dysmorphia. NS is caused by germ-line mutations in several components of the RAS-RAF-MEK-ERK MAPK pathway, including both kinase-activating and kinase-impaired alleles of \(\text{RAF1} \) (~3-5%), which encodes a serine-threonine kinase for MEK1/2. To investigate how kinase-impaired RAF1 mutants cause NS, we generated knock-in mice expressing \(\text{Raf1}^{D486N} \). \(\text{Raf1}^{D486N/+} \) (hereafter D486N/+) female mice exhibited a mild growth defect. Male and female D486N/D486N mice developed concentric cardiac hypertrophy and incompletely penetrant, but severe, growth defects. Remarkably, Mek/Erk activation was enhanced in Raf1\(D486N\)-expressing cells compared with controls. \(\text{RAF1}^{D486N} \), as well as other kinase-impaired RAF1 mutants, showed increased heterodimerization with BRAF, which was necessary and sufficient to promote increased MEK/ERK activation. Furthermore, kinase-activating \(\text{RAF1} \) mutants also required heterodimerization to enhance MEK/ERK activation. Our results suggest that increased heterodimerization ability is the common pathogenic mechanism for NS-associated \(\text{RAF1} \) mutations.

### 4.2 Background

Noonan syndrome (NS) is a relatively common (1 in 1,000–2,500 live births) autosomal dominant disorder (127, 462, 463), characterized by short stature, craniofacial dysmorphia, a wide spectrum of congenital cardiac anomalies, and an increased risk of hematopoietic malignancy. Although NS is genetically heterogeneous (31, 32, 144), all known cases are caused by germ-line mutations in conserved components of the canonical RAS-RAF-MEK-ERK MAPK (hereafter, RAS/ERK) cascade, a key regulator of cell proliferation, differentiation and survival (26, 27). Mutations in \(\text{PTPN11} \), which encodes the protein-tyrosine phosphatase SHP2, account for approximately half of NS cases (145). Other known NS genes include \(\text{SOS1} \) (~10%) (146, 147), \(\text{RAF1} \) (3-5%) (148, 149), \(\text{KRAS} \) (<2%) (150, 151), \(\text{NRAS} \) (152) and \(\text{SHOC2} \) (<1-2%). Mutations in some of these genes, as well as in genes encoding other RAS/ERK pathway components, also cause phenotypically related disorders, such as neurofibromatosis type 1 (NF1), Costello syndrome, cardio-facio-cutaneous (CFC) syndrome, and LEOPARD syndrome; together with NS, these syndromes are now termed “RASopathies” (32). How mutations in the
same signaling pathway cause similar, yet clearly distinct, phenotypes remains unclear. Consequently, detailed understanding of RASopathy pathogenesis should yield new insights into RAS/ERK pathway regulation.

RAF family serine-threonine kinases (250-252) function as key RAS effectors, phosphorylating and activating the dual specificity kinases MEK1 and MEK2, which in turn promote the activation of the MAPKs, ERK1 and ERK2. The three mammalian RAF family members (RAF1, BRAF and ARAF) differ in their expression profiles and regulatory mechanisms, and have distinct roles during development. RAF1 (also known as CRAF) is the most intensively studied isoform. Nevertheless, controversy and disagreement surround the precise molecular events required for RAF1 activation, which include RAS-dependent membrane recruitment, conformational changes, dimerization or oligomerization, scaffold protein binding, and distinct phosphorylation/dephosphorylation events. RAF1 also has important kinase-independent functions. For example, it interacts with and inhibits apoptosis signal-regulating kinase 1 (ASK1) (227, 338), Mammalian STE20-like kinase 2 (MST2) (228, 339) and Rok-α (229).

Two groups (148, 149) identified multiple missense mutations of RAF1 in NS, which cluster in three regions. Approximately 70% of NS-associated RAF1 alleles alter the motif flanking S259 within the so-called CR2 domain, which binds to 14-3-3 proteins and is critical for auto-inhibition (163, 164). The second group of mutations (~15%) affects residues within the activation segment of the kinase domain (D486 and T491). The remaining alleles (~15%) involve two adjacent residues (S612 and L613) located C-terminal to the kinase domain. Transient transfection studies indicate that mutations affecting the 14-3-3 binding motif or the C-terminus of the protein enhance RAF1 kinase activity and increase MEK/ERK activation in cells. By contrast, mutations that cluster in the activation segment are kinase-impaired and reportedly act as dominant negative or null alleles (148, 149). Previous work suggested that the increased kinase activity of NS-associated CR2 domain mutants results from decreased S259 phosphorylation and consequent dissociation from 14-3-3 (149, 165, 166), but the mechanism underlying increased kinase activity of the RAF1 C-terminal mutants remains unclear. Likewise, how kinase-defective RAF1 alleles cause NS has remained obscure, if not paradoxical. Studies of kinase-defective BRAF alleles strongly implicate enhanced MEK/ERK activation and heterodimerization with RAF1 in human melanoma pathogenesis (167, 168). The paradoxical
activation of the MEK/ERK pathway in wild type cells treated with selective small molecule BRAF inhibitors also has been attributed to the ability of these inhibitors to induce BRAF/RAF1 heterodimer formation (169, 170). The relevance of these observations for RAF1 alleles expressed at physiological expression levels remains to be determined.

Kinase-activating and kinase-impaired RAF1 alleles also are associated with different syndromic phenotypes. NS patients with RAF1 mutations have a much higher incidence (~75%) of hypertrophic cardiomyopathy (HCM) than is found in the overall NS population (~20%). However, only RAF1 alleles encoding kinase-activated mutants are highly associated (~95%) with HCM. Recently, we reported that knock-in mice expressing the kinase-activated allele Raf1L613V develop typical NS features (short stature, facial dysmorphia, haematological abnormalities), as well as HCM (464). As expected, agonist-evoked MEK/ERK activation was enhanced in multiple cell types expressing Raf1L613V. Moreover, postnatal MEK inhibition normalized the growth, facial, and cardiac defects in L613V/+ mice, showing that enhanced MEK/ERK activation is critical for evoking RAF1-mutant NS phenotypes.

Whether kinase-defective Raf1 alleles also faithfully model human NS, and if so, how kinase-activating and kinase-defective mutants can cause similar phenotypes, remains to be resolved. To investigate this paradox, we generated and analyzed knock-in mice expressing the kinase-impaired NS mutant Raf1D486N, and also re-examined the effects of various other NS mutants expressed at more physiological levels than in previous transient transfection studies. Our results strongly implicate increased heterodimerization ability as the common pathogenic mechanism for NS-associated RAF1 mutations.

4.3 Results

4.3.1 Generation of Raf1D486N mice

To avoid the possibility that expression of kinase-impaired Raf1 might cause embryonic lethality, we designed an inducible Raf1D486N “knock-in” allele (D486Nfl; Figure 4-1A). The targeting vector included a cassette containing a splice acceptor sequence, a Raf1 cDNA fragment encoding WT exons 13-16, and a pGK-Neo gene. The fusion cDNA/Neo cassette was flanked by LoxP sites and was positioned between Raf1 exons 12 and 13, with the D486N
Figure 4-1. Generation of Raf1^{D486N} knock-in mice.

(A) Targeting strategy. Structures of the Raf1 locus, targeting vector, mutant allele and location of probes for Southern blotting are shown. (B) Selection of targeted ES cells. To assay for inclusion of the 5’ loxP site, a PCR product, obtained by using primers within exon 11 and the Raf1 cDNA, was digested with NotI. To ensure inclusion of the D486N mutation, a PCR product, obtained by using primers around exon 13, was digested with ApoI. To assay for inclusion of the Neo cassette, a PCR product was obtained by using primers within the PGK Neo gene. (C) ES cells are targeted correctly. Genomic DNA from WT ES cells and PCR-positive D486Nfl/+ ES clones was digested with XbaI (5’ and Neo probe) or BamHI (3’ probe) and subjected to Southern blotting with 5’, 3’ or Neo probes, respectively. (D) Inducible expression of the Raf1^{D486N} allele. RNA was isolated from WT and D486Nfl/+ ES cells with or without prior transfection of pMSCV-Cre-GFP plasmid and reverse transcribed. A PCR product, obtained by using primers within exon 11 and at the end of exon 16 of the Raf1 cDNA, was digested with ApoI. Note that the mutant allele is silent until Cre is introduced, and then is expressed efficiently. (E) Progeny from D486N/+ matings with the indicated littermates.
mutation introduced into exon 13 and an HSV-TK cassette for negative selection. In the absence of Cre recombinase (Cre), Raf1 exon 12 should be spliced to the cDNA (exons 13-16), resulting in the expression of WT Raf1. When Cre is present, the floxed cassette is excised, leading to the transcription of the mutant Raf1 allele (D486N).

The targeting construct was electroporated into G4 ES cells, and correctly targeted clones (D486Nfl/+ ) were identified by PCR (Figure 4-1B) and confirmed by Southern blotting (Figure 4-1C). To test whether the mutant Raf1 allele could be induced in D486Nfl/+ ES cells, a Cre expression vector (pMSCV-GFP-Cre) was introduced; as expected, this resulted in efficient transcription of the mutant allele (Figure 4-1D). Chimeras were generated by outbred morula aggregation, and germ-line transmission was obtained. D486Nfl/+ progeny were crossed to EIIa-Cre mice, which express Cre ubiquitously, and then to WT mice, thereby generating mice with global Raf1D486N expression (referred to as D486N/+ mice) on a 129Sv × C57BL/6 mixed background. D486N/+ littermates were intercrossed to generate mice homozygous for Raf1D486N (referred to as D486N/D486N mice). D486N/+ and D486N/D486N mice were obtained at the expected Mendelian ratios at weaning (Figure 4-1E), indicating that Raf1D486N expression is compatible with embryonic development.

