c-Kit modifies the inflammatory status of smooth muscle cells

Lei Song [1] , Laisel Martinez [2] , Zachary M Zigmond [1] , Diana R Hernandez [2] , Roberta M Lassance-Soares [2] , Guillermo Selman [2] , Roberto I Vazquez-Padron Corresp. [2]

[1] Department of Molecular and Cellular Pharmacology, University of Miami, Miami, FL, United States

[2] Department of Surgery, University of Miami, Miami, FL, United States

Corresponding Author: Roberto I Vazquez-Padron
Email address: rvazquez@med.miami.edu

Background: c-Kit is a receptor tyrosine kinase present in multiple cell types including vascular smooth muscle cells (SMC). However, little is known about how c-Kit influences SMC biology and vascular pathogenesis. **Methods:** High-throughput microarray assays and *in silico* pathway analysis were used to identify differentially expressed genes between primary c-Kit deficient ($Kit^{W/W-v}$) and control ($Kit^{+/+}$) SMC. Quantitative real-time RT-PCR and functional assays further confirmed the differences in gene expression and pro-inflammatory pathway regulation between both SMC populations. **Results:** The microarray analysis revealed elevated NF-κB gene expression secondary to the loss of c-Kit that affects both the canonical and alternative NF-κB pathways. Upon stimulation with an oxidized phospholipid as pro-inflammatory agent, c-Kit deficient SMC displayed enhanced NF-κB transcriptional activity, higher phosphorylated/total p65 ratio, and increased protein expression of NF-κB regulated pro-inflammatory mediators with respect to cells from control mice. The pro-inflammatory phenotype of mutant cells was ameliorated after restoring c-Kit activity using lentiviral transduction. Functional assays further demonstrated that c-Kit suppresses NF-κB activity in SMC in a TGFβ-activated kinase 1 (TAK1) and Nemo-like kinase (NLK) dependent manner. **Discussion:** Our study suggests a novel mechanism by which c-Kit suppresses NF-κB regulated pathways in SMC to prevent their pro-inflammatory transformation.

1 **c-Kit Modifies the Inflammatory Status of Smooth Muscle Cells**

2

3 Lei Song[a], Laisel Martinez[b], Zachary M. Zigmond[a], Diana R. Hernandez[b], Roberta M. Lassance-

4 Soares[b], Guillermo Selman[b], and Roberto I. Vazquez-Padron [b,*]

5 [a] Molecular and Cellular Pharmacology Department, Leonard M. Miller School of Medicine,

6 University of Miami, Miami, FL 33136

7 [b] DeWitt Daughtry Family Department of Surgery, Division of Vascular Surgery, Leonard M.

8 Miller School of Medicine, University of Miami, Miami, FL 33136

9

10

11

12

13 [*] **Corresponding Author:**

14 Roberto I. Vazquez-Padron, PhD

15 1600 NW 10th Ave, RMSB 1048

16 Miami, FL 33136, United States

17 Tel: (305) 243-1154

18 Fax: (305) 243-3134

19 Email: RVazquez@med.miami.edu

20 **ABSTRACT**

21   **Background:** c-Kit is a receptor tyrosine kinase present in multiple cell types including

22 vascular smooth muscle cells (SMC). However, little is known about how c-Kit influences SMC

23 biology and vascular pathogenesis. **Methods:** High-throughput microarray assays and *in silico*

24 pathway analysis were used to identify differentially expressed genes between primary c-Kit

25 deficient ($Kit^{W/W-v}$) and control ($Kit^{+/+}$) SMC. Quantitative real-time RT-PCR and functional

26 assays further confirmed the differences in gene expression and pro-inflammatory pathway

27 regulation between both SMC populations. **Results:** The microarray analysis revealed elevated

28 NF-κB gene expression secondary to the loss of c-Kit that affects both the canonical and

29 alternative NF-κB pathways. Upon stimulation with an oxidized phospholipid as pro-

30 inflammatory agent, c-Kit deficient SMC displayed enhanced NF-κB transcriptional activity,

31 higher phosphorylated/total p65 ratio, and increased protein expression of NF-κB regulated pro-

32 inflammatory mediators with respect to cells from control mice. The pro-inflammatory

33 phenotype of mutant cells was ameliorated after restoring c-Kit activity using lentiviral

34 transduction. Functional assays further demonstrated that c-Kit suppresses NF-κB activity in

35 SMC in a TGFβ-activated kinase 1 (TAK1) and Nemo-like kinase (NLK) dependent manner.

36 **Discussion:** Our study suggests a novel mechanism by which c-Kit suppresses NF-κB regulated

37 pathways in SMC to prevent their pro-inflammatory transformation.

38

39

40

41

42 **INTRODUCTION**

43   The c-Kit receptor tyrosine kinase is a proto-oncogene and stem cell marker that has been

44 recently implicated in vascular pathogenesis. Widely recognized for its proliferative and anti-

45 apoptotic role in hematopoietic stem and progenitor cells (Bernstein et al. 1991), c-Kit signaling

46 is now known to increase endothelial permeability (Kim et al. 2014; Im et al. 2016), and regulate

47 the phenotype of smooth muscle cells (SMC) in the vasculature (Wang et al. 2007; Davis et al.

48 2009). On one hand, animal models indicate that c-Kit activation by its ligand the stem cell

49 factor (SCF) plays an important role in the development of both arterial and venous intimal

50 hyperplasia (IH) (Hollenbeck et al. 2004; Wang et al. 2006; Wang et al. 2007; Skartsis et al.

51 2014). On the other hand, c-Kit expression preserves the SMC contractile phenotype (Davis et al.

52 2009), and protects arteries from excessive atherosclerosis (Song et al. 2016). Therefore, whether

53 c-Kit is beneficial or detrimental for the vasculature is still a matter of debate and warrants

54 further investigations.

55   Expression of c-Kit and SCF in vascular myofibroblasts and SMC seems to be tightly

56 regulated by a variety of physiological and pathological triggers. c-Kit positive SMC populate

57 the intima of arteries and veins after vascular injury in models of angioplasty and vein grafting

58 (Hollenbeck et al. 2004; Wang et al. 2006; Wang et al. 2007). Temporal upregulation of both c-

59 Kit and SCF are reported after injury in these models (Hollenbeck et al. 2004; Wang et al. 2006;

60 Wang et al. 2007), where c-Kit dependent induction of the Akt-Bcl-2 cascade is thought to

61 mediate the anti-apoptotic and migratory SMC phenotype responsible for IH (Wang et al. 2007).

62 The formation of venous IH in arteriovenous fistulas (AVF) also occurs secondary to the

63 activation of c-Kit expressing adventitial progenitors and migration of c-Kit positive

64 myofibroblasts to the intima (Skartsis et al. 2014). Both animal and human AVF demonstrate

65   higher numbers of c-Kit expressing SMC after surgery compared to preoperative veins, along

66   with a transitional increase in SCF levels in animal models after AVF creation (Skartsis et al.

67   2014).

68   A small population of c-Kit expressing myofibroblasts/SMC was also found in

69   pulmonary arteries of patients with idiopathic pulmonary arterial hypertension but not in healthy

70   controls (Montani et al. 2011). In line with this evidence, both SCF and c-Kit were upregulated

71   in pulmonary arterioles of experimental animals with pulmonary hypertension, where c-Kit

72   colocalized with cells in the endothelium, media and adventitia (Young et al. 2016). In the latter

73   model, SCF/c-Kit signaling promotes pathological remodeling and pulmonary vascular cell

74   proliferation after hypoxic stimulation via activation of the ERK1/2 pathway (Young et al.

75   2016). Paradoxically, the presence of c-Kit in human primary pulmonary artery SMC upregulates

76   the transcription factor myocardin and preserves the contractile SMC phenotype (Davis et al.

77   2009), suggesting a protective role of c-Kit under certain vascular conditions.

78   Along with the upregulation of c-Kit, the above evidence indicates that SCF is expressed

79   and released in the vasculature in response to different insults (Miyamoto et al. 1997; Hollenbeck

80   et al. 2004; Wang et al. 2007). Vascular SCF exists in both membrane-bound and soluble forms,

81   thus its ability to promote cell recruitment and elicit autocrine and paracrine responses, as well as

82   cell-to-cell stimulation (Hollenbeck et al. 2004; Lennartsson and Ronnstrand 2012; Skartsis et al.

83   2014). The soluble form of SCF is generated by alternative splicing or released through the

84   proteolytic action of matrix metallopeptidase 9 (MMP-9) (Hollenbeck et al. 2004; Bengatta et al.

85   2009; Lennartsson and Ronnstrand 2012; Klein et al. 2015). This latter enzyme is also

86   upregulated in vascular remodeling processes, thereby perpetuating the local effects of the

87   SCF/c-Kit pathway (Hollenbeck et al. 2004; Skartsis et al. 2014). Interestingly, the soluble and

88   membrane-bound SCF isoforms seem to have different effects on c-Kit activation (Miyazawa et

89   al. 1995). The former causes rapid and transient stimulation and autophosphorylation of the

90   receptor as well as fast degradation, whereas the latter leads to sustained activation (Miyazawa et

91   al. 1995). This observation suggests that cell-to-cell interactions between SCF and c-Kit

92   expressing SMC have the potential to significantly modify their respective phenotypes and

93   nearby microenvironment. The concomitant expression of SCF and c-Kit in various cell types

94   (Lennartsson and Ronnstrand 2006; Zakiryanova et al. 2014), including myofibroblasts/SMC

95   (Hollenbeck et al. 2004; Wang et al. 2007; Skartsis et al. 2014), also implies the presence of an

96   autocrine loop for the activation of this receptor. Unfortunately, the available data on the role of

97   c-Kit in vascular remodeling processes is still scarce, and more information is particularly

98   needed on the c-Kit mediated pathways that regulate the SMC phenotypic transformation (Wang

99   et al. 2007).

100   In this work, we used high-throughput microarray analyses to identify differentially

101   expressed genes as a result of c-Kit loss of function in arterial SMC isolated from mutant and

102   littermate control mice. We combined *in silico* pathway analyses and confirmatory assays to

103   further investigate the gene expression profiles of stimulated SMC under both experimental

104   conditions. We showed increased NF-κB activation in c-Kit deficient SMC compared to their

105   wild type counterparts. Furthermore, we demonstrated that these changes were associated with a

106   heightened state of vascular inflammation, as indicated by the elevated protein expression of pro-

107   inflammatory mediators in c-Kit deficient SMC. Outcomes from this study challenge the existing

108   belief that vascular c-Kit expression is pathological and suggest instead a beneficial contribution

109   of this signaling axis for the preservation of SMC's anti-inflammatory status under adverse

110   conditions.

111   **MATERIALS AND METHODS**

112   *Smooth Muscle Cell Isolation and Culture*

113   Primary aortic SMC were isolated from c-Kit deficient ($Kit^{W/W-v}$) mice and control

114   littermate mice ($Kit^{+/+}$) (Stock #100410, The Jackson Laboratories, Bar Harbor, ME) (Bernstein

115   et al. 1990) using the explant technique (Metz et al. 2012) with minor modifications. Briefly,

116   mouse aortas were digested with collagenase type II (5 mg/mL, Worthington, Lakewood, NJ) at

117   37°C for 1 hour, after which they were transferred to 10% FBS and cut with a scalpel into small

118   pieces. Individual SMC migrate out of the explants within 1 week of culture. Cells were

119   maintained in DMEM-F12-FBS (5:3:2; Thermo Fisher Scientific, Waltham, MA) supplemented

120   with 100 µg/ml penicillin, 100 µg/ml streptomycin, 0.1 mM glutamine, 10 mM sodium pyruvate,

121   and 0.75% sodium bicarbonate (Metz et al. 2012). Primary cells were maintained at ~90%

122   confluency and used within three passages to avoid fibroblast-like phenotypic switch. All animal

123   procedures were performed according to the National Institutes of Health guidelines (Guide for

124   the Care and Use of Laboratory Animals) and approved by the University of Miami Miller

125   School of Medicine Institutional Animal Care and Use Committee (protocol 15-114).

126   *RNA Microarray and Pathway Analysis*

127   Total RNA was isolated from $Kit^{+/+}$ and $Kit^{W/W-v}$ SMC using the Quick-RNA MiniPrep

128   kit (Zymo Research, Irvine, CA). RNA quality was validated in the Agilent 2100 Bioanalyzer

129   (Agilent Technologies, Santa Clara, CA) before being sent to Ocean Ridge Biosciences (Palm

130   Beach Gardens, FL) for Mouse MI-Ready Gene Expression Microarray analysis. Once in Ocean

131   Ridge Biosciences, RNA processing included a 30-minute digestion with RNase-free DNase I

132   (Epicentre, Madison, WI) at 37°C followed by purification using the AgenCourt RNAClean XP

133   bead method (Beckman Coulter, Indianapolis, IN). Biotin-labeled complementary RNA (cRNA)

134   was prepared from 2 μg per sample of re-purified RNA by the method of Van Gelder *et al*. (Van

135   Gelder's Multi-gene expression profile - US Patent 7049102). Eighteen micrograms of

136   biotinylated cRNA per sample were fragmented, diluted in formamide-containing hybridization

137   buffer, and loaded onto the surface of the Mouse MI-Ready microarray slides enclosed in custom

138   hybridization chambers. The slides were hybridized for 16-18 hours under constant rotation in a

139   Model 400 hybridization oven (Scigene, Sunnyvale, CA). After hybridization, the microarray

140   slides were washed under stringent conditions, stained with Streptavidin-Alexa-647 (Life

141   Technologies, Waltham, MA), and scanned using an Axon GenePix 4000B scanner (Molecular

142   Devices, Sunnyvale, CA). Probe intensities were calculated for each feature on each microarray

143   by subtracting the median local background from the median local foreground for each probe.

144   Data for all manufacturer-flagged probes and visually flagged probes impacting >25% of

145   samples were removed. Data for visually flagged probes impacting < 25% of samples were

146   replaced with the sample average for the probe. Probe intensities were transformed by taking the

147   base 2 logarithm of each value. Array-specific detection thresholds (T) were calculated by

148   adding 3 times the standard deviation of the median local background and the mean negative

149   control probe signal. Probe intensity and T were normalized by subtracting the 70th percentile of

150   the mouse probe intensities and adding back the mean of the 70th percentile across all samples as

151   a scaling factor. The data were filtered to select for mouse probes showing signal above the

152   normalized T in at least 25% of the samples; data for control sequences and other non-mouse

153   probes were removed. Mouse probe sequences were annotated using a BLAST analysis of the

154   Ensembl Mouse cDNA database version 84 (2016). Gene expression differences between $Kit^{+/+}$

155   and $Kit^{W/W-v}$ SMC were considered statistically significant if $p<0.05$ by *t-test*.

156   For pathway analysis, genes with statistically significant expression differences in

157   microarray analysis were imported into the Ingenuity Pathway Analysis software

158   (www.ingenuity.com; Ingenuity Systems, Redwood City, CA). The Core Analysis was used to

159   identify the canonical pathways associated with the differentially expressed genes. Pathway

160   overlap and p-value calculations were performed using the reference gene set in the Ingenuity

161   Knowledge Base, where only molecular relationships (direct and indirect) that have been

162   experimentally observed were considered. The Molecule Activity Predictor tool was used to

163   estimate activation or inhibition of pathway branches based on the observed gene expression fold

164   changes in $Kit^{W/W-v}$ vs. $Kit^{+/+}$ SMC.

165   ***Quantitative Real-Time PCR (qPCR)***

166   Relative gene expression of selected mRNA transcripts was evaluated using TaqMan

167   Gene Expression Assays (Applied Biosystems, Foster City, CA). Total RNA was isolated as

168   described above, and cDNA synthesized with the High-Capacity cDNA Reverse Transcription

169   kit (Applied Biosystems). Real-time RT-PCR was performed on an ABI Prism 7500 Fast Real-

170   Time PCR System (96-well plate) (Applied Biosystems) using primers/probe sets

171   complementary to the genes of interest (*ActB*, Mm00607939_m1; *Ccl2*, Mm00441242_m1;

172   *Ikbka*, Mm00432529_m1; *Ikbkb*, Mm01222247_m1; *Ikbkg*, Mm00494927_m1; *Il6*,

173   Mm00446190_m1; *Kit*, Mm00445212_m1; *Kitl*, Mm00442972_m1; *Map3k14*,

174   Mm0048444166_m1; *Mmp2*, Mm00439498_m1; *Mmp9*, Mm00442991_m1; *Nfkb2*,

175   Mm00479807_m1; *Nfkbia*, Mm00477798_m1; *Nos2*, Mm00440502_m1; *Ptgs2*,

176   Mm00478374_m1; *RelB*, Mm00485664-m1; *Tnf*, Mm00443258_m1). Relative gene expression

177   was determined using the ΔΔCT method (Livak and Schmittgen 2001) and normalized with

178   respect to *ActB*.

179   ***Gene Rescue and Knockdown***

180   Gene rescue in c-Kit deficient (Kit[W/W-v]) SMC was performed using a lentiviral vector

181   (pRVPG24). This rescue vector was constructed by inserting a blunted BsrBI-NotI digested

182   DNA fragment (3.6 Kb), containing the coding region of the mouse Kit cDNA under the murine

183   phosphoglycerate kinase (PGK) promoter, into the blunted EcoRV-ClaI digested pLenti CMV

184   PuroDest vector (Addgene Inc., Cambridge, MA). Third generation lentiviral stocks were

185   produced in HEK-293 cells co-transfected with the lentiviral vector and the packaging and

186   envelope plasmids psPAX2 and pMD2.G (Addgene Inc.). Transfections were done with the

187   jetPRIME transfection kit (Polyplus, New York, NY). Infected cells (100 MOI) were selected in

188   DMEM-F12-FBS (5:3:2; Thermo Fisher Scientific) supplemented with 100 μg/ml penicillin, 100

189   μg/ml streptomycin, 0.1 mM glutamine, 10 mM sodium pyruvate, 0.75% sodium bicarbonate,

190   and 10 μg/ml puromycin (Sigma, St Louis, MO).

191   Knockdown of TAK1 or NLK in c-Kit wild type (Kit^{+/+}) SMC was performed using

192   pooled lentiviral particles carrying different target siRNAs (Supplementary Table 1; Applied

193   Biological Materials, Richmond, Canada). An anti-GFP siRNA was used as control. Transduced

194   cells were puromycin-selected as described above. All gene modifications were confirmed by

195   analytical flow cytometry or Western blot (WB).

196

197

198 ***Flow Cytometry Analysis***

199   c-Kit surface expression was evaluated by flow cytometry in SMC stained with an anti-

200 CD117 antibody (CD117-PE, Cat# 130-091730, Miltenyi Biotec, San Diego, CA). Analytical

201 flow cytometry was performed on a BD FACS Canto II (BD Biosciences, San Jose, CA) using

202 the BD FACSDiva software (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed using

203 the FlowJo software (Ashland, OR).

204 ***Western Blot and Immunoprecipitation (IP)***

205   Whole cell protein lysates were prepared in RIPA buffer supplemented with 200 mM

206 phenylmethylsulfonyl fluoride (PMSF), 100mM sodium orthovanadate (Santa Cruz

207 Biotechnology, Dallas, TX), and a complete protease inhibitor cocktail (Roche Life Science,

208 Indianapolis, IN). Lysate concentration was determined using a commercial Bradford's protein

209 assay kit (BioRad, Hercules, CA). For WB analysis, ~50 μg of sample was loaded into a

210 NuPAGE 4-12% Bis-Tris SDS-polyacrylamide gel (Thermo Fisher Scientific) and subsequently

211 transferred to a PVDF membrane (GE Healthcare, Marlborough, MA). Specific proteins were

212 detected using antibodies against c-Kit (1:1000, Cat# sc-1494, Santa Cruz Biotechnology), MCP-

213 1, MMP-2, TAK1 (1:500, Cat# sc-1785, sc-1839, and sc-6838, Santa Cruz Biotechnology), NLK

214 (1:1000, Cat# ab26050, Abcam, San Francisco, CA), Src (1:500, Cat# 2108S, Cell Signaling

215 Technology, Danvers, MA), and β-Actin (1:5000, Cat# A5316, Sigma). Bound antibodies were

216 detected after sequentially incubating the membranes with HRP-conjugated secondary

217 antibodies. The Amersham ECL Western Blotting Detection Reagent (GE Healthcare) or

218 SuperSignal West Femto Maximum Sensitivity Substrate Reagent (Thermo Fisher Scientific)

219 were used for signal detection. Images were analyzed using ImageJ Pro 5.0.

220   For co-IP, ~200 µg of protein lysate was incubated at 4°C for 4 hours with 1 µg of anti- c-

221   Kit (Cat# A4502, Dako, Santa Clara, CA) or TAK1 antibodies and 20 µl of Protein A/G PLUS-

222   Agarose microbeads (Santa Cruz Biotechnologies). Microbeads were washed with cold RIPA

223   buffer before WB analysis for c-Kit, TAK1, or NLK as indicated above.