4.3.2 Phenotypes of D486N/+ and D486N/D486N mice

Major features of NS include short stature, facial dysmorphia, cardiovascular abnormalities and often, some form of myeloproliferative disease (MPD) (438). D486N/+ female mice exhibited a mild, but reproducible growth defect compared with WT littermates, although male heterozygotes had normal body size (Figure 4-2A). Male and female D486N/D486N mice showed two distinct patterns of growth: about two thirds of these animals had a normal growth pattern but in the remaining one third, body length and weight were markedly decreased (~50% smaller than littermate controls). Hereafter, we refer to the normal sized mice as n-D486N/D486N, and the smaller ones as s-D486N/D486N. The majority of s-D486N/D486N mice died shortly after weaning, while those that survived continued to have reduced body size, a hunched appearance with ruffled fur and frequent tremors, and ultimately, died between 4-8 months of age. Consistent with their decreased body size, the skulls of s-D486N/D486N mice were significantly shorter than those of WT mice. However, skull width was decreased only slightly, resulting in a significant increase in width/length ratio and a “triangular” facial
appearance (Figure 4-2B). By contrast, n-D486N/D486N mice had normal lifespan, body size and facial morphology (Figure 4-2B). Because D486N/D486N mice were maintained on a mixed strain background, genetic modifiers presumably account for the variable phenotype.

Similar to RAFT1D486N/+ NS patients, D486N/+ mice had normal heart size. By contrast, n-D486N/D486N mice showed cardiac enlargement, manifested by a significantly increased heart weight to body weight ratio compared with WT and D486N/+ littermates (Figure 4-2C). Neonatal cardiomyocytes prepared from D486N/D486N mice showed significant increased surface area compared with WT cardiomyocytes, indicating that cardiac enlargement was due to hypertrophy (Figure 4-2D). Echocardiography performed on 4 month-old n-D486N/D486N mice showed increased left ventricular diastolic posterior wall thickness (LVPWd) as expected, while D486N/+ mice had normal LVPWd (Figure 4-2E). In contrast to Raf1L613V/+ mice, which develop cardiac dilatation (464), left ventricular internal end-diastolic dimension (LVIDd) remained normal in D486N/+ and n-D486N/D486N mice, while left ventricular internal end-systolic dimension (LVIDs) tended to be reduced in D486N/+ hearts and was decreased significantly in n-D486N/D486N hearts (Figure 4-2F). Stroke volume (SV), fractional shortening (FS), cardiac output (CO), and ejection fraction (EF) also were increased in n-D486N/D486N mice (Figure 4-2G). Cardiac parameters could not be assessed in s-D486N/D486N mice because of their size and general ill health. Overall, these findings indicate that D486N/D486N mice have concentric cardiac hypertrophy with enhanced cardiac function.

Unlike other mouse models of NS (204, 210, 464, 465), D486N/+ and D486N/D486N mice did not develop splenomegaly (Figure 4-3A), nor they show overt hematological defects (Figure 4-3B and Figure 4-3C).

4.3.3 Raf1D486N expression increases Mek/Erk activation in response to multiple stimuli

NS-associated RAFT1 mutations include kinase-activating mutations (e.g., S257L and L613V) and mutations with impaired kinase activity (e.g., D486N and T491I/R) (148, 149). To begin to investigate why kinase-impaired and -activated RAFT1 mutants cause similar phenotypes, we assessed Mek and Erk activation in mouse embryonic fibroblasts (MEFs) prepared from heterozygous and homozygous Raf1D486N embryos and stimulated with various
Figure 4-2. Phenotypes of D486N/+ and D486N/D486N mice.

(A) D486N/+ females have mild growth defect. Growth curves of male (top) and female (bottom; p<0.0001, two-way repeated measure ANOVA) WT (n=25) and D486N/+ (n=25) mice. (B) D486N/D486N mice with severe growth defect (s-D486N/D486N) have facial dysmorphia. Morphometric measurements from microCT scans of a cohort of 2-month-old WT (n=12), normal size D486N/D486N (n=D486N/D486N) (n=10) and s-D486N/D486N (n=3) male mice. (C) Representative gross appearance and heart weight/body weight (mg/g) ratios of WT (n=15), D486N/+ (n=20) and D486N/D486N (n=15) male mice at 4 months. (D) Surface area of isolated neonatal cardiomyocytes from WT and D486N/D486N mice. For each genotype, 350 cells were analyzed using ImageJ. ### p<0.001, 2-tailed Student’s t-test. (E) Left ventricular diastolic posterior wall thickness (LVPWd) at 4 months, measured by echocardiography (n=12 for WT; n=20 for D486N/+; n=12 for D486N/D486N). (F) Left ventricular chamber dimensions of 4 month-old WT (n=12), D486N/+ (n=20) and D486N/D486N (n=12) hearts. LVIDs, left ventricular internal end-systolic dimension; LVIDd, left ventricular internal end-diastolic dimension. (G) Echocardiographic parameters of 4 month-old WT (n=12), D486N/+ (n=20) and D486N/D486N (n=12) hearts. SV, stroke volume; FS, fractional shortening; CO, cardiac output; EF, ejection fraction. * p<0.05; ** p<0.01; *** p<0.001 (Bonferroni post-test when ANOVA is significant). ns, not significant.
(A) Spleen weight to body weight ratios in WT, D486N/+ and D486N/D486N male mice at 5 month (n=15 for each genotype). (B) White blood counts (WBC) in WT, D486N/+ and D486N/D486N male mice at 1 year (n=6 for each genotype). (C) Myeloid colony formation in the absence of cytokines of bone marrow cells from WT, D486N/+, and D486N/D486N mice. Bulk bone marrow cells were extracted at 1 year (n=3 for each genotype). Colonies were enumerated 7 days after plating. Bone marrow from an induced Mx-Cre:KrasG12D mouse (n=1), serves as a positive control for cytokine-independent colony formation. ns, not significant.
agonists. As reported previously (464), Raf1<sup>L613V</sup> expressing cells show enhanced Mek/Erk activation in response to agonists for receptor tyrosine kinases (RTKs), cytokine receptors and G protein-coupled receptors (GPCRs). Notably, in D486N/+ MEFs, Raf1 protein levels were only ~60% of WT levels, a finding consistent with previous work showing that Raf1 kinase activity is required to prevent ubiquitin-mediated proteolysis of Raf1 (297). Nevertheless, D486N/+ MEFs showed sustained Mek and Erk activation in response to EGF (Figure 4-4A) stimulation. D486N/D486N MEFs had only ~30% of WT Raf1 levels, but showed enhanced and sustained Mek and Erk activation (Figure 4-4A). Mek and Erk activation also were enhanced in D486N/D486N neonatal cardiomyocytes stimulated with cytokine receptor (IL-6), GPCR (Ang-II), or RTK (IGF-I, EGF and NRG) ligands (Figure 4-4B and Figure 4-5), providing a potential explanation for cardiac hypertrophy in D486N/D486N mice (Figure 4-2). Cardiac fibroblasts also are implicated in hypertrophy pathogenesis (452, 466), and compared with their WT counterparts, neonatal D486N/D486N cardiac fibroblasts showed enhanced Mek/Erk activation in response to multiple agonists (Figure 4-4C and Figure 4-6).

### 4.3.4 Quantitative differences in effects of NS-associated Raf1 D486N and L613V mutants on Mek/Erk activation

Kinase-activating (e.g., L613V) and kinase-impaired (e.g., D486N) Raf1 mutants cause NS, but HCM is only highly associated with the former (148, 149, 467). Remarkably, our Raf1<sup>L613V</sup> (L613V+/+) (464) and Raf1<sup>D486N</sup> mouse models showed analogous genotype-dependent phenotypic differences (Table 4-1). L613V/+ mice exhibit HCM that progresses to chamber dilatation (464). By contrast, D486N/+ mice had no obvious cardiac phenotype, but doubling the dosage of this kinase-defective Raf1 allele (e.g., in D486N/D486N mice) resulted in concentric cardiac hypertrophy with decreased left ventricular end-systolic dimension and normal end-diastolic dimension (Figure 4-2).

We asked whether these phenotypic differences correlated with differential effects on Mek/Erk activation. Indeed, Raf1<sup>L613V</sup> caused a dramatic increase of the magnitude of EGF-evoked Mek/Erk activation in primary MEFs, whereas Raf1<sup>D486N</sup> resulted mainly in sustained Mek/Erk activation (Figure 4-7A). More importantly, Mek activation was enhanced only slightly in hearts from D486N/D486N mice, whereas L613V/+ hearts showed a more profound increase
**Figure 4-4. Raf1D486N mutant increases Mek and Erk activation.**

(A) Primary mouse embryo fibroblasts (MEFs) from WT, D486N/+ and D486N/D486N mice were serum-starved for 16 hr, and then stimulated with EGF for the indicated times. Cell lysates were immunoblotted with the indicated antibodies. A representative blot and quantification of blots from all experiments are shown (n=6; two independent experiments using three different MEF strains). * p<0.05; ** p<0.01; *** p<0.001 (left bottom). * p<0.05; ** p<0.01; *** p<0.001 (right; D486N/D486N vs. WT). # p<0.05; ## p<0.01 (right; D486N/+ vs. WT); Bonferroni post-test, when ANOVA is significant. (B) Neonatal cardiomyocytes from WT and D486N/D486N mice were starved for 24 hr, and then stimulated for the indicated times with the indicated agonists. AngII, Angiotensin II. NRG, heregulin-β1. Cell lysates were immunoblotted, as indicated. (C) Cardiac fibroblasts from WT and D486N/D486N neonates were starved for 16 hr, and then stimulated for the indicated times with various agonists. Cell lysates were immunoblotted with the indicated antibodies.
Figure 4-5. Mek/Erk activation in WT and D486N/D486N neonatal cardiomyocytes.

Cardiomyocytes prepared from neonatal WT and D486N/D486N mice were starved for 24 hr, and then stimulated for the indicated times with different agonists, as indicated. Mek/Erk activation (n=2) was analyzed by immunoblotting with specific antibodies and quantified using Odyssey software.
Figure 4-6. Mek/Erk activation in WT and D486N/D486N neonatal cardiac fibroblasts.