224   *NF-κB Promoter Activity*

225   Primary SMC were transfected with a commercial mix of NF-κB Luc-reporter plasmids

226   (Qiagen, Germantown, MD) using the Axama Basic Nucleofector Primary Smooth Muscle Cells

227   electroporation kit (Cat# VPI-1004, Lonza, Walkersville, MD). Transfected cells were incubated

228   with 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC; Avanti Polar Lipids,

229   Alabaster, AL) for 24 hours in serum-free medium as previously described (Pidkovka et al.

230   2007) before lysis using the Passive Lysis Buffer (Promega, Madison, WI). NF-κB promoter

231   activity was determined using the Dual-Luciferase Reporter Assay System (Cat# E1910,

232   Promega) in a Turner Biosystems Luminometer model Glomax 20/20 (Mountain View, CA), and

233   normalized to the Renilla luciferase activity of the kit's internal control. Promoter activity was

234   expressed as folds of control activity.

235   *Enzyme-Linked Immunosorbent Assay (ELISA)*

236   The levels of cellular NF-κB p65 and phosphorylated protein (p-p65) were measured in

237   SMC treated with POVPC as described above. Cells were lysed using the 1X Cell Extraction

238   Buffer PTR provided in the ELISA kit (Abcam, Cambridge, MA). The ELISA was performed

239   using the NF-κB p65 (pS536 + Total) SimpleStep Kit (Abcam) following the manufacturer's

240   protocol. Protein levels were measured using an endpoint reading at OD 455 nm in an Ultramark

241   Microplate Reader (BioRad).

242   *Statistics*

243   Results are presented as mean ± standard deviation. A two-tailed student *t-test* was used

244   to compare the difference between two groups, and one-way ANOVA followed by a Newman-

245   Keuls test was applied to compare the difference among multiple groups. A *p* value <0.05 was

246   considered significant.

247

248   **RESULTS**

249   ***Different gene expression profiles in c-Kit positive and deficient smooth muscle cells***

250   Considering the reported contribution of c-Kit to vascular remodeling processes

251   (Hollenbeck et al. 2004; Wang et al. 2006; Wang et al. 2007; Skartsis et al. 2014; Young et al.

252   2016), we sought for differentially expressed genes between primary SMC isolated from c-Kit

253   deficient (Kit$^{W/W-v}$) and control littermate (Kit$^{+/+}$) mice (n=3 per strain). Out of a total of 34,265

254   mouse probes queried by microarray, 18,224 yielded a detectable signal above threshold and

255   1,086 genes were found differentially expressed between SMC from both experimental groups

256   (p<0.05) (Figure 1A-B). Specifically, 564 and 522 transcripts were significantly up- and

257   downregulated, respectively, with the loss of c-Kit activity with respect to control SMC (Figure

258   1A). No statistically significant differences in expression were detected by microarray in the

259   remaining 17,138 genes.

260   Table 1 presents selected differentially expressed genes in c-Kit deficient SMC that are

261   relevant for inflammation such as *Ilf2*, *Ifna14*, and *Tnfsf9* (Zhao et al. 2005; Chan et al. 2006;

262   Croft 2009). We also show decreased expression of the anti-inflammatory genes *Foxo1*, *Gdf6*,

263   *Igf1*, *Igf2r*, and *Lpl* (Ziouzenkova et al. 2003; Sukhanov et al. 2007; Savai et al. 2014; Hisamatsu

264   et al. 2016). Lipoprotein lipase (*Lpl*), for example, is 14-fold lower in c-Kit deficient cells than in

265   those isolated from littermate controls. Additional changes in c-Kit deficient SMC are associated

266   with a downregulation of the contractile SMC phenotype (increased *Tnfaip3* and reduced *Sirt1*)

267   (Damrauer et al. 2010; Huang et al. 2015) and higher susceptibility to calcification (decreased

268   *Foxo1* and *Pth1r*) (Cheng et al. 2010; Deng et al. 2015). Finally, we found significant

269   expression differences in genes that code for cell adhesion proteins and for receptors and

270   enzymes that regulate vasomotor responses (Table 1).

271   Confirmatory real-time RT-PCR assays were performed for selected inflammation-

272   related genes that showed a trend by microarray analysis. Tumor necrosis factor (*Tnf*),

273   interleukin 6 (*Il6*), C-C motif chemokine ligand 2 (*Ccl2*), metalloproteinases 2 and 9 (*Mmp2*,

274   *Mmp9*), inducible nitric oxide synthase (*Nos2*), and cyclooxygenase 2 (*Ptgs2*) were significantly

275   upregulated in c-Kit deficient SMC compared to controls (Figure 1C).

276   ***Predicted activation of NF-κB signaling in c-Kit deficient cells by pathway analysis***

277   *In silico* pathway analysis was used to predict the molecular pathways affected by the

278   loss of c-Kit in SMC. A total of 71 statistically significant pathways were identified (p<0.05) by

279   the software, 42 of which with a biologically relevant function in SMC. These pathways covered

280   cellular processes such as cell survival and apoptosis, inflammation, cell adhesion, nitric oxide

281   signaling, and lipid metabolism (Table 2). Interestingly, 10 independent molecular pathways

282   were associated with NF-κB signaling, and all of them showed either predicted activation of the

283   entire pathway (5/10; z-scores ranging from 0.258 to 1.265) or of the NF-κB branch (5/10) in c-

284   Kit deficient SMC (Table 2).

Upregulation of NF-κB pathway genes in c-Kit deficient smooth muscle cells

286   The NF-κB pathway plays a fundamental role in SMC differentiation, inflammation, and

287   response to stress signals (Zahradka et al. 2002; Ramana et al. 2004; Mehrhof et al. 2005; Mack

288   2011). Therefore, we confirmed the upregulation of components of this pathway in c-Kit

289   deficient cells by real-time RT-PCR (Figure 1D). We found significantly higher expression

290   levels of genes that are part of both the canonical (*Ikbka, Ikbkb, Ikbkg, Nfkbia*) and alternative

291   (*Ikbka, Map3k14, Nfkb2, RelB*) NF-κB signaling pathways in Kit$^{W/W-v}$ vs. Kit$^{+/+}$ SMC.

Increased activity of the canonical NF-κB pathway in stimulated c-Kit deficient cells

293   Given that the inhibitor of the canonical NF-κB pathway (*Nfkbia*) and the negative

294   regulator *Tnfaip3* are upregulated in c-Kit deficient SMC (Table 1, Figure 1D), we turned to

295   demonstrate the relationship between c-Kit expression and functional activity of the NF-κB

296   signaling pathway. To further validate our findings, we rescued c-Kit expression in Kit$^{W/W-v}$

297   SMC by lentiviral transduction (Supplementary Figure 1).

298   POVPC-stimulated SMC with deficient c-Kit expression showed higher NF-κB

299   transcriptional activity compared to wild type and c-Kit rescued cells as determined by a dual

300   luciferase reporter assay (Figure 2A). Accordingly, a significantly higher ratio of the S536-

301   phosphorylated/total p65 factor was detected in Kit$^{W/W-v}$ SMC vs. wild type and rescued cells

302   (Figure 2B), demonstrating increased availability of active p65 in c-Kit deficient SMC for

303   nuclear translocation and promoter binding (Lawrence 2009). Finally, we evaluated the protein

304   concentrations of the pro-inflammatory mediators MMP-2 and MCP-1 in POVPC-stimulated

305   SMC, two factors that are regulated by NF-κB (Lee et al. 2008; Song et al. 2016). In agreement

306   with the enhanced transcriptional activity shown above, the protein expressions of both MMP-2

307   and MCP-1 were significantly higher in Kit$^{W/W-v}$ SMC compared to wild type and rescued cells

308   (Figure 2C). To control for off-target effects of POVPC stimulation on c-Kit expression, we

309   demonstrated that this treatment did not modify the cellular levels of SCF or the receptor

310   (Supplementary Figure 2).

311   ***c-Kit regulates NF-κB activation through TAK1/NLK in smooth muscle cells***

312   Previous studies indicate an association between c-Kit, Lyn (a member of the Src family

313   of non-receptor tyrosine kinases), and TAK1 (Drube et al. 2015), a negative regulator of NF-κB

314   signaling (Ajibade et al. 2012). Therefore, we assessed whether this latter factor or its

315   downstream partner NLK (Yasuda et al. 2004; Li et al. 2014) were responsible for the observed

316   inhibition of the NF-κB pathway in c-Kit expressing SMC. We found that the protein

317   expressions of both TAK1 and NLK were reduced or lost in Kit$^{W/W-v}$ SMC compared to wild

318   type or c-Kit rescued cells (Figure 3A). Next, we selectively knocked down TAK1 or NLK in

319   Kit$^{+/+}$ SMC (Figure 3B-C), and showed that this genetic manipulation restored the NF-κB

320   transcriptional activity, phosphorylated/total p65 ratio, and protein expressions of MMP-2 and

321   MCP1 in POVPC-stimulated c-Kit wild type SMC (Figure 3D-F). Lastly, we demonstrated by

322   co-IP a physical interaction between all c-Kit, Src, TAK1, and NLK (Figure 4), further

323   supporting a direct relationship in NF-κB regulation. Altogether, these experiments demonstrate

324   that c-Kit inhibits NF-κB signaling in SMC through the actions of TAK1 and NLK.

325

326

327   **DISCUSSION**

328   Vascular SMC are characterized by tremendous phenotypic diversity (Yoshida and

329   Owens 2005). Moreover, their contribution to vascular pathogenesis greatly depends on their

330   phenotype and state of differentiation (Archer 1996; Yoshida and Owens 2005). Expression of

331   the c-Kit receptor in SMC has been associated with various vascular pathologies in both animal

332   models (Wang et al. 2006; Wang et al. 2007; Skartsis et al. 2014; Young et al. 2016) and human

333   samples (Hollenbeck et al. 2004; Skartsis et al. 2014). In contrast, it has also proven protective in

334   models of atherosclerosis (Song et al. 2016). In light of this evidence, there is little information

335   on how c-Kit influences the phenotypes of SMC or the mechanisms by which they contribute to

336   pathology. Our work reveals that the absence of c-Kit modified the expression of approximately

337   6% of the genes that were detected by microarray in SMC from c-Kit mutant and littermate

338   control mice. Furthermore, we provide evidence that c-Kit suppresses NF-κB signaling in SMC

339   and decreases the production of pro-inflammatory mediators under stimulus.

340   Using *in silico* pathway analysis, we first demonstrated that c-Kit signaling influences a

341   wide variety of cellular processes in SMC. Specifically, we found evidence of a pro-synthetic

342   and pro-inflammatory phenotype in SMC secondary to the loss of this receptor. This is

343   particularly evident by the potential dysregulation of lipid metabolism as indicated by a 14-fold

344   decrease in *Lpl* gene expression. While increased vascular lipoprotein lipase can be pro-

345   atherogenic (Clee et al. 2000), it is also believed to have anti-inflammatory properties both by

346   generating metabolic PPAR agonists and inhibiting NF-κB activity (Ziouzenkova et al. 2003;

347   Kota et al. 2005). c-Kit deficient cells also have decreased expression of the anti-inflammatory

348   and anti-atherogenic factor IGF-1 (Sukhanov et al. 2007) and increased susceptibility to

349   calcification due to the downregulation of the *Foxo1* and *Pth1r* genes (Cheng et al. 2010; Deng

350 et al. 2015). It is possible that these changes explain the increased severity of atherosclerosis in

351 c-Kit mutant animals (Song et al. 2016). In addition, c-Kit deficient SMC appear to respond

352 differently to vasomotor stimuli. Their gene expression profile indicates a significantly lower

353 expression of vasoconstrictive G-protein coupled receptors such as the angiotensin II receptor

354 type 1B and the arginine vasopressin receptor 1A. The response to nitric oxide may be also

355 impaired in these cells due to a lower expression of guanylate cyclase 1 soluble subunit beta and

356 cGMP-dependent protein-kinase type I. In the absence of functional experiments, it is not clear

357 what is the biological impact of the above differences in c-Kit deficient SMC compared to their

358 wild type counterparts. However, these observations warrant further investigations.

359 Interestingly, 24% of the differentially regulated pathways identified were associated

360 with NF-κB signaling. Furthermore, both the *in silico* analysis and our experimental data

361 demonstrated activation of this pathway in c-Kit deficient SMC with respect to those from

362 littermate controls. NF-κB signaling is critical for the regulation of proliferation, differentiation,

363 stress responses, and inflammatory processes in vascular SMC (Zahradka et al. 2002; Ramana et

364 al. 2004; Mehrhof et al. 2005; Mack 2011). Whether NF-κB activation is associated with

365 increased proliferation or apoptosis in SMC is dependent on the upstream stimuli and the type of

366 vessel (Zahradka et al. 2002; Mehrhof et al. 2005; Ogbozor et al. 2015). A recent study

367 demonstrated that NF-κB activation led to increased proliferation in fibroblasts, while inducing

368 apoptosis and inflammation in SMC (Mehrhof et al. 2005). On the other hand, NF-κB was shown

369 to be an important intracellular mediator of angiotensin II responses, leading to SMC

370 proliferation and migration under these conditions (Zahradka et al. 2002). In terms of cell

371 differentiation, NF-κB is known to repress myocardin activity and cause downregulation of SMC

372 contractile genes (Tang et al. 2008). This molecular interaction has been implicated in the origin

373   of synthetic SMC under inflammatory processes such as atherosclerosis (Mack 2011).

374   Interestingly, the reduced expression of *Sirt* and increased mRNA level of *Tnfaip3* in c-Kit

375   deficient cells are independently associated with downregulation of contractile genes in SMC

376   (Damrauer et al. 2010; Huang et al. 2015). These observations are in agreement with the

377   predicted de-differentiated phenotype of c-Kit deficient SMC (Davis et al. 2009) and with the

378   reported atheroprotective role of the c-Kit receptor (Song et al. 2016). TNFAIP3 normally

379   provides a negative regulatory loop for the NF-κB pathway, including the decreased downstream

380   production of the MCP-1 inflammatory mediator (Patel et al. 2006; Giordano et al. 2014).

381   Downregulation of the *Crebbp* transcription factor is also thought to reduce NF-κB

382   transcriptional activity (Yang et al. 2010). Nonetheless, neither higher *Tnfaip3* expression nor

383   less *Crebbp* in c-Kit deficient SMC seem to have an appreciable inhibitory effect on NF-κB

384   signaling, as demonstrated by our functional experiments and the increased protein expressions

385   of the MMP-2 and MCP-1 factors.

386   Typical stimuli for NF-κB activation include cytokines, endotoxins, lipids, and

387   mechanical stress (Maziere et al. 1996; De Martin et al. 2000; Kumar and Boriek 2003). For

388   example, the oxidized phospholipid POVPC has been previously used to induce inflammation in

389   vascular SMC (Pidkovka et al. 2007; Lu et al. 2013) and NF-κB activation (Pegorier et al. 2006;

390   Vladykovskaya et al. 2011; Lu et al. 2013). As predicted by the *in silico* analysis, c-Kit

391   deficiency in SMC led to higher levels of NF-κB transcriptional activity, phosphorylation of its

392   key subunit p65, and expression of the NF-κB regulated inflammatory mediators MMP-2 and

393   MCP-1 under POVPC challenge. Gene members of the non-canonical NF-κB pathway and other

394   inflammatory mediators were also upregulated in mutant SMC.

395   The role of c-Kit as a negative regulator of the NF-κB pathway and related inflammation

396   has been previously observed in other cell types and under different stimuli (Jin et al. 2013;

397   Micheva-Viteva et al. 2013). Pharmacological inhibition of c-Kit results in increased activation

398   of NF-κB in HEK293 cells and secretion of TNFα in dendritic cells and the THP-1 monocytic

399   cell line in response to bacterial infection (Micheva-Viteva et al. 2013). Lower expressions of

400   SCF and c-Kit were also associated with increased NF-κB signaling and oxidative stress in

401   gastric smooth muscle (Jin et al. 2013).

402   Our experiments further revealed that c-Kit reduces NF-κB mediated inflammation via a

403   direct molecular interaction with the NF-κB negative regulators TAK1 and NLK (Yasuda et al.

404   2004; Ajibade et al. 2012; Li et al. 2014). The physical association between c-Kit, Lyn (a

405   member of the Src family of non-receptor tyrosine kinases), and TAK1 has been previously

406   observed in the HEK293T cell line, where these proteins form a signalosome that interacts with

407   IKKβ, one of the catalytic units of the IκB kinase (IKK) complex (Drube et al. 2015).

408   Nonetheless, the inhibitory activity of TAK1 on the NF-κB signaling pathway appears to be cell-

409   specific, since in some cells it can be activating (Israel 2010; Ajibade et al. 2012). In the

410   inhibitory instances, TAK1 blocks the phosphorylation and inactivates IKK (Ajibade et al.

411   2012), which in turn is unable to phosphorylate and induce proteosomal degradation of the IκB

412   inhibitors of the NF-κB pathway (Karin 1999; Israel 2010). When active, IκB proteins prevent

413   the nuclear translocation of p65/RelA complexes (Karin 1999; Israel 2010). NLK also functions

414   as an inhibitor of IKK phosphorylation (even in cells where TAK1 acts as an activator) (Li et al.

415   2014). Therefore, our data indicate that in SMC the roles of TAK1 and NLK may be redundant.

416   In conclusions, our study demonstrates that c-Kit expression in SMC has an anti-

417   inflammatory role. Our mechanistic studies contradict the existing belief about the noxious effect

418   of SCF/c-Kit signaling to the vasculature. It is noteworthy to recognize that such idea originated

419   from descriptive studies and models of post injury IH. The current knowledge describes the

420   expression of SCF and its receptor c-Kit in endothelial cells and SMC (Hollenbeck et al. 2004;

421   Matsui et al. 2004; Wang et al. 2007; Skartsis et al. 2014), and suggests a key role for this

422   signaling pathway in myofibroblast mobilization towards the neointima (Hollenbeck et al. 2004;

423   Skartsis et al. 2014). Increased survival of SCF-treated SMC through Akt has also been

424   demonstrated (Wang et al. 2007). In contrast, one recent study revealed that SCF/c-Kit signaling

425   protects hyperlipidemic ApoE$^{-/-}$ mice from excessive atherosclerotic plaque deposition (Song et

426   al. 2016). This apparent discrepancy may reflect the existing differences between IH (restenosis)

427   and atherosclerosis in terms of etiology, natural history, culprit lesions, and progenitor cell

428   contribution to disease progression. Therefore, our results suggests that while c-Kit positive cells

429   have a detrimental effect on proliferative vascular lesions, their presence may prove protective in

430   inflammatory conditions such as atherosclerosis. In addition, we propose a novel pathway for

431   NF-κB regulation downstream of c-Kit activation. This information could be relevant in the

432   setting of atherosclerosis disease development and complications, and may shed light on new

433   proliferation control mechanisms to address IH after vascular injury.

434 **FIGURE LEGENDS**

435 **Figure 1. Loss of c-Kit function accounts for significant gene expression differences**

436 **between c-Kit deficient and wild type smooth muscle cells (SMC). A)** Venn diagram

437 indicating the numbers of differentially upregulated genes in primary SMC isolated from c-Kit

438 deficient (blue; $Kit^{W/W-v}$) and littermate control mice (red; $Kit^{+/+}$) as determined by microarray

439 analysis. The group of genes in the interception (black area) did not show statistically significant

440 differences by *t-test* between the two strains (n=3 per group). **B)** Heat map of differentially

441 expressed genes in primary SMC from c-Kit deficient and littermate control mice. **C-D)** Relative

442 expression of inflammatory **(C)** and NF-κB related **(D)** genes in c-Kit deficient vs. control SMC

443 as determined by real-time RT-PCR. Values are shown as fold change over expression in $Kit^{+/+}$

444 cells; * p<0.05 and ** p<0.01 using a two-tailed *t-test* assuming unequal variance, n=3 per

445 group.

446 **Figure 2. Loss of c-Kit function in primary smooth muscle cells (SMC) is associated with**

447 **increased NF-κB activity. A)** NF-κB transcriptional activity in c-Kit deficient ($Kit^{W/W-v}$),

448 control ($Kit^{+/+}$), and c-Kit rescued SMC (Kit^{R}) after 24-hour treatment with POVPC (50 μg/ml),

449 as determined by dual-luciferase reporter assay. Transcriptional activity is represented as the

450 mean ± standard deviation (SD) of the Firefly/Renilla luciferase ratio normalized with respect to

451 the control group ($Kit^{+/+}$) (n=3 independent experiments). **B)** Phosphorylated (pS536) protein

452 levels of the NF-κB p65 subunit in POVPC-treated c-Kit deficient, control, and c-Kit rescued

453 SMC as determined by ELISA. Values are expressed as the mean ± SD of the p-p65/total p65

454 ratio normalized with respect to the control group ($Kit^{+/+}$) (n=3 independent experiments). **C)**

455 Protein expression of the NF-κB related pro-inflammatory mediators MMP-2 and MCP-1 in

456 POVPC-treated c-Kit deficient, control, and c-Kit rescued SMC as determined by Western blot.

457   Molecular weight markers are shown on the right side of the gel. Protein expression is expressed

458   as the mean ± SD of the MMP-2/β-actin and MCP-1/β-actin signal ratios normalized with

459   respect to the control group (Kit$^{+/+}$) (n=3 per cell type). * $p<0.05$ and ** $p<0.01$ using a one-way

460   ANOVA followed by a Newman-Keuls test.

461   **Figure 3. c-Kit inhibits NF-κB activity in smooth muscle cells (SMC) through the actions of**

462   **TAK1 and NLK. A)** Protein expression of the TAK1 and NLK regulatory proteins in c-Kit

463   deficient (Kit$^{W/W-v}$), control (Kit$^{+/+}$), and c-Kit rescued SMC (Kit^{R}) as determined by Western