Cardiac fibroblasts prepared from neonatal WT and D486N/D486N mice were starved for 24 hr, and then stimulated for the indicated times with different agonists, as indicated. Mek/Erk activation (n=2) was analyzed by immunobloting with specific antibodies and quantified using Odyssey software.
<table>
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<th>WT (n=20)</th>
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<th>L613V/+ (n=11)</th>
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<td>LVPWd (mm)</td>
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<td>0.81±0.02 *</td>
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<td>LVIDd (mm)</td>
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<td>4.03±0.08###</td>
<td>4.47±0.14**</td>
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<td>LVIDs (mm)</td>
<td>2.87±0.06</td>
<td>2.59±0.09** #</td>
<td>2.96±0.12</td>
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<tr>
<td>SV (µl)</td>
<td>41.4±0.9</td>
<td>47.9±3.4** #</td>
<td>57.5±3.3***</td>
</tr>
<tr>
<td>CO (ml/min)</td>
<td>20.3±0.5</td>
<td>23.8±1.2** #</td>
<td>27.8±2.1***</td>
</tr>
<tr>
<td>FS%</td>
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<td>35.3±1.6**</td>
<td>34.0±1.0*</td>
</tr>
<tr>
<td>EF%</td>
<td>56.8±1.4</td>
<td>64.3±2.2*</td>
<td>62.9±1.5*</td>
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<tr>
<td>dP/dt Max (mmHg/s)</td>
<td>10390±347</td>
<td>10707±690###</td>
<td>12486±414**</td>
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Table 4-1. Comparison of cardiac phenotypes in D486N/D486N and L613V/+ mice.

Echocardiographic and invasive hemodynamic parameters of 4 month-old WT (n=12), D486N/D486N (n=12) and L613V/+ (n=11) hearts. LVPWD, left ventricular diastolic posterior wall thickness; LVIDs, left ventricular internal end-systolic dimension; LVIDd, left ventricular internal end-diastolic dimension; SV, stroke volume; FS, fractional shortening; CO, cardiac output; EF, ejection fraction. Phenotypic data for L613V/+ mice are derived from our previous publication (464). * p<0.05; ** p<0.01; *** p<0.001 (WT vs. L613V/+ or WT vs. D486N/D486N). # p<0.05; ## p<0.01 (D486N/D486N vs. L613V/+); Bonferroni post-test when ANOVA is significant.
Figure 4-7. NS-associated Raf1 mutants differentially activate Mek/Erk.

(A) Primary WT, D486N/D486N and L613V/+ MEFs were starved for 16 hr, and then stimulated with EGF, as indicated. Cell lysates were immunoblotted with the indicated antibodies. The top panel shows a representative blot; quantification of blots from all experiments (n=4) is shown below. ** p<0.01; *** p<0.001 (D486N/D486N vs. WT); ## p<0.01; ### p<0.001 (L613V/+ vs. WT); Bonferroni post-test when ANOVA is significant. (B) Mek activation in heart tissues. Lysates from WT (n=7), L613V/+ (n=4) and D486N/D486N (n=4) hearts were analyzed by immunoblotting with anti-pMek antibodies with Erk2 as a loading control. Representative samples for each genotype are shown on the left. Each lane represents an individual animal. Quantification of all samples is shown on the right. *p<0.05 (2-tailed Student’s t-test).
in Mek activation (Figure 4-7B). Although Raf1\textsuperscript{L613V} and Raf1\textsuperscript{D486N} enhanced Mek/Erk activation in neonatal cardiomyocytes and cardiac fibroblasts, their effects on the response to various stimuli differed (Figure 4-8). In some cases (e.g., IL-6 and FGF2), Raf1\textsuperscript{L613V} and Raf1\textsuperscript{D486N} had similar effects on pathway activation, but in many others (e.g., AngII, NRG, EGF, PDGF), Raf1\textsuperscript{L613V} had a much stronger effect. Both the magnitude and the duration of Mek/Erk activation were affected differentially by Raf1\textsuperscript{L613V} and Raf1\textsuperscript{D486N}. Such differences likely reflect important, if sometimes subtle, differences in the regulation of the RAS/ERK pathway in response to distinct agonists and in different cell types. However, given that Mek inhibitor treatment reverses HCM in L613V/+ mice (464), these data are consistent with the idea that the ability of kinase-activating Raf1 mutants to more profoundly enhance Mek/Erk pathway activation underlies their distinct phenotypic effects (see Discussion).

4.3.5 Kinase-impaired Raf1 mutants enhance Mek/Erk activation by promoting heterodimerization with Braf

To study the biochemical properties of RAF1 mutants in a more biochemically tractable cell system, while avoiding the marked over-expression seen in transient transfection experiments, we generated stable mammalian Flp-In T-REx 293 (T-REx 293) cell lines. Such cells allow tetracycline-inducible expression of Flag-tagged WT and mutant RAF1 from the same genomic locus at levels comparable to endogenous RAF1. As in primary MEFs, cardiomyocytes and cardiac fibroblasts, expression of RAF1\textsuperscript{D486N} in T-REx 293 cells resulted in increased MEK/ERK activation, compared with the effects of RAF1\textsuperscript{WT} expression (Figure 4-9A). Transient transfection studies have shown that RAF1 can form heterodimers with BRAF, that RAF1/BRAF heterodimers have increased kinase activity compared with the respective homodimers or monomers, and that a single kinase-competent RAF isoform can confer high catalytic activity to the heterodimer (168, 298, 302, 303). These findings suggested that RAF1\textsuperscript{D486N} might enhance MEK/ERK activation by promoting heterodimer formation. To test this possibility, we immunoprecipitated Flag-tagged RAF1 from induced T-REx 293 cell lysates, and subjected the immunoprecipitates to immunoblotting with anti-BRAF antibodies. Following EGF stimulation, low levels of RAF1/BRAF heterodimers were found in WT RAF1-expressing T-REx 293 cell lysates. By contrast, heterodimerization was enhanced dramatically in RAF1\textsuperscript{D486N}-expressing cells, even though, as in MEFs, RAF1\textsuperscript{D486N} accumulated to levels considerably lower than WT RAF1 (Figure 4-9A). Increased heterodimerization was not due to
Figure 4-8. Severity of cardiac phenotype in D486N/D486N and L613V/+ mice correlates with Mek/Erk activation.

Quantification of Mek/Erk activation from immunoblots (n=2) of cardiomyocytes (A) and cardiac fibroblasts (B). Lysates from neonatal WT and D486N/D486N or WT and L613V/+ cells, starved for 24 hr and then stimulated as indicated. Values indicate fold change in Mek/Erk activity (assessed by immunoblotting with phospho-specific antibodies) in D486N/D486N or L613V/+ cells, compared with the corresponding WT control at 5 min post-stimulation.
defective negative feedback (244), as heterodimerization was increased even when ERK activation was blocked by MEK inhibitor treatment (Figure 4-9B). These results were confirmed using primary D486N/+ and D486N/D486N MEFs, despite their markedly lower expression of Raf1D486N (Figure 4-9C). Furthermore, enhanced ability to form RAF1/BRAF heterodimers was a common feature of kinase-impaired, NS-associated RAF1 mutants: the T491I and T491R kinase domain mutants also showed enhanced heterodimerization, and increased and sustained MEK/ERK activation in EGF-stimulated T-REx 293 cells (Figure 4-10).

4.3.6 Heterodimerization with BRAF is required for RAF1D486N to enhance MEK/ERK activation

We next asked whether heterodimerization with BRAF is required for enhanced MEK/ERK activation in these cells. Indeed, infection of T-REx 293 cells expressing RAF1D486N with a lentivirus expressing BRAF shRNA abolished MEK/ERK hyperactivation in these cells (Figure 4-11A). Similar experiments were performed on primary D486N/D486N MEFs. Again, Mek activation and Raf1/Braf heterodimer levels were reduced significantly in Braf knock-down cells (Figure 4-11B and Figure 4-11C). Surprisingly, Erk activation was not affected, although this could reflect significant up-regulation of Erk1 protein levels in Braf knock-down MEFs (Figure 4-11B).

Mammals express three RAF family members, RAF1, BRAF and ARAF (252), all of which share MEK1/2 as substrates. Co-immunoprecipitation experiments using lysates from D486N/D486N MEFs showed that Araf also was present in Raf1 immunoprecipitates (Figure 4-11D). However, Araf knockdown did not reduce Mek activation significantly in these cells, compared with the marked effects of Braf depletion. Braf knockdown did cause enhanced Araf/Raf1 heterodimerization, which led us to test whether heterodimerization with Araf helps to explain persistent Mek/Erk activation in Braf knockdown cells. As expected, combined depletion of Araf and Braf in these cells further impaired Mek/Erk activation. Taken together, these results suggest that at least in MEFs, Raf1D486N enhances Mek/Erk activation primarily by promoting heterodimerization with Braf, but raise the possibility that Araf/Raf1 heterodimers might contribute to the effects of Raf1D486N in other cell types.
Figure 4-9. RAF1^{D486N} forms more heterodimers with BRAF.

(A) T-REx 293 cell lines expressing Flag-tagged human RAF1 WT or D486N were incubated with 1µg/ml Tetracycline for 20 hours in serum-free medium, and then stimulated with EGF for the indicated times. Heterodimers were detected by immunoprecipitation with anti-Flag antibody, followed by blotting for endogenous BRAF. MEK and ERK activation in the same lysates were assessed by immunoblotting. (B) T-REx 293 cell lines expressing Flag-tagged human RAF1 WT or D486N were incubated with 1µg/ml Tetracycline for 20 hours in serum-free medium, and then treated with 10µM PD0325901 for 1 hr before stimulation with EGF for the indicated times. Heterodimers were detected by immunoprecipitation with anti-Flag antibody, followed by blotting for BRAF. Total cell lysates from the same experiment were immunoblotted with the indicated antibodies. (C) Primary MEFs from WT, D486N/+ and D486N/D486N mice were starved for 16 hr, and then stimulated with EGF, as indicated. Endogenous heterodimers were detected by immunoprecipitation with RAF1 antibody, followed by blotting for Braf. Mek and Erk activation in the same lysates were assessed by immunoblotting.
Figure 4-10. Kinase-impaired RAF1 mutants associated with NS enhance MEK/ERK activation and form more RAF1/BRAF heterodimers.

(A) T-REx 293 cell lines expressing Flag-tagged human RAF1 WT, D486N, T491I or T491R were incubated with 1µg/ml Tetracycline for 20 hours in serum-free medium, and then stimulated with EGF. Cell lysates from a representative experiment were subjected to immunoblotting with the indicated antibodies. Quantification of blots from all experiments (n=4) is shown on the right. *** p<0.001 (WT vs. D486N in blue; WT vs. T491I in red; WT vs. T491R in green); Bonferroni post-test when ANOVA is significant. (B) T-REx 293 cell lines expressing Flag-tagged human RAF1 WT or the indicated mutants were incubated with 1µg/ml Tetracycline for 20 hours in serum-free medium, and then stimulated with EGF. Heterodimers were detected by immunoprecipitation with anti-Flag antibody, followed by immunoblotting for BRAF. The same lysates were immunoblotted with the indicated antibodies.
Figure 4-11. BRAF is required for RAF1<sup>D486N</sup> to enhance MEK/ERK activation.

(A) T-REx 293 cell lines expressing Flag-tagged human WT or D486N mutant RAF1 were infected with a lentivirus expressing BRAF shRNA (BRAF-KD) or a control (Luciferase) shRNA (shluc). At 48 hours post-puromycin selection, cells were incubated with 1µg/ml Tetracycline for 20 hours in serum-free medium, and then stimulated with EGF. Cell lysates were immunoblotted with the indicated antibodies. A representative experiment is shown at the left, and blots from all experiments (n=3) are quantified on the right. * p<0.05; ** p<0.01; *** p<0.001 (WT-shluc vs. D486N-shluc); ### p<0.001 (D486N-shluc vs. D486N-BRAF-KD); Bonferroni post-test when ANOVA is significant. (B) Primary MEFs from D486N/D486N mice were infected with a lentivirus expressing Braf shRNA (Braf-KD) or a control shRNA (shluc). Cell lysates were immunoblotted with the indicated antibodies. A representative blot is shown on the top (arrow indicates position of Erk1). Quantification of blots from all experiments (n=4) for Mek activation is shown at the bottom. *** p<0.001; Bonferroni post-test when ANOVA is significant. (C) Primary WT and D486N/D486N MEFs were infected with a lentivirus expressing Braf shRNA (Braf-KD) or a control shRNA (shluc). Heterodimers were detected by immunoprecipitation with RAF1 antibody, followed by immunoblotting for BRaf. Total cell lysates were immunoblotted with the indicated antibodies. (D) Primary D486N/D486N MEFs were infected with lentiviruses expressing shRNAs against Braf (Braf-KD) and/or Araf (Araf-KD) or a control shRNA (shluc). Heterodimers were detected by immunoprecipitations with RAF1 antibody, followed by blotting for Braf or Araf. Total cell lysates also were immunoblotted with the indicated antibodies.
Although these experiments showed that Braf, and to a lesser extent, Araf, is necessary for the effects of Raf1\textsuperscript{D486N}, and that these effects correlate with the ability of this mutant to increase heterodimerization, they did not establish a causal relationship between heterodimerization and Mek/Erk hyperactivation. Structural studies of \textit{Drosophila} Raf (299) showed that Arg 481 (Arg401 in RAF1 and Arg509 in BRAF) is at the center of a side-to-side RAF dimer interface, and directly participates in these interactions (Figure 4-12A). As in \textit{Drosophila} Raf, introduction of the R401H mutation into WT RAF1 or RAF1\textsuperscript{D486N} abolished RAF1/BRAF heterodimerization (Figure 4-12B). Moreover, the compound R401H/D486N mutant no longer enhanced EGF-evoked MEK/ERK activation (Figure 4-12C). Similar results were obtained when we tested another mutation (F408A) within the dimer interface (Figure 4-12A and Figure 4-12D). Furthermore, re-expression of Myc-tagged WT Braf in Braf knock-down D486N/D486N MEFs restored Mek and Erk activation, whereas Braf\textsuperscript{R509H} could not rescue (Figure 4-12E).

Finally, to test unambiguously the effects of dimerization per se (as opposed to other, unanticipated structural consequences of dimer-interface mutants), we asked if forced heterodimerization could restore the ability of RAF1R401H/D486N to promote MEK/ERK activation. We fused Flag-RAF1R401H/D486N, which cannot form heterodimers with BRAF, to FKBP, while FRB was fused to BRAF (Figure 4-13A). Upon co-transfection, these two proteins can heterodimerize only upon addition of a rapamycin analog (A/C heterodimerizer). Remarkably, MEK and ERK activation were restored upon A/C heterodimerizer addition (Figure 4-13B); importantly, the heterodimerizer itself had no effect (Figure 4-13C). Taken together, these results show that heterodimerization with BRAF is necessary and sufficient for the NS-associated RAF1\textsuperscript{D486N} mutant to enhance MEK/ERK activation.

Surprisingly, we also noticed that the kinase-activating mutant RAF1L613V also formed more RAF1/BRAF heterodimers in response to growth factor stimulation (Figure 4-12B and Figure 4-14A) and, as for the kinase-defective Raf1 alleles, knock-down of Braf in primary MEFs expressing Raf1L613V significantly reduced Mek activation in these cells (data not shown). Moreover, the compound L613V and dimer mutant (R401H) abolished RAF1/BRAF heterodimerization and no longer caused enhanced MEK/ERK activation (Figure 4-12B and
Figure 4-12. Heterodimerization with BRAF is required for RAF1D486N mutant to enhance MEK/ERK activation.

(A) Side-to-side dimer interface in Drosophila Raf (299). One protomer is displayed as a surface representation in orange, while the other is shown as a ribbon representation in violet. The inset shows a close-up of hydrogen bonding interactions involving R481 and F488 (R401 and F408 in human RAF1). (B) T-REx 293 cell lines expressing Flag-tagged human RAF1 WT or the indicated mutants were incubated with 1µg/ml Tetracycline for 20 hours in serum-free medium, and then either left unstimulated (-) or stimulated with EGF for 5 min (+). Heterodimers were detected by immunoprecipitation with anti-Flag antibody, followed by immunoblotting with BRAF antibodies. Total cell lysates also were immunoblotted with the indicated antibodies. (C) T-REx 293 cell lines expressing Flag-tagged human RAF1 WT or the indicated mutants were incubated with 1µg/ml Tetracycline for 20 hours in serum-free medium, and then stimulated with EGF for the times indicated. Cell lysates were immunoblotted with the indicated antibodies. A representative immunoblot is shown, and quantification of the blots from all experiments (n=4) is shown at the bottom. * p<0.05; ** p<0.01; *** p<0.001 (WT vs. D486N). ### p<0.001 (R401H/D486N vs. WT); Bonferroni post-test when ANOVA is significant. (D) T-REx 293 cell lines expressing Flag-tagged human RAF1 WT, D486N and/or F408A were incubated with 1µg/ml Tetracycline for 20 hours in serum-free medium, and then stimulated with EGF for the times indicated. Cell lysates were immunoblotted with the indicated antibodies. (E) Immortalized D486N/D486N MEFs were infected with a lentivirus expressing Braf shRNA. At 72 hours post-puromycin selection, cells were infected with MCSV-based retroviruses expressing GFP alone or GFP plus myc-BRAFWT or myc-BRAFR509H. GFP+ cells, obtained by FACS, were serum-starved for 16 hours, and then stimulated with EGF. Cell lysates were immunoblotted with the indicated antibodies.
Figure 4-13. Induced RAF1/BRAF heterodimerization restores activity of RAF1<sup>R401H/D486N</sup> mutant to enhance MEK/ERK activation.

(A) RAF1<sup>R401H/D486N</sup> and BRAF coding sequences were sub-cloned into FKBP or PRB expression vectors, respectively, as shown. (B) T-REx 293 cells were co-transfected with Flag-RAF1<sup>R401H/D486N</sup>-FKBP and BRAF-FRB-HA expression plasmids for 24 hours, and then left untreated (0h), or treated with A/C heterodimerizer (500nM) for the indicated times. Cell lysates were immunoblotted with the indicated antibodies. (C) T-REx 293 host cells were co-transfected with Flag-RAF1<sup>D486N</sup> or Flag-RAF1<sup>R401H/D486N</sup> and Myc-BRAF expression plasmids, and 24 hours later were left untreated (-), or treated with A/C heterodimerizer (500nM) (+) for 30 min. Cell lysates were immunoblotted with the indicated antibodies.
Figure 4-14. RAFl<sup>L613V</sup> mutant enhances MEK/ERK activation via RAFl/BRAF heterodimer formation.

(A) Primary WT, L613V/+ and L613V/L613V MEFs were starved for 16 hr, and then stimulated with EGF for the indicated times. Endogenous heterodimers were detected by immunoprecipitation with RAFl antibody, followed by blotting for Braf. Total cell lysates from the same experiments were immunoblotted with the indicated antibodies. (B) T-REx 293 cell lines expressing Flag-RAFl WT, L613V or R401H/L613V were incubated with 1µg/ml Tetracycline for 20 hours in serum-free medium, and then stimulated with EGF for the times indicated. MEK and ERK activation were assessed by immunoblotting with the indicated antibodies. A representative immunoblot is shown, and quantification of the blots from all experiments (n=3) is shown on the right. * p<0.05; ** p<0.01; *** p<0.001 (WT vs. L613V). ## p<0.01; ### p<0.001 (R401H/L613V vs. WT); Bonferroni post-test when ANOVA is significant.
These data provide an explanation for the ability of this class of kinase-activated alleles to promote MEK/ERK hyper-activation.