464   blot. Protein expression is expressed as the mean ± standard deviation (SD) of the TAK1/β-actin

465   and NLK/β-actin signal ratios normalized with respect to the control group (Kit$^{+/+}$) (n=3 per cell

466   type). **B-C)** Protein expression of TAK1 **(B)** and NLK **(C)** in Kit$^{+/+}$ cells transduced with

467   lentivirus-encoded siRNAs of the corresponding targets or GFP control. Protein expression is

468   expressed as the mean ± SD of the TAK1/β-actin and NLK/β-actin signal ratios normalized with

469   respect to the siGFP-treated group (n=3 independent experiments). **D)** NF-κB transcriptional

470   activity in Kit$^{+/+}$ SMC transduced with lentivirus-encoded siRNAs complementary to TAK1,

471   NLK, or GFP after 24-hour treatment with POVPC (50 μg/ml), as determined by dual-luciferase

472   assay. Transcriptional activity is represented as the mean ± SD of the Firefly/Renilla luciferase

473   ratio normalized with respect to the siGFP-treated group (n=3 independent experiments). **E)**

474   Phosphorylated (pS536) protein levels of NF-κB p65 in POVPC-treated Kit$^{+/+}$ SMC transduced

475   with lentivirus-encoded siRNAs as determined by ELISA. Values are expressed as the mean ±

476   SD of the p-p65/total p65 ratio normalized with respect to the siGFP-treated group (n=3

477   independent experiments). **F)** Protein expression of the pro-inflammatory mediators MMP-2 and

478   MCP-1 in POVPC-treated Kit$^{+/+}$ SMC transduced with lentivirus-encoded siRNAs as determined

479   by Western blot. Protein expression is expressed as the mean ± SD of the MMP-2/β-actin and

480   MCP-1/β-actin signal ratios normalized with respect to the siGFP-treated group (n=3

481   independent experiments). Molecular weight markers are shown on the right side of the gels. *

482   p<0.05 and ** p<0.01 using a one-way ANOVA followed by a Newman-Keuls test.

483   **Figure 4. c-Kit forms a molecular complex with the regulatory proteins TAK1, Src, and**

484   **NLK in smooth muscle cells (SMC). A)** Diagram illustrating the proposed molecular complex

485   between c-Kit, TAK1, Src, and NLK in SMC and their inhibitory function on NF-κB regulated

486   transcriptional activity. **B-C)** Co-immunoprecipitation experiments in control (Kit$^{+/+}$) and c-Kit

487   deficient (Kit$^{W/W\text{-}v}$) SMC using anti-c-Kit **(B)** and anti-TAK1 antibodies **(C)** to pull down protein

488   complexes. Molecular weight markers are shown on the right side of the gels, while antibodies

489   used to detect eluted proteins are indicated on the left. IP, immunoprecipitation; IB, immunoblot.

490

491

492

493

494

495

496

497

498

499

500 **REFERENCES**

501 Ajibade, AA, Q Wang, J Cui, J Zou, X Xia, M Wang, Y Tong, W Hui, D Liu, B Su, HY Wang
502   and RF Wang. 2012. Tak1 negatively regulates nf-kappab and p38 map kinase activation
503   in gr-1+cd11b+ neutrophils. *Immunity* 36: 43-54.
504 Archer, SL. 1996. Diversity of phenotype and function of vascular smooth muscle cells. *J Lab*
505   *Clin Med* 127: 524-529.
506 Bengatta, S, C Arnould, E Letavernier, M Monge, HM de Preneuf, Z Werb, P Ronco and B
507   Lelongt. 2009. Mmp9 and scf protect from apoptosis in acute kidney injury. *J Am Soc*
508   *Nephrol* 20: 787-797.
509 Bernstein, A, B Chabot, P Dubreuil, A Reith, K Nocka, S Majumder, P Ray and P Besmer. 1990.
510   The mouse w/c-kit locus. *Ciba Found Symp* 148: 158-166; discussion 166-172.
511 Bernstein, A, L Forrester, AD Reith, P Dubreuil and R Rottapel. 1991. The murine w/c-kit and
512   steel loci and the control of hematopoiesis. *Semin Hematol* 28: 138-142.
513 Chan, A, DL Newman, AM Shon, DH Schneider, S Kuldanek and C Ober. 2006. Variation in the
514   type i interferon gene cluster on 9p21 influences susceptibility to asthma and atopy.
515   *Genes Immun* 7: 169-178.
516 Cheng, SL, JS Shao, LR Halstead, K Distelhorst, O Sierra and DA Towler. 2010. Activation of
517   vascular smooth muscle parathyroid hormone receptor inhibits wnt/beta-catenin signaling
518   and aortic fibrosis in diabetic arteriosclerosis. *Circ Res* 107: 271-282.
519 Clee, SM, N Bissada, F Miao, L Miao, AD Marais, HE Henderson, P Steures, J McManus, B
520   McManus, RC LeBoeuf, JJ Kastelein and MR Hayden. 2000. Plasma and vessel wall
521   lipoprotein lipase have different roles in atherosclerosis. *J Lipid Res* 41: 521-531.
522 Croft, M. 2009. The role of tnf superfamily members in t-cell function and diseases. *Nat Rev*
523   *Immunol* 9: 271-285.
524 Damrauer, SM, CR Peterson, C Eva, P Studer and C Ferran. 2010. Partial loss of a20 (tnfaip3)
525   promotes resistance to abdominal aortic aneurysms through altering smooth muscle cell
526   differentiation. *Journal of the American College of Surgeons* 211: S137.
527 Davis, BN, AC Hilyard, PH Nguyen, G Lagna and A Hata. 2009. Induction of microrna-221 by
528   platelet-derived growth factor signaling is critical for modulation of vascular smooth
529   muscle phenotype. *J Biol Chem* 284: 3728-3738.
530 De Martin, R, M Hoeth, R Hofer-Warbinek and JA Schmid. 2000. The transcription factor nf-
531   kappa b and the regulation of vascular cell function. *Arterioscler Thromb Vasc Biol* 20:
532   E83-88.
533 Deng, L, L Huang, Y Sun, JM Heath, H Wu and Y Chen. 2015. Inhibition of foxo1/3 promotes
534   vascular calcification. *Arterioscler Thromb Vasc Biol* 35: 175-183.
535 Drube, S, F Weber, C Gopfert, R Loschinski, M Rothe, F Boelke, MA Diamanti, T Lohn, J Ruth,
536   D Schutz, N Hafner, FR Greten, R Stumm, K Hartmann, OH Kramer, A Dudeck and T
537   Kamradt. 2015. Tak1 and ikk2, novel mediators of scf-induced signaling and potential
538   targets for c-kit-driven diseases. *Oncotarget* 6: 28833-28850.
539 Giordano, M, R Roncagalli, P Bourdely, L Chasson, M Buferne, S Yamasaki, R Beyaert, G van
540   Loo, N Auphan-Anezin, AM Schmitt-Verhulst and G Verdeil. 2014. The tumor necrosis
541   factor alpha-induced protein 3 (tnfaip3, a20) imposes a brake on antitumor activity of cd8
542   t cells. *Proc Natl Acad Sci U S A* 111: 11115-11120.

543 Hisamatsu, D, M Ohno-Oishi, S Nakamura, Y Mabuchi and H Naka-Kaneda. 2016. Growth
544   differentiation factor 6 derived from mesenchymal stem/stromal cells reduces age-related
545   functional deterioration in multiple tissues. *Aging (Albany NY)* 8: 1259-1275.
546 Hollenbeck, ST, K Sakakibara, PL Faries, B Workhu, B Liu and KC Kent. 2004. Stem cell factor
547   and c-kit are expressed by and may affect vascular smcs through an autocrine pathway. *J*
548   *Surg Res* 120: 288-294.
549 Huang, K, ZQ Yan, D Zhao, SG Chen, LZ Gao, P Zhang, BR Shen, HC Han, YX Qi and ZL
550   Jiang. 2015. Sirt1 and foxo mediate contractile differentiation of vascular smooth muscle
551   cells under cyclic stretch. *Cell Physiol Biochem* 37: 1817-1829.
552 Im, JE, SH Song and W Suh. 2016. Src tyrosine kinase regulates the stem cell factor-induced
553   breakdown of the blood-retinal barrier. *Mol Vis* 22: 1213-1220.
554 Israel, A. 2010. The ikk complex, a central regulator of nf-kappab activation. *Cold Spring Harb*
555   *Perspect Biol* 2: a000158.
556 Jin, QH, HX Shen, H Wang, QY Shou and Q Liu. 2013. Curcumin improves expression of scf/c-
557   kit through attenuating oxidative stress and nf-kappab activation in gastric tissues of
558   diabetic gastroparesis rats. *Diabetol Metab Syndr* 5: 12.
559 Karin, M. 1999. How nf-kappab is activated: The role of the ikappab kinase (ikk) complex.
560   *Oncogene* 18: 6867-6874.
561 Kim, JY, JS Choi, SH Song, JE Im, JM Kim, K Kim, S Kwon, HK Shin, CK Joo, BH Lee and W
562   Suh. 2014. Stem cell factor is a potent endothelial permeability factor. *Arterioscler*
563   *Thromb Vasc Biol* 34: 1459-1467.
564 Klein, G, O Schmal and WK Aicher. 2015. Matrix metalloproteinases in stem cell mobilization.
565   *Matrix Biol* 44-46: 175-183.
566 Kota, RS, CV Ramana, FA Tenorio, RI Enelow and JC Rutledge. 2005. Differential effects of
567   lipoprotein lipase on tumor necrosis factor-alpha and interferon-gamma-mediated gene
568   expression in human endothelial cells. *J Biol Chem* 280: 31076-31084.
569 Kumar, A and AM Boriek. 2003. Mechanical stress activates the nuclear factor-kappab pathway
570   in skeletal muscle fibers: A possible role in duchenne muscular dystrophy. *FASEB J* 17:
571   386-396.
572 Lawrence, T. 2009. The nuclear factor nf-kappab pathway in inflammation. *Cold Spring Harb*
573   *Perspect Biol* 1: a001651.
574 Lee, SJ, KW Seo, MR Yun, SS Bae, WS Lee, KW Hong and CD Kim. 2008. 4-hydroxynonenal
575   enhances mmp-2 production in vascular smooth muscle cells via mitochondrial ros-
576   mediated activation of the akt/nf-kappab signaling pathways. *Free Radic Biol Med* 45:
577   1487-1492.
578 Lennartsson, J and L Ronnstrand. 2006. The stem cell factor receptor/c-kit as a drug target in
579   cancer. *Curr Cancer Drug Targets* 6: 65-75.
580 Lennartsson, J and L Ronnstrand. 2012. Stem cell factor receptor/c-kit: From basic science to
581   clinical implications. *Physiol Rev* 92: 1619-1649.
582 Li, SZ, HH Zhang, JB Liang, Y Song, BX Jin, NN Xing, GC Fan, RL Du and XD Zhang. 2014.
583   Nemo-like kinase (nlk) negatively regulates nf-kappa b activity through disrupting the
584   interaction of tak1 with ikkbeta. *Biochim Biophys Acta* 1843: 1365-1372.
585 Livak, KJ and TD Schmittgen. 2001. Analysis of relative gene expression data using real-time
586   quantitative pcr and the 2(-delta delta c(t)) method. *Methods* 25: 402-408.
587 Lu, Y, L Zhang, X Liao, P Sangwung, DA Prosdocimo, G Zhou, AR Votruba, L Brian, YJ Han,
588   H Gao, Y Wang, K Shimizu, K Weinert-Stein, M Khrestian, DI Simon, NJ Freedman and