4.4 Discussion

We generated and analyzed a knock-in mouse model for the NS-associated, kinase-impaired \( \text{Raf}1^{D486N} \) mutation. Unlike the kinase-activating mutation \( \text{Raf}1^{L613V} \) (464), which causes most major features of NS, including proportional short stature, facial dysmorphia, hematological defects and HCM, \( \text{Raf}1^{D486N} \) heterozygosity results only in a mild growth defect in female mice. \( \text{Raf}1^{D486N} \) homozygous mice (on a 129Sv × C57BL/6 mixed background) exhibit concentric cardiac hypertrophy (but not HCM) and an incompletely penetrant severe growth defect that is accompanied by facial dysmorphia, failure to thrive and early death. Detailed analysis of the mechanism underlying the effects of this \( \text{Raf}1 \) allele, as well as other kinase-defective \( \text{RAF1} \) mutants, indicate that they paradoxically hyper-activate the RAS/ERK pathway by promoting heterodimerization with BRAF and, to a lesser extent, ARAF. Finally, we unexpectedly found that \( \text{Raf}1^{L613V} \), whose activation mechanism had remained unclear, also promotes increased heterodimer formation. Taken together, these data identify increased heterodimerization capacity as the common theme in the pathogenesis of \( \text{RAF1} \) mutant-associated NS.

The incompletely penetrant growth/facial dysmorphia/viability phenotype of \( \text{D486N/D486N} \) mice suggests the existence of modifier gene(s) that vary between the 129Sv and C57BL/6 strains. Indeed, preliminary mapping studies have identified a 129Sv locus on mouse chromosome 8 that is strongly linked (LOD score ~15) to the s-D486N/D486N phenotype (see 5.2 Future directions). Like s-D486N/D486N mice, \( \text{Araf} \)-deficient mice have severe growth defects, neurological abnormalities and post-natal lethality (234); notably, these phenotypes also are sensitive to genetic background. The identification of loci that modify \( \text{Raf}1 \) function could provide important insights into the regulation of Raf-dependent signaling, and it will be interesting to see if the same modifier(s) affect the \( \text{Raf}1^{D486N} \) and \( \text{Araf} \) mutant phenotypes. Primary lung fibroblasts prepared from s- and n- \( \text{D486N/D486N} \) mice show comparable Mek/Erk activation (data not shown), suggesting that the putative modifier(s) likely acts downstream of Erk or parallel to the RAS/ERK pathway. Our previous NS mouse models, \( \text{Shp2}^{D61G} \) (204) and \( \text{Raf}1^{L613V} \) (464), also show strain-specific differences in phenotype.
However, different modifiers are likely to be involved, because increasing C57BL/6 content causes lethality in these two mouse models. Given the marked genetic heterogeneity of the human population, and the differences in penetrance of RASopathy phenotypes within a single affected family, not to mention between unrelated patients with the same mutant allele, our mouse models provide excellent opportunities to investigate the relative effects of RASopathy mutations and genetic background.

Kinase-activating \( \text{RAF1} \) mutants are strongly associated with HCM in human NS patients, a phenotype reproduced by our \( \text{Raf1}^{L613V} \) mouse model (464). Similarly, \( \text{D486N}^{+/-} \) mice, like NS patients with kinase-impaired \( \text{RAF1} \) mutations, do not exhibit HCM. Notably, \( \text{Raf1}^{D486N} \) homozygosity (which is not seen in humans) results in concentric cardiac hypertrophy that does not progress to heart failure within one year of observation (Figure 4-2), unlike the eccentric cardiac hypertrophy seen in \( \text{L613V}^{+/-} \) mice (464). Biochemical analysis revealed that, in contrast to initial reports based on transient transfection experiments (149), expression of kinase-impaired \( \text{Raf1}^{D486N} \) at normal (endogenous) levels causes sustained and/or enhanced Mek/Erk activation in multiple cell types in response to a variety of stimuli. In order to transmit signals downstream, RAF proteins must assemble into multi-protein complexes (252). Presumably, the substantial over-expression that occurs in transiently transfected cells results in artifactual dominant negative effects of kinase-deficient \( \text{RAF1} \) mutants that are not seen when these proteins are expressed at appropriate, endogenous levels. Importantly, however, \( \text{Raf1}^{D486N} \) is less potent than \( \text{Raf1}^{L613V} \) in enhancing Mek/Erk activation in response to most stimuli, both in cardiomyocytes and in cardiac fibroblasts (Figure 4-8). Furthermore, \( \text{Raf1}^{L613V} \) generally increases the magnitude of Mek/Erk activation, whereas \( \text{Raf1}^{D486N} \) most often prolongs signal duration. Given our previous finding that HCM can be prevented or reversed by MEK inhibitor treatment of \( \text{Raf1}^{L613V^+} \) mice, these data strongly suggest that differences in the ability of the \( \text{D486N} \) and \( \text{L613V} \) mutants (and by inference, between kinase-impaired and kinase-activated \( \text{RAF1} \) in general) to promote Mek/Erk activation in the heart explains their distinct cardiac phenotypes. If so, then agonists that show differential effects on Mek/Erk pathway activation in \( \text{Raf1}^{L613V^+} \) and \( \text{Raf1}^{D486N^+D486N} \) cardiomyocytes (e.g., AngII, NRG, but not IL6) and/or cardiac fibroblasts (e.g., EGF, IGF, PDGF, but not FGF2) might be particularly important for cardiac hypertrophy in NS. \( \text{Raf1}^{D486N} \) accumulates to much lower levels than \( \text{Raf1}^{L613V} \), consistent with a previous report that kinase activity is required to prevent Raf1 degradation (297). Consequently, we cannot
ascertain whether lower levels of the Raf1\textsuperscript{D486N} protein or its lower kinase activity accounts for the decreased Mek/Erk hyperactivation seen in D486N/D486N, compared with L613V/+, mice (also, see below).

The carboxyl oxygen of the highly conserved aspartic acid 486 (the “D” of the DFG motif) plays a critical role in chelating Mg\textsuperscript{2+} and stabilizing ATP binding in the active site of protein kinases (468). Mutation of this residue to alanine creates a kinase-inactive protein (297), whereas mutation to asparagine severely impairs (but does not eliminate) activity (149). All RAF family members require phosphorylation of key activation loop residues for maximal kinase activation (244, 469), and T491 is one of these residues. As expected, NS-associated mutants affecting T491 also show impaired kinase activity (149).

Our results clearly establish how all such mutants (D486N, T491I and T491R) enhance downstream MEK/ERK activation. Previous studies showed that RAF1/BRAF heterodimerization occurs during normal growth factor signaling, and indicated that heterodimers have increased catalytic activity compared with homodimers or monomers (298). Although some have argued that RAF1 inhibits BRAF activation in a kinase domain-dependent fashion (470), other studies reported that human cancer-associated BRAF mutants with impaired kinase activity promote MEK activation by binding to and trans-activating RAF1 (167, 168). Of note, these kinase-impaired mutations alter the activation segment of BRAF, and some (D594G and T599I) are analogous to NS-associated RAF1 mutants (471, 472). Marais and colleagues recently reported that mice with a conditional kinase-dead \textit{Braf} (\textit{Braf}\textsuperscript{LSL-D594A}) allele, when combined with oncogenic \textit{Ras} (\textit{Kras}\textsuperscript{G12D}), induced melanomas in mice. In tumor cells from these mice, Braf\textsuperscript{D594A} was bound to Raf1 constitutively (303). By analogy, we suspected that kinase-impaired, NS-associated RAF1 mutants hyperactivated MEK/ERK by promoting heterodimerization with BRAF. Indeed, we found that all of the kinase-impaired RAF1 mutants form more heterodimers with BRAF upon growth factor stimulation (Figure 4-9 and Figure 4-10). Furthermore, the level of MEK/ERK hyperactivation depends on the level of RAF1/BRAF heterodimerization, as \textit{RAF1}\textsuperscript{T491I} and \textit{RAF1}\textsuperscript{T491R} formed more heterodimers and caused more MEK/ERK activation than did \textit{RAF1}\textsuperscript{D486N} (Figure 4-10).

Several subsequent lines of evidence establish a causal relationship between heterodimerization and MEK/ERK activation. First, knockdown of \textit{BRAF} in T-REx293 cells
expressing Flag-RAF1\textsuperscript{D486N} reduces MEK/ERK activation. Braf knockdown in primary D486N/D486N MEFs also impairs Mek activation, although Erk activation is unaffected (Figure 4-11B). This difference might be more apparent than real, however, as total Erk1 levels are increased in Braf-knockdown MEFs, suggesting potential compensation during the selection of stable knockdown clones. Such feedback regulation presumably is impaired (or less effective) in T-REx 293 cells. Second, mutation of either of two key residues (R401H, F408A) in the dimer interface of the RAF1 kinase domain, as revealed by the crystal structures of BRAF (167) and Drosophila Raf (299), abolishes the ability of RAF1\textsuperscript{D486N} to enhance EGF-evoked MEK/ERK activation (Figure 4-12). Most compellingly, forced heterodimerization of Raf1\textsuperscript{D486N/R401H} and BRAF by means of an inducible FKBP-FRB interaction system restored the ability of Raf1\textsuperscript{D486N/R401H} to enhance EGF-evoked MEK/ERK hyperactivation (Figure 4-13). The latter result shows unambiguously that Raf1\textsuperscript{D486N/R401H} cannot hyperactivate MEK/ERK hyperactivation solely because it has lost its ability to promote RAF1/BRAF heterodimerization, as opposed to some unanticipated effect of the second site R401H mutation on the conformation of the D486N mutant. Taken together, our results demonstrate that heterodimerization with BRAF is necessary and sufficient for such mutants to enhance MEK/ERK activation. Although our knockdown studies show that RAFl\textsuperscript{D486N} enhances MEK/ERK activation (at least in MEFs and T-REx 293 cells) mainly through heterodimerization and transactivation of BRAF, we also find that kinase-defective RAFl mutants can heterodimerize with ARAF. Consequently, we do not exclude the possibility that RAFl/ARAF heterodimers play an important role in the pathogenesis of NS caused by kinase-defective RAFl alleles in tissues where ARAF is a major isoform (e.g., the brain).