589   MK Jain. 2013. Kruppel-like factor 15 is critical for vascular inflammation. *J Clin Invest*
590   123: 4232-4241.
591 Mack, CP. 2011. Signaling mechanisms that regulate smooth muscle cell differentiation.
592   *Arterioscler Thromb Vasc Biol* 31: 1495-1505.
593 Matsui, J, T Wakabayashi, M Asada, K Yoshimatsu and M Okada. 2004. Stem cell factor/c-kit
594   signaling promotes the survival, migration, and capillary tube formation of human
595   umbilical vein endothelial cells. *J Biol Chem* 279: 18600-18607.
596 Maziere, C, M Auclair, M Djavaheri-Mergny, L Packer and JC Maziere. 1996. Oxidized low
597   density lipoprotein induces activation of the transcription factor nf kappa b in fibroblasts,
598   endothelial and smooth muscle cells. *Biochem Mol Biol Int* 39: 1201-1207.
599 Mehrhof, FB, R Schmidt-Ullrich, R Dietz and C Scheidereit. 2005. Regulation of vascular
600   smooth muscle cell proliferation: Role of nf-kappab revisited. *Circ Res* 96: 958-964.
601 Metz, RP, JL Patterson and E Wilson. 2012. Vascular smooth muscle cells: Isolation, culture,
602   and characterization. *Methods Mol Biol* 843: 169-176.
603 Micheva-Viteva, SN, Y Shou, KL Nowak-Lovato, KD Rector and E Hong-Geller. 2013. C-kit
604   signaling is targeted by pathogenic yersinia to suppress the host immune response. *BMC*
605   *Microbiol* 13: 249.
606 Miyamoto, T, Y Sasaguri, T Sasaguri, S Azakami, H Yasukawa, S Kato, N Arima, K Sugama
607   and M Morimatsu. 1997. Expression of stem cell factor in human aortic endothelial and
608   smooth muscle cells. *Atherosclerosis* 129: 207-213.
609 Miyazawa, K, DA Williams, A Gotoh, J Nishimaki, HE Broxmeyer and K Toyama. 1995.
610   Membrane-bound steel factor induces more persistent tyrosine kinase activation and
611   longer life span of c-kit gene-encoded protein than its soluble form. *Blood* 85: 641-649.
612 Montani, D, F Perros, N Gambaryan, B Girerd, P Dorfmuller, LC Price, A Huertas, H Hammad,
613   B Lambrecht, G Simonneau, JM Launay, S Cohen-Kaminsky and M Humbert. 2011. C-
614   kit-positive cells accumulate in remodeled vessels of idiopathic pulmonary arterial
615   hypertension. *Am J Respir Crit Care Med* 184: 116-123.
616 Ogbozor, UD, M Opene, LS Renteria, S McBride and BO Ibe. 2015. Mechanism by which
617   nuclear factor-kappa beta (nf-kb) regulates ovine fetal pulmonary vascular smooth
618   muscle cell proliferation. *Mol Genet Metab Rep* 4: 11-18.
619 Patel, VI, S Daniel, CR Longo, GV Shrikhande, ST Scali, E Czismadia, CM Groft, T Shukri, C
620   Motley-Dore, HE Ramsey, MD Fisher, ST Grey, MB Arvelo and C Ferran. 2006. A20, a
621   modulator of smooth muscle cell proliferation and apoptosis, prevents and induces
622   regression of neointimal hyperplasia. *FASEB J* 20: 1418-1430.
623 Pegorier, S, D Stengel, H Durand, M Croset and E Ninio. 2006. Oxidized phospholipid: Povpc
624   binds to platelet-activating-factor receptor on human macrophages. Implications in
625   atherosclerosis. *Atherosclerosis* 188: 433-443.
626 Pidkovka, NA, OA Cherepanova, T Yoshida, MR Alexander, RA Deaton, JA Thomas, N
627   Leitinger and GK Owens. 2007. Oxidized phospholipids induce phenotypic switching of
628   vascular smooth muscle cells in vivo and in vitro. *Circ Res* 101: 792-801.
629 Ramana, KV, B Friedrich, S Srivastava, A Bhatnagar and SK Srivastava. 2004. Activation of
630   nuclear factor-kappab by hyperglycemia in vascular smooth muscle cells is regulated by
631   aldose reductase. *Diabetes* 53: 2910-2920.
632 Savai, R, HM Al-Tamari, D Sedding, B Kojonazarov, C Muecke, R Teske, MR Capecchi, N
633   Weissmann, F Grimminger, W Seeger, RT Schermuly and SS Pullamsetti. 2014. Pro-

634   proliferative and inflammatory signaling converge on foxo1 transcription factor in
635   pulmonary hypertension. *Nat Med* 20: 1289-1300.
636 Skartsis, N, L Martinez, JC Duque, M Tabbara, OC Velazquez, A Asif, F Andreopoulos, LH
637   Salman and RI Vazquez-Padron. 2014. C-kit signaling determines neointimal hyperplasia
638   in arteriovenous fistulae. *Am J Physiol Renal Physiol* 307: F1095-1104.
639 Song, L, C Kang, Y Sun, W Huang, W Liu and Z Qian. 2016. Crocetin inhibits
640   lipopolysaccharide-induced inflammatory response in human umbilical vein endothelial
641   cells. *Cell Physiol Biochem* 40: 443-452.
642 Song, L, G Selman, N Santos, L Martinez, RM Lassance-Soares, K Webster and RI Vazquez-
643   Padron. 2016. C-kit expression in vascular smooth muscle cells protects mice against
644   excessive atherosclerosis. *Circulation* 134: A14889.
645 Sukhanov, S, Y Higashi, SY Shai, C Vaughn, J Mohler, Y Li, YH Song, J Titterington and P
646   Delafontaine. 2007. Igf-1 reduces inflammatory responses, suppresses oxidative stress,
647   and decreases atherosclerosis progression in apoe-deficient mice. *Arterioscler Thromb*
648   *Vasc Biol* 27: 2684-2690.
649 Tang, RH, XL Zheng, TE Callis, WE Stansfield, J He, AS Baldwin, DZ Wang and CH Selzman.
650   2008. Myocardin inhibits cellular proliferation by inhibiting nf-kappab(p65)-dependent
651   cell cycle progression. *Proc Natl Acad Sci U S A* 105: 3362-3367.
652 Vladykovskaya, E, E Ozhegov, JD Hoetker, Z Xie, Y Ahmed, J Suttles, S Srivastava, A
653   Bhatnagar and OA Barski. 2011. Reductive metabolism increases the proinflammatory
654   activity of aldehyde phospholipids. *J Lipid Res* 52: 2209-2225.
655 Wang, CH, N Anderson, SH Li, PE Szmitko, WJ Cherng, PW Fedak, S Fazel, RK Li, TM Yau,
656   RD Weisel, WL Stanford and S Verma. 2006. Stem cell factor deficiency is
657   vasculoprotective: Unraveling a new therapeutic potential of imatinib mesylate. *Circ Res*
658   99: 617-625.
659 Wang, CH, S Verma, IC Hsieh, A Hung, TT Cheng, SY Wang, YC Liu, WL Stanford, RD
660   Weisel, RK Li and WJ Cherng. 2007. Stem cell factor attenuates vascular smooth muscle
661   apoptosis and increases intimal hyperplasia after vascular injury. *Arterioscler Thromb*
662   *Vasc Biol* 27: 540-547.
663 Yang, J, H Jiang, SS Chen, J Chen, SK Xu, WQ Li and JC Wang. 2010. Cbp knockdown inhibits
664   angiotensin ii-induced vascular smooth muscle cells proliferation through downregulating
665   nf-kb transcriptional activity. *Mol Cell Biochem* 340: 55-62.
666 Yasuda, J, H Yokoo, T Yamada, I Kitabayashi, T Sekiya and H Ichikawa. 2004. Nemo-like
667   kinase suppresses a wide range of transcription factors, including nuclear factor-kappab.
668   *Cancer Sci* 95: 52-57.
669 Yoshida, T and GK Owens. 2005. Molecular determinants of vascular smooth muscle cell
670   diversity. *Circ Res* 96: 280-291.
671 Young, KC, E Torres, D Hehre, S Wu, C Suguihara and JM Hare. 2016. Antagonism of stem cell
672   factor/c-kit signaling attenuates neonatal chronic hypoxia-induced pulmonary vascular
673   remodeling. *Pediatr Res* 79: 637-646.
674 Zahradka, P, JP Werner, S Buhay, B Litchie, G Helwer and S Thomas. 2002. Nf-kappab
675   activation is essential for angiotensin ii-dependent proliferation and migration of vascular
676   smooth muscle cells. *J Mol Cell Cardiol* 34: 1609-1621.
677 Zakiryanova, G, O Karpova, O Khamdiyeva, D Kopytina, N Urazalieva, E Kustova and Z
678   Biyasheva. 2014. C-kit/scf autocrine loop in human nk cells (ccr5p.254). *J Immunol* 192:
679   181.188.

680   Zhao, G, L Shi, D Qiu, H Hu and PN Kao. 2005. Nf45/ilf2 tissue expression, promoter analysis,
681   and interleukin-2 transactivating function. *Exp Cell Res* 305: 312-323.
682   Ziouzenkova, O, S Perrey, L Asatryan, J Hwang, KL MacNaul, DE Moller, DJ Rader, A
683   Sevanian, R Zechner, G Hoefler and J Plutzky. 2003. Lipolysis of triglyceride-rich
684   lipoproteins generates ppar ligands: Evidence for an antiinflammatory role for lipoprotein
685   lipase. *Proc Natl Acad Sci U S A* 100: 2730-2735.