Another cluster of NS-associated RAFl mutations maps to the C-terminus (S612 and L613) of RAFl. Like NS-associated CR2 domain mutants, these mutants have increased kinase activity (149), even though S612 and L613 lie distal to the RAFl kinase domain. Previous studies have not implicated these residues in RAFl regulation, so it has been unclear how such mutations enhance kinase activity. Moreover, phosphorylation of Ser259 (an inhibitory binding site for 14-3-3), and Ser 621 (149), a 14-3-3 binding site that promotes RAFl activation (297), are unaffected in RAFl\textsuperscript{L613V}. We found that, compared with WT RAFl, RAFl\textsuperscript{L613V} also forms more heterodimers with BRAF upon growth factor stimulation. Moreover, superimposing the


dimer interface mutant (R401H) on L613V blocked its ability to enhance MEK/ERK activation (Figure 4-14).

These data strongly suggest that RAF1^{L613V} also promotes MEK/ERK hyper-activation via enhanced BRAF heterodimerization ability. As the kinase domain of RAF1^{L613V} is not altered, and this mutant is expressed at normal levels unlike kinase-deficient RAF1 alleles, heterodimerization with BRAF presumably results in a greater increase in MEK/ERK activation, compared with that evoked by kinase-impaired RAF1 alleles. The NS-associated CR2 domain mutant RAF1^{S257L} and RAF1^{P261T} (98) (data not shown) also forms more heterodimers with BRAF. Taken together, these results argue that increased ability to heterodimerize with BRAF (and possibly ARAF) represents a general pathogenic mechanism for NS-associated RAF1 mutations, and suggest that agents that interfere with dimerization might have general utility for the treatment of RAF1 mutant NS.
Chapter 5

Summary and future directions
5.1 Summary and Key Findings

NS is one of several autosomal dominant “RASopathies” caused by mutations in components of the RAS/ERK pathway. Germ line mutations in \textit{RAF1} account for ~3-5% of NS, and unlike other NS alleles, \textit{RAF1} mutations that confer increased kinase activity are highly associated with hypertrophic cardiomyopathy (HCM). Surprisingly, some NS-associated \textit{RAF1} mutations show normal or decreased kinase activity. These observations raised the question of why both kinase-activating and kinase-impaired \textit{RAF1} mutants can cause similar diseases, and how NS-associated \textit{RAF1} mutations cause HCM. In this thesis, these issues were addressed by generating and analyzing two lines of “knock-in” mice that express kinase-activating (L613V) and impaired (D486N) \textit{RAF1} mutants, respectively.

In Chapter 3, I described a knock-in mouse model for NS caused by the kinase-activating \textit{Raf1}^{L613V} mutation. Similar to mouse models of \textit{Ptpn11} mutation-associated NS, \textit{Raf1}^{L613V} heterozygosity (L613V/+) causes proportional short stature, facial dysmorphia, and hematological defects. Unlike phosphatase-activating \textit{Ptpn11} alleles, which cause valvuloseptal abnormalities, L613V/+ mice have normal valvuloseptal development and instead exhibit eccentric cardiac hypertrophy that decompensates upon pressure overload. Agonist-evoked Mek/Erk activation is enhanced in multiple cell types without changes in several other signaling pathways implicated in cardiac hypertrophy/HCM. Remarkably, post-natal MEK inhibition normalizes the growth, facial and cardiac defects in L613V/+ mice, demonstrating that elevated MEK/ERK activity is critical for causing HCM and other NS phenotypes and identifying MEK inhibitors as potential therapeutic agents for the treatment of NS.

Whether kinase-defective \textit{Raf1} alleles also faithfully model human NS, and if so, how kinase-activating and kinase-defective mutants can cause similar phenotypes, remained to be resolved. In Chapter 4, I generated and analyzed knock-in mice expressing the kinase-impaired NS mutant \textit{Raf1}^{D486N}, and also re-examined the effects of various other NS mutants expressed at more physiological levels than in previous transient transfection studies. Unlike the kinase-activating mutation \textit{Raf1}^{L613V}, which causes most major features of NS, \textit{Raf1}^{D486N} heterozygosity results in only a mild growth defect in female mice. \textit{Raf1}^{D486N} homozygous mice (on a 129Sv × C57BL/6 mixed background) exhibit concentric cardiac hypertrophy (but not HCM) and an
incompletely penetrant severe growth defect that is accompanied by facial dysmorphia, failure to thrive and early death. Detailed analysis of the mechanism underlying the effects of this Raf1 allele, as well as other kinase-defective RAF1 mutants, indicate that they paradoxically hyper-activate the RAS/ERK pathway by promoting heterodimerization with BRAF and, to a lesser extent, ARAF. Finally, I unexpectedly found that Raf1L613V also promotes increased heterodimer formation. Taken together, these data identify increased heterodimerization capacity as the common theme in the pathogenesis of RAF1 mutant-associated NS.

5.2 Future Directions

5.2.1 Optimizing therapy for NS-associated HCM

RASopathies are rare, “orphan diseases”, so extensive pre-clinical evaluations are needed to prioritize potential therapeutic approaches for these patients. Post-natal therapy for 6 weeks with the MEK inhibitor PD0325901 (PD) rescues all NS phenotypes, including HCM, in L613V/+ mice (Figure 3-17, Figure 3-19 and Figure 3-20; (464)). Whether the dose used for our initial experiments (5mg/kg BW/day) is optimal is uncertain, nor is it clear whether HCM recurs upon drug withdrawal. Furthermore, obesity was an unanticipated side effect of PD treatment. Therefore, it is worthy to perform more extensive pre-clinical studies with PD treatment on our NS mouse models, to ask whether its body mass effects are on- or off-target by testing a structurally unrelated MEK inhibitor, and to test whether other agents can be used for L613V/+ HCM.

Our initial MEK inhibitor treatment studies were done with PD at 5mg/kg BW/day, a dose below that used in most mouse xenograft tumor models of this agent for cancer therapy (453). In humans, PD has side effects such as skin rash, diarrhea, fatigue, nausea, and visual disturbances (473, 474) that may be tolerable for treatment of patients with advanced cancers, but not for a developmental disorder such as NS, particularly if long term, continuous treatment is required. Although we did not observe severe side effects except weight gain in our original study, we would nevertheless like to use the lowest effective dose in patients. Therefore, I performed a lower dose MEK inhibitor treatment (3mg/kg BW/day PD) in our L613V/+ NS mouse model, beginning at 4 weeks of age and continuing for 6 weeks, as in our original study (464). Mice were weighed and measured weekly. At the end of the treatment period, the hearts of
these mice were analyzed by echocardiography. The results showed that the lower dose of MEK inhibitor could still rescue the growth defect in L613V/+ mice (Figure 5-1A), although it takes longer for L613V/+ mice to catch up with the WT controls (Figure 3-17). The increased body mass effect also was reduced by decreasing the dose of PD (Figure 5-1B). However, at this dose, MEK inhibitor treatment could not rescue the cardiac hypertrophy, nor the chamber dilatation of the hearts (Figure 5-1C, D and E). Interestingly, MEK inhibitor treatment did caused a mild but significant increase in LVIDs (Figure 5-1F), which is consistent with a decrease in FS and EF in L613V/+ mice after treatment (Figure 5-2). However, other parameters of cardiac function (SV and CO) did not change upon lower dose MEK inhibitor treatment (Figure 5-2). These data suggest that there may be a different threshold of the level of Mek/Erk activation for causing the growth defects and HCM in NS, and/or that an off-target effect of PD might contribute to the rescue of HCM. Either way, we cannot lower the dose of PD in order to fully rescue the HCM in our L613V/+ NS mouse model.

Future work should investigate whether growth retardation or HCM recur after drug cessation. In order to test this, the same general design can be used as in our original study (464), except that body size would be measured continuously post-treatment, and echocardiography will be performed both at the end of the treatment period and at different time points post-withdrawal. Another interesting question is whether we can rescue NS phenotypes in adult mice (e.g., starting treatment at 10 weeks of age or later) instead of treating the mice at puberty as in our original study (464) by MEK inhibitor treatment. All of these findings would provide important guidelines for potential clinical treatment of NS patients with MEK inhibitors.

Finally, PD treatment causes a rapid and significant increase in body mass/body fat, an obviously undesirable side effect. This could be an on-target effect of Mek-inhibition; notably, deletion of Ptpn11 in the forebrain causes marked obesity (461), and Shp2, of course, is a major regulator of Erk activation. Alternatively, it could be an idiosyncratic effect of PD. To distinguish between these possibilities, we can perform similar studies to those described above using the structurally unrelated MEK inhibitors, such as the compound ARRY162, which is available commercially.
Figure 5-1. Lower dose MEK inhibitor treatment rescues growth defect, but not cardiac hypertrophy and chamber dilatation in L613V/+ mice.

Mice were injected IP daily with PD0325901 (PD; 3mg/kg) or vehicle, starting at 4 weeks of age and for the succeeding 6 weeks. Body length (A) and body weight (B) were measured weekly. # p<0.05, ### p<0.0001, two-way repeated-measures ANOVA; * p<0.05, Bonferroni post-test when ANOVA was significant (black symbols, WT PD vs. WT control; red symbols, L613V/+ PD vs. L613V/+ control). (C) Heart weight/body weight (HW/BW) ratio, (D) LVPWd and (E) LVIDd were not restored to within normal limits in lower dose MEK inhibitor-treated mice. (F) LVIDs increased after MEK inhibitor treatment. LVPWd, LVIDd and LVIDs were also normalized by BW^{1/3}. ns, not significant. # p<0.05, ## p<0.005 1-tailed Student’s t test; * p<0.05, ** p<0.005, *** p<0.0001, Bonferroni post-test when ANOVA was significant. n= 9 (WT); 10 (WT PD); 10 (L613V/+); 11 (L613V/+ PD).
Figure 5-2. Lower dose MEK inhibitor treatment partially normalizes cardiac function in L613V/+ mice.

Echocardiographic parameters at 4 month of age. ns, not significant. * p<0.05, ** p<0.005, *** p<0.0001, Bonferroni post-test when ANOVA was significant. n= 9 (WT); 10 (WT PD); 10 (L613V/+); 11 (L613V/+ PD).
Although PD treatment reverses all of the phenotypes in Raf1 mutant NS, no MEK inhibitor is approved for use in children (or adults); therefore, it would be difficult to bring MEK inhibitor therapy to the clinic quickly. Besides increased Mek/Erk activation, S6 phosphorylation also is enhanced in Raf1 mutant NS (Figure 5-3). S6 can be a downstream target of the Erk target Rsk (107); although p70S6K activation (and presumably mTorC1 activity) is normal in these mice (Figure 3-16), reducing mTorC1 activity with Rapamycin might be effective for Raf1 mutant HCM. Rapamycin is approved for the pediatric population, and could be tested in NS patients with HCM more rapidly than a MEK inhibitor. In order to test this hypothesis, the same general design was used as in our original MEK inhibitor treatment study (464), and Rapamycin (2 mg/kg BW/day), a dose that reverses HCM in LS mice (404), was used to treat L613V/+ mice. Our preliminary data showed that, at this dose, rapamycin treatment caused significant growth retardation and weight loss in both L613V/+ and WT control mice (Figure 5-4A and B), which forced us to terminate the treatment. It could not rescue the cardiac hypertrophy after 4 weeks of treatment (Figure 5-4C). These data suggest that excess S6 phosphorylation is not a major part of HCM pathogenesis in NS. Alternatively, many Erk-dependent pathways might have to be reversed to ameliorate Raf1 mutant HCM.

5.2.2 Downstream target(s) of the RAS/ERK signaling in NS and HCM

Many pathways are implicated in HCM pathogenesis (Figure 1-14), in addition to the RAS/ERK pathway demonstrated by our studies, yet the key targets of excessive Erk activation in HCM pathogenesis remain to be determined. Specific transcription factors can mediate cardiac hypertrophy. For example, Gata4 activates hypertrophy-associated genes and causes cardiomyopathy, in part via Nfat family members, themselves key transducers of the hypertrophic response (392). Gata4 interacts with Nfat2 and Nfat3 to strengthen DNA binding, thereby synergistically activating endothelin-1 (475) and BNP (476) transcription. Notably, Gata4 is an Erk1/2 target (419, 477). Therefore, these transcription factors are attractive candidates for mediating the hypertrophic effects of NS-associated Raf1. To test the possible involvement of Gata4 in Raf1 mutant HCM, we can assess phosphorylation at its Erk site, S105, by immunoblotting. We also can cross L613V/+ mice to knock-in mice expressing Gata4S105A, which lack the Erk-dependent phosphorylation site, and assess whether HCM can be attenuated in these mice.
Figure 5-3. Increased pS6 (S235/236) in cardiomyocytes and cardiac fibroblasts from L613V/+ mice.

(A) Neonatal cardiomyocytes were starved for 24 hr, and then stimulated as indicated with AngII or IL-6. (B) Neonatal cardiac fibroblasts were starved for 16 hr, and then stimulated as indicated with EGF or IGF-I. ERK2 serves as a loading control.
Figure 5-4. Rapamycin treatment does not rescue growth defect and cardiac hypertrophy in L613V/+ mice.

Mice were injected IP daily with Rapamycin (Rapa; 2mg/kg) or vehicle, starting at 4 weeks of age and for the succeeding 4 weeks. Body length (A) and body weight (B) were measured weekly. # p<0.05, ### p<0.0001, two-way repeated-measures ANOVA; * p<0.05, ** p<0.005, *** p<0.0001, Bonferroni post-test when ANOVA was significant (black symbols, WT Rapa vs. WT control; red symbols, L613V/+ Rapa vs. L613V/+ control). (C) Heart weight/body weight (HW/BW) ratio was measured by the end of treatment. ns, not significant. *** p<0.0001, Bonferroni post-test when ANOVA was significant. n= 11 (WT); 10 (WT Rapa); 13 (L613V/+); 12 (L613V/+ Rapa).
Global Raf1\textsuperscript{L613V} expression causes eccentric cardiac hypertrophy with enhanced cardiac contractility and cardiac fetal gene re-expression (464). Under basal conditions, fibrosis is minimal, but TAC causes massive fibrosis in L613V/+ hearts and functional decompensation. Recent Langendorf preparations (data not shown) reveal increased contractility in the L613V/+ heart, indicating a cardiac-intrinsic process, but the detailed physiological effects of mutant Raf1 at the single cell level are unclear. Furthermore, both cardiomyocytes (CM) and cardiac fibroblasts (CF) from L613V/+ mice show increased Mek/Erk activation in response to multiple agonists, including growth factors, cytokines, and GPCR agonists (464), so either cell type might contribute to HCM pathogenesis in NS. Indeed, CF are found throughout the heart and account for up to two-thirds of the cells in adult hearts (478). Moreover, recent studies indicate that CF play key roles in myocardial development and function (457, 458). Embryonic CF induce myocyte proliferation (457), whereas adult CF promote myocyte hypertrophy (457) and evoke pathological hypertrophy and fibrosis in response to disease stimuli (451, 452). Notably, enhanced Ras/Erk activation in CF is critical for pathological hypertrophy and fibrosis caused by overexpression of the β-adrenergic receptor in CM (452). In a neonatal rat cell co-culture model (479), Ang II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-β\textsubscript{1} and endothelin-1 from CF. Studying the cell(s)-of-origin for RAF1 mutant-induced HCM would advance our knowledge on the pathogenesis of HCM, and also provide important information for therapeutic investigations (e.g., drug screening on the relevant cell type).

In order to ask whether CM and/or CF expression of mutant Raf1 is/are important for the cardiac phenotype in L613V/+ mice, I have begun to induce Raf1\textsuperscript{L613V} expression selectively in CM or CF by crossing inducible L613Vfl/+ mice with tissue-specific Cre lines, and monitor effects on basal and pressure overload-induced cardiac hypertrophy. For CM-specific expression, L613Vfl/+ mice were first crossed to Mlc2v-Cre mice, in which Cre is expressed beginning at ~E9.5 (480, 481), to generate L613Vfl/+::Mlc2vCre/+ mice. The morphology of the L613Vfl/+::Mlc2vCre/+ and control hearts was analyzed by echocardiography at 10 weeks of age, at what time L613V/+ mice already have a significant cardiac hypertrophy and chamber dilatation. However, L613Vfl/+::Mlc2vCre/+ mice did not show the same cardiac phenotypes as L613V/+ mice. Instead, they exhibited a trend towards, but not a significant, increase in LVPWd
Figure 5-5. Cardiomyocyte-specific expression of Raf1^{L613V} does not cause significant cardiac hypertrophy.

LVPWd (A) and LV chamber dimension (B), as measured by echocardiography at 10 weeks of age. ns, not significant. * p<0.05, Bonferroni post-test when ANOVA was significant. n= 9 (WT); 11 (L613Vfl/+); 9 (Mlc2vCre/+); 11 (L613Vfl/+:Mlc2vCre/+).
at 10 weeks of age (Figure 5-5A). There was no chamber dilatation in these mice, although they showed a significant decrease in LVIDs (Figure 5-5B), which was consistent with a mild increase in cardiac function (Figure 5-6) and contractility (Figure 5-7). In total, L613Vfl/+:Mlc2vCre/+ mice showed a mild concentric cardiac hypertrophy instead of the eccentric cardiac hypertrophy observed in L613V/+ mice. However, we cannot conclude that CMs are not the only cell-of-origin for the HCM phenotype because our preliminary data showed that expression of Mlc2v-Cre was not universal in all ventricular CMs at E12.5, although studies from other groups demonstrate that Mlc2v-Cre efficiently targets the ventricular myocardium (481, 482). The big variation in the LVPWd measurement (Figure 5-5A) could reflect differential Mlc2v-Cre expression and Raf1L613V induction within the ventricle. The cardiac phenotype of these L613Vfl/+:Mlc2vCre/+ mice should be monitored at a later stage (e.g., 4 months of age) to see whether they would progress to more severe cardiac hypertrophy or even chamber dilatation. Alternatively, another CM-specific Cre line (e.g., α-MHC-MerCreMer (483)) might be needed to determine the contribution of CM in Raf1 mutant-induced HCM.

To date, there is no Cre line for specifically targeting cardiac fibroblasts. However, several Cre lines targeting the general fibroblast population have been used to study the function of CF. Considering that studies have shown that CF are heterogeneous (45-49), different CF-specific Cre lines, such as Cre expressed under the control of fibroblast-specific protein 1 promoter (Fsp1-Cre) (484-486) and the Coll1a2 promoter (Coll12a-Cre) (487, 488), should be used to monitor the effects of CF-specific Raf1L613V expression.

There are several potential outcomes to these experiments. HCM might be mediated entirely via CM or CF; alternatively, both might contribute. In the latter case, CM or CF might be particularly important for different aspects of the HCM phenotype (e.g., effects on wall thickness vs. contractility). Our preliminary data above for CM-specific expression of NS-associated Raf1 mutant suggests that CM might not be the only cell-of-origin for RAF1 mutant-induced HCM. We strongly suspect that CF will be important (if not required) for the severe fibrosis post-TAC found in L613V/+ mice, but also expect effects on basal function. If CF-specific expression of the Raf1 mutant does not cause detectable HCM, it would imply a complex interaction between CM and CF in HCM pathogenesis. We would then evaluate mice expressing mutant Raf1 in both cell types by generating compound CF/CN-Cre:L613Vfl/+
Figure 5-6. Cardiomyocyte-specific expression of Raf1^{L613V} causes mild increase in cardiac function.