686

Table 1**(on next page)**

Table 1. Selected list of differentially expressed genes in c-Kit deficient vs. wild type smooth muscle cells

Table 1. List of select differentially expressed genes in c-Kit deficient vs. wild type smooth muscle cells

Gene Symbol	Gene Product	Fold Change [a]	P-Value
Transcription Factors			
Crebbp	CREB binding protein	-1.18	0.010
Foxo1	Forkhead box O1	-2.05	0.007
Ilf2	Interleukin enhancer binding factor 2	1.38	0.034
Irf3	Interferon regulatory factor 3	-1.14	0.029
Nfatc1	Nuclear factor of activated T-cells 1	-1.36	0.040
Nfatc2	Nuclear factor of activated T-cells 2	2.01	0.033
Nfatc4	Nuclear factor of activated T-cells 4	-2.32	0.009
Cell Adhesion Proteins			
Cdh5	Cadherin 5	-5.00	0.043
Itga9	Integrin subunit alpha 9	-2.91	0.047
Itga11	Integrin subunit alpha 11	-13.28	0.034
Pcdh7	Protocadherin 7	-2.71	0.015
Pcdha1	Protocadherin alpha 1	-1.44	0.042
Pcdha8	Protocadherin alpha 8	2.26	0.002
Selplg	P-selectin glycoprotein ligand 1	-3.94	0.006
Cytokines/Growth Factors			
Ccl6	Chemokine (C-C motif) ligand 6	-8.76	0.005
Gdf6	Growth differentiation factor 6	-4.10	0.019
Ifna14	Interferon alpha 14	1.27	0.043
Igf1	Insulin-like growth factor 1	-5.06	0.027
Pdgfb	Platelet-derived growth factor subunit B	-2.59	0.011
Pgf	Placental growth factor	-4.96	0.037
Tnfsf9	Tumor necrosis factor ligand superfamily member 9	2.97	0.048
Enzymes			
Bmp1	Bone morphogenetic protein 1	-2.36	0.016
Casp3	Caspase 3	2.06	0.040
Ccnd1	Cyclin D1	2.20	0.043
Gucy1b3	Guanylate cyclase 1 soluble subunit beta	-8.95	0.033
Ikbkb	Inhibitor of nuclear factor kappa-B kinase subunit beta	1.34	0.001
Lpl	Lipoprotein lipase	-14.24	0.022
Mmp23	Matrix metallopeptidase 23	-6.61	0.049
Pde1a	Ca2+/calmodulin dependent phosphodiesterase 1A	-4.73	0.028
Pde2a	Phosphodiesterase 2A	-1.91	0.048
Prkg1	cGMP-dependent protein kinase 1 (PKG)	-9.09	0.034
Ptgs1	Prostaglandin-endoperoxide synthase 1 (COX-1)	2.22	0.027
Sirt1	Sirtuin 1	-1.52	0.034
Tnfaip3	TNF alpha induced protein 3	3.46	<0.001
Receptors			
Adra2a	Adrenoceptor alpha 2A	-6.49	0.002
Agtr1b	Angiotensin II type 1b receptor	-9.88	0.043
Avpr1a	Arginine vasopressin receptor 1A	-6.62	0.010
Cxcr4	Chemokine (C-X-C motif) receptor 4	-6.55	0.040
Igf2r	Insulin like growth factor 2 receptor	-1.48	0.012
Il3ra	Interleukin 3 receptor subunit alpha	-2.38	0.023
Il20ra	Interleukin 20 receptor alpha	-3.52	0.003
Pdgfrb	Platelet-derived growth factor receptor beta	-2.89	0.016
Pth1r	Parathyroid hormone 1 receptor	-5.42	0.002

[a] *Average fold gene expression change in c-Kit deficient smooth muscle cells compared to wild type cells.*

1

Table 2(on next page)

Table 2. Selected canonical pathways with differentially expressed genes in c-Kit deficient vs. wild type smooth muscle cells

Table 2. Select canonical pathways with differentially expressed genes in c-Kit deficient vs. wild type smooth muscle cells

Pathway	Biological Function	P-Value	Z-Score[a]	Predicted Status[a]	Differentially Expressed Genes
PTEN signaling[b]	Proliferation, apoptosis, de-differentiation, cell migration, inflammation	<0.001	0.258	Activation	Akt2, Casp3, Rac1, Ccnd1, Igf2r, Rac3, Ddr1, Shc1, Ikbkb, Inpp5f, Foxo1, Bmpr1a, Magi2, Magi3, Pdgfrb
Death receptor signaling[b]	Apoptosis	0.003	1.265	Activation	Map2k4, Gas2, Rock1, Diablo, Ikbkb, Casp3, Htra2, Tbk1, Parp1, Birc2
TNFR2 signaling[b]	Cell survival, inflammation	0.005	1.000	Activation	Map2k4, Ikbkb, Tnfaip3, Tbk1, Birc2
Wnt/β-catenin signaling	Proliferation, cell survival, cell migration	0.008	0.577	Activation	Sfrp4, Akt2, Crebbp, Csnk1a1, Fzd9, Ccnd1, Rarg, Fzd8, Cdh5, Dkk3, Sox18, Ppp2r5e, Sfrp1, Wnt5b
IRF activation pathway[b]	Inflammation	0.011	1.134	Activation	Map2k4, Ikbkb, Crebbp, Tbk1, Ifna14, Irf3, Atf2
ERK/MAPK signaling	Proliferation, cell migration, vasoconstriction	0.028	1.069	Activation	Crebbp, Rac1, Ppp1r14a, Mknk2, Rac3, Nfatc1, Atf2, Pla2g4e, Shc1, Pla2g6, Prkar2b, Prkag2, Rps6ka1, Ppp2r5e
TNFR1 signaling[b]	Cell survival, inflammation	0.044	1.000	Activation	Map2k4, Ikbkb, Casp3, Tnfaip3, Birc2
Wnt/Ca²⁺ pathway[b]	Proliferation, cell migration	<0.001	-1.000	Inhibition	Fzd8, Plcb4, Crebbp, Nfatc2, Fzd9, Nfatc4, Wnt5b, Nfatc1, Atf2
AMPK signaling[b]	Cellular senescence, anti-inflammatory, differentiation, vasoconstriction	<0.001	-0.535	Inhibition	Pbrm1, Akt2, Crebbp, Ccnd1, Slc2a4, Elavl1, Atf2, Ak6, Prkar2b, Foxo1, Adra2a, Ppm1b, Sirt1, Prkag2, Ppm1a, Ppp2r5e, Ppat, Camkk2
Apoptosis signaling[b]	Apoptosis	<0.001	-0.302	Inhibition	Map2k4, Gas2, Rock1, Diablo, Ikbkb, Casp3, Htra2, Rps6ka1, Bcl2a1, Parp1, Birc2
Phospholipase C signaling	Vasoconstriction, stress responses	0.001	-0.378	Inhibition	Rala, Arhgef12, Pld3, Fcgr2a, Arhgef15, Crebbp, Rac1, Ppp1r14a, Nfatc4, Fcgr2b, Rhoh, Nfatc1, Atf2, Pla2g6, Shc1, Pla2g4e, Plcb4, Itpr3, Fcer1g, Nfatc2
Nitric oxide/GC signaling	Vasodilation	0.005	-0.302	Inhibition	Bdkrb2, Kng1, Pde2a, Akt2, Prkg1, Prkar2b, Itpr3, Prkag2, Pde1a, Gucy1b3, Pgf
Integrin signaling	Cell adhesion, cell migration, proliferation, apoptosis, stress responses, differentiation	0.029	-1.387	Inhibition	Map2k4, Akt2, Rala, Rac1, Rac3, Rhoh, Pdgfb, Rock1, Arhgap5, Shc1, Itga11, Itga9, Actn4, Tspan6, Nedd9
Adipogenesis pathway	Lipid synthesis and storage	<0.001	N.D.	Could not be predicted	Nr2f2, Sin3b, Fzd9, Nfatc4, Rbp1, Slc2a4, Fzd8, Cdk5, Foxo1, Bmpr1a, Lpl, Sirt1, Ctbp2, Clock, Fabp4, Rps6ka1, Stat5b
Fibroblast inflammatory pathway[b]	Proliferation, cell migration, differentiation, inflammation	0.012	N.D.	Could not be predicted	Map2k4, Sfrp4, Akt2, Crebbp, Csnk1a1, Rac1, Fzd9, Nfatc4, Ccnd1, Nfatc1, Pdgfb, Pgf, Atf2, Rock1, Ikbkb, Fzd8, Plcb4, Dkk3, Nfatc2, Sfrp1, Wnt5b
Gαq signaling[b]	Proliferation, cell migration, vasoconstriction	0.026	0.000	Could not be predicted	Rock1, Ikbkb, Plcb4, Akt2, Pld3, Agtr1b, Itpr3, Nfatc2, Nfatc4, Avpr1a, Rhoh, Nfatc1

[a] *Z-score and predicted functional status in c-Kit deficient smooth muscle cells compared to wild type cells. The z-score measures how well the gene expression data matches the experimentally observed direction of pathway regulation in the literature. A positive z-score predicts activation, while a negative z-score indicates inhibition. N.D., could not be determined.*
[b] *NF-κB associated signaling pathway*

1

Figure 1

Figure 1. Loss of c-Kit function accounts for significant gene expression differences between c-Kit deficient and wild type smooth muscle cells (SMC).

A) Venn diagram indicating the numbers of differentially upregulated genes in primary SMC isolated from c-Kit deficient (blue; $Kit^{W/W-v}$) and littermate control mice (red; $Kit^{+/+}$) as determined by microarray analysis. The group of genes in the interception (black area) did not show statistically significant differences by *t-test* between the two strains (n=3 per group). **B)** Heat map of differentially expressed genes in primary SMC from c-Kit deficient and littermate control mice. **C)** Expression of NF-κB related genes in c-Kit deficient vs. control SMC as determined by real-time PCR. Values are shown as fold change over expression in $Kit^{+/+}$ cells; * p<0.05 and ** p<0.01 using a two-tailed *t-test* assuming unequal variance, n=3 per group.

Figure 2

Figure 2. Loss of c-Kit function in primary smooth muscle cells (SMC) is associated with increased NF-κB activity.

A) NF-κB transcriptional activity in c-Kit deficient (Kit$^{W/W-v}$), control (Kit$^{+/+}$), and c-Kit rescued SMC (Kit^{R}) after 24-hour treatment with POVPC (50 μg/ml), as determined by dual-luciferase reporter assay. Transcriptional activity is represented as the mean ± standard deviation (SD) of the Firefly/Renilla luciferase ratio normalized with respect to the control group (Kit$^{+/+}$) (n=3 independent experiments). **B)** Phosphorylated (pS536) protein levels of the NF-κB p65 subunit in POVPC-treated c-Kit deficient, control, and c-Kit rescued SMC as determined by ELISA. Values are expressed as the mean ± SD of the p-p65/total p65 ratio normalized with respect to the control group (Kit$^{+/+}$) (n=3 independent experiments). **C)** Protein expression of the NF-κB related pro-inflammatory mediators MMP-2 and MCP-1 in POVPC-treated c-Kit deficient, control, and c-Kit rescued SMC as determined by Western blot. Molecular weight markers are shown on the right side of the gel. Protein expression is expressed as the mean ± SD of the MMP-2/β-actin and MCP-1/β-actin signal ratios normalized with respect to the control group (Kit$^{+/+}$) (n=3 per cell type). $* p<0.05$ and $** p<0.01$ using a one-way ANOVA followed by a Newman-Keuls test.
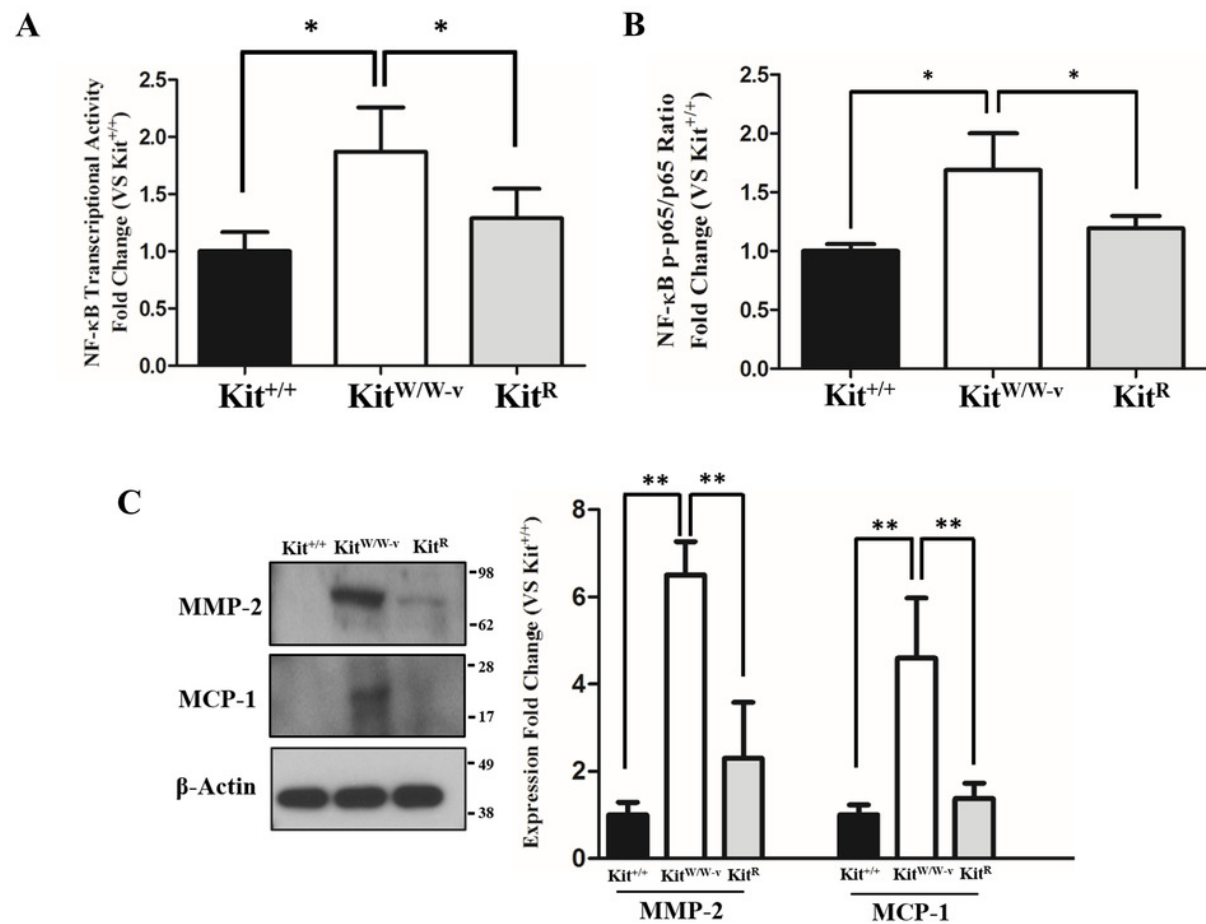
Figure 3

Figure 3. c-Kit inhibits NF-κB activity in smooth muscle cells (SMC) through the actions of TAK1 and NLK.

A) Protein expression of the TAK1 and NLK regulatory proteins in c-Kit deficient (Kit$^{W/W-v}$), control (Kit$^{+/+}$), and c-Kit rescued SMC (Kit^{R}) as determined by Western blot. Protein expression is expressed as the mean ± standard deviation (SD) of the TAK1/β-actin and NLK/β-actin signal ratios normalized with respect to the control group (Kit$^{+/+}$) (n=3 per cell type). **B-C)** Protein expression of TAK1 **(B)** and NLK **(C)** in Kit$^{+/+}$ cells transduced with lentivirus-encoded siRNAs of the corresponding targets or GFP control. Protein expression is expressed as the mean ± SD of the TAK1/β-actin and NLK/β-actin signal ratios normalized with respect to the siGFP-treated group (n=3 independent experiments). **D)** NF-κB transcriptional activity in Kit$^{+/+}$ SMC transduced with lentivirus-encoded siRNAs complementary to TAK1, NLK, or GFP after 24-hour treatment with POVPC (50 μg/ml), as determined by dual-luciferase assay. Transcriptional activity is represented as the mean ± SD of the Firefly/Renilla luciferase ratio normalized with respect to the siGFP-treated group (n=3 independent experiments). **E)** Phosphorylated (pS536) protein levels of NF-κB p65 in POVPC-treated Kit$^{+/+}$ SMC transduced with lentivirus-encoded siRNAs as determined by ELISA. Values are expressed as the mean ± SD of the p-p65/total p65 ratio normalized with respect to the siGFP-treated group (n=3 independent experiments). **F)** Protein expression of the pro-inflammatory mediators MMP-2 and MCP-1 in POVPC-treated Kit$^{+/+}$ SMC transduced with lentivirus-encoded siRNAs as determined by Western blot. Protein expression is expressed as the mean ± SD of the MMP-2/β-actin and MCP-1/β-actin signal ratios normalized with respect to the siGFP-treated group (n=3 independent experiments). Molecular weight markers are shown on the right side of the gels. * $p<0.05$ and ** $p<0.01$ using a one-way ANOVA followed by a Newman-Keuls test.
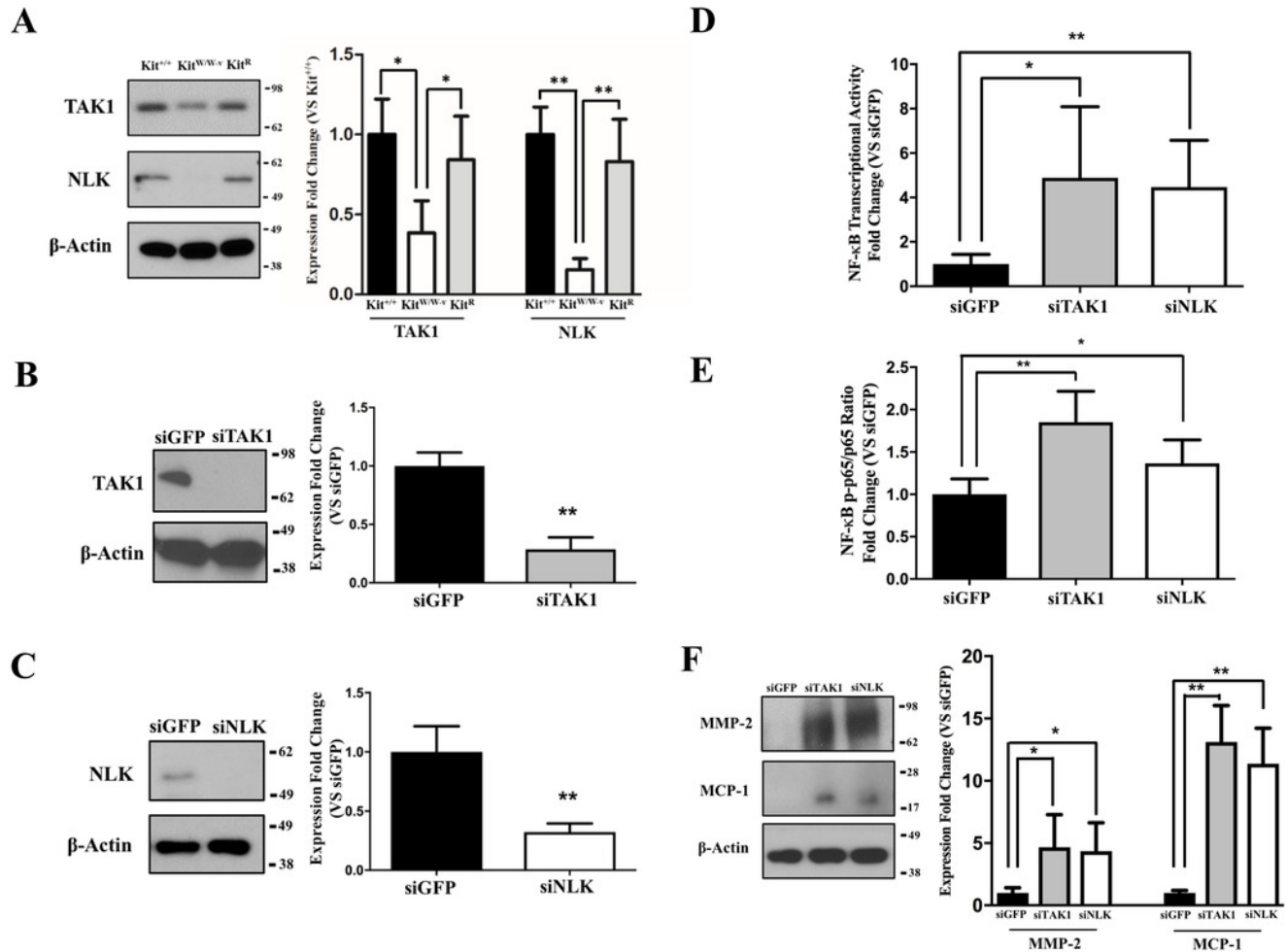
Figure 4

Figure 4. c-Kit forms a molecular complex with the regulatory proteins TAK1, Src, and NLK in smooth muscle cells (SMC).

A) Diagram illustrating the proposed molecular complex between c-Kit, TAK1, Src, and NLK in SMC and their inhibitory function on NF-κB transcriptional activity. **B-C)** Co-immunoprecipitation experiments in control (Kit$^{+/+}$) and c-Kit deficient (Kit$^{W/W-v}$) SMC using anti-c-Kit **(B)** and anti-TAK1 antibodies **(C)** to pull down protein complexes. Molecular weight markers are shown on the right side of the gels, while antibodies used to detect eluted proteins are indicated on the left. IP, immunoprecipitation; IB, immunoblot.