Echocardiographic parameters at 10 weeks of age. ns, not significant. * p<0.05, ** p<0.005, Bonferroni post-test when ANOVA was significant. n= 9 (WT); 11 (L613Vfl/+); 9 (Mlc2vCre/+); 11 (L613Vfl/+::Mlc2vCre/+).
Figure 5-7. Cardiomyocyte-specific expression of Raf1<sub>L613V</sub> causes mild increase in cardiac contractility.

Cardiac contractility (A) and relaxation (B) of 4-month-old hearts as measured by invasive hemodynamic analysis. ns, not significant. * p<0.05, Bonferroni post-test when ANOVA was significant. n= 3 (WT); 3 (L613Vfl/+); 5 (Mlc2vCre/+); 6 (L613Vfl/+;Mlc2vCre/+).
mice. In the unlikely event that even the latter does not result in HCM, we would explore the possibility that other cell types contribute to the HCM phenotype, such as endothelial cells (Tie2-Cre) (93). Surprisingly, recent preliminary data indicate that L613Vfl/+::Tie2-Cre/+ mice showed a significant cardiac hypertrophy (data not shown), although whether it was primary or secondary to valve defects needs to be further investigated.

5.2.4 The roles of specific Erk isoforms in Raf1-induced HCM

I found that post-natal MEK inhibition normalizes the growth, facial and cardiac defects in L613V/+ mice, indicating that elevated MEK/ERK activity is critical for causing HCM and other NS phenotypes. To further investigate the role of specific Erks in HCM pathogenesis, I took a genetic approach by knocking-out Erk1 and/or Erk2 in L613V/+ mice and monitoring their cardiac phenotypes.

In initial experiments, I analyzed L613V/+:Erk1+/- and L613V/+::Erk1-/- mice (Figure 5-8, Figure 5-9, and Figure 5-10). Remarkably, Erk1 deficiency normalized the increased contractility in L613V/+ mice, but did not rescue the cardiac hypertrophy. On the other hand, preliminary data showed that knocking-out one allele of Erk2 in L613V/+ mice could not rescue the cardiac hypertrophy, and might even cause more severe hypertrophy (Figure 5-11). The contractility of L613V/+:Erk2+/- hearts needs to be analyzed further.

There are several potential outcomes of these experiments. If reducing Erk2 levels can rescue cardiac contractility as does Erk1 deficiency, the total Erk activity likely is critical for inducing cardiac phenotypes in NS, and there may be a different threshold of the level of Erk activation for regulating cardiac contractility vs. hypertrophy. If knocking-out one allele of Erk2 does not alter the contractility in L613V/+ mice, there is likely to be a specific role for Erk1 in regulating cardiac contractility, considering that Erk2 levels are higher than Erk1 in both CM and CF (464, 489). To further investigate the specific roles of Erk1/2 in cardiac contractility vs. hypertrophy conclusively, complete deletion of Erk2 needs to be carried out. Due to the embryonic lethality caused by full Erk2 depletion, cardiac-specific depletion of Erk2 by Nkx2.5-Cre line (490), or knocking-out of Erk2 in the relevant cell type(s), which are the cell(s)-of-origin for Raf1 mutant-induced HCM, needs to be done. These mouse models with specific depletion of Erk1 or Erk2, or both, also will help us to investigate the downstream target(s) of
Figure 5-8. Reducing Erk1 levels in L613V/+ mice does not rescue the cardiac hypertrophy and chamber dilatation.

HW/BW ratio (A), LVPWd (B) and LV chamber dimension (C and D) at 4 month of age. ns, not significant. * p<0.05, ** p<0.005, Bonferroni post-test when ANOVA was significant. n= 12 (WT); 15 (Erk1+/+); 11 (Erk1/-); 12 (L613V/+); 15 (L613V+/+;Erk1+/+); 8 (L613V+/+;Erk1-/-).
Figure 5-9. Reducing Erk1 levels in L613V/+ mice does not rescue the cardiac function.

Echocardiographic parameters at 4 month of age. ns, not significant. # p<0.05, 1-tailed Student’s t test; ** p<0.005, *** p<0.0001, Bonferroni post-test when ANOVA was significant. n= 12 (WT); 15 (Erk1+/-); 11 (Erk1-/-); 12 (L613V/+); 15 (L613V/+:Erk1+/-); 8 (L613V/+:Erk1-/-).
Figure 5-10. Reducing Erk1 levels in L613V/+ mice rescues the cardiac contractility.

Cardiac contractility (A) and relaxation (B) of 4-month-old hearts as measured by invasive hemodynamic analysis. ns, not significant. # p<0.05, ## p<0.005, 1-tailed Student’s *t* test; *p*<0.05, **p*<0.005, ***p*<0.0001, Bonferroni post-test when ANOVA was significant. n= 14 (WT); 18 (Erk1+/-); 8 (Erk1-/-); 14 (L613V/+); 13 (L613V/+;Erk1+/-); 6 (L613V/+;Erk1-/-).
Figure 5-11. Reducing Erk2 levels in L613V/+ mice does not rescue the cardiac hypertrophy.

HW/BW ratio at 4 month of age. * p<0.05, *** p<0.0001, Bonferroni post-test when ANOVA was significant. n= 4 for each genotype.
RAS/ERK signaling in the pathogenesis of NS and HCM, and in the regulation of cardiac contractility vs. hypertrophy.

5.2.5 Genetic modifiers in D486N/D486N mice

The incompletely penetrant growth/facial dysmorphia/viability phenotype of D486N/D486N mice on a 129Sv × C57BL/6 mixed background suggests the existence of modifier gene(s) that vary between the 129Sv and C57BL/6 strains. To screen genetic loci linked to the s-D486N/D486N phenotypes, genomic DNA from s-D486N/D486N and n-D486N/D486N mice was collected (Figure 5-12) and subjected to linkage analysis using the Illumina Mouse Medium Density linkage panel. This panel contains 1449 SNP markers, among which 877 are informative (different) between 129Sv and C57BL/6 mice. LOD (logarithm of odds) scores were calculated by R/qtl (491) for mapping quantitative trait loci (QTLs) in experimental populations derived from inbred lines (Figure 5-13). The trait was also binarized using a cutoff of body weight at 10g (Figure 5-14 and Figure 5-15).

These preliminary mapping studies identified a 129Sv locus on mouse chromosome 8 that is strongly linked (LOD score ~15 for quantitative trait; LOD score ~9 for binary trait) to the s-D486N/D486N phenotype (Figure 5-14, Figure 5-15 and Figure 5-16). However, this region covers more than 30 megabase pairs (Mbps), and contains numerous genes. The Mouse SNP Query from Mouse Genome Informatics (MGI) reveals that there are hundreds of SNPs, varying between 129Sv and C57BL/6 strains, within this region, the majority of which are coding-synonymous or located within introns or non-coding sequences. Most likely, the modifier(s) are differentially regulated transcriptionally or translationally between 129Sv and C57BL/6 mice.

Further studies need to be performed in order to find the critical genetic modifier(s) linked to the s-D486N/D486N phenotypes. More genomic DNA samples from s-D486N/D486N and n-D486N/D486N mice could be collected and thereby increase the sample size of the screening in order to narrow the region. Alternatively, this can be achieved by genotype imputation without including more samples. The number of SNPs assessed in Illumina Mouse Medium Density genotyping panels provides very low genomic coverage. Statistically, it is therefore more likely for phenotype-associated SNPs to be in linkage disequilibrium (LD) with
Figure 5-12. Distributions of the body weight data for all the 56 samples.
Figure 5-13. Genome-wide LOD score plot of the quantitative trait.

Produced by Pingzhao Hu
Figure 5-14. Genome-wide LOD score plot of the binary trait.

The trait is binarized using a cutoff of body weight: 10g. Produced by Pingzhao Hu
Figure 5-15. LOD score plot of the binary trait on Chromosome 8.

The trait is binarized using a cutoff of body weight: 10g. Black dots indicate the individual SNPs tested in the analysis.
rs6237645 and rs3662808 are the two SNPs with highest LOD score from the array.
causal variants than to be causal themselves (492). Therefore, a list of all SNPs that are in strong LD with each SNP strongly linked to the s-D486N/D486N phenotype could be generated using data from the laboratory mouse haplotype map (493). Through this imputation approach, we could extend the coverage of phenotype-associated SNPs to include a more comprehensive list of putative functional SNPs. On the other hand, gene expression profiles for the genes coded within this region could be compared in available databases or be analyzed by qPCR in the relevant tissues (e.g., pituitary, hypothalamus and liver for growth retardation) between the two mouse strains. The gene(s) that is(are) strongly down-regulated on the 129/Sv background, could be the critical genetic modifier(s) linked to the s-D486N/D486N phenotypes.

5.3 Concluding Remarks

In this thesis, I have carried out detailed studies leading to a better understanding of the molecular pathogenesis of NS-associated \textit{RAF1} mutations. Using knock-in mouse models and a variety of biochemical and molecular biology approaches, I have demonstrated that elevated MEK/ERK activation is critical for causing HCM and other NS phenotypes. My work also indicates that increased BRAF heterodimerization is the common theme in the pathogenesis of NS-associated \textit{RAF1} mutations. The role of RAS/ERK signaling in regulating pathological cardiac hypertrophy revealed in this study resolves a prior controversy in the field. I further identified MEK inhibitors as potential therapeutic agents for the treatment of \textit{RAF1} mutant-associated NS. In comparison with other RASopathy mouse models, I propose that different RASopathies lead to distinct alterations in signaling, and a personalized, mutant gene-specific strategy may be needed for treating the RASopathies. Further studies are required to clarify the cell(s)-of-origin underlying HCM in NS, the role of specific Erk family members and modifier gene(s) in Raf1 mutant NS.
References


