Myocardial KRAS$^{G12D}$ expression does not cause cardiomyopathy in mice

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Aims
Germ-line mutations in genes encoding components of the RAS/mitogen-activated protein kinase (MAPK) pathway cause developmental disorders called RASopathies. Hypertrophic cardiomyopathy (HCM) is the most common myocardial pathology and a leading cause of death in RASopathy patients. KRAS mutations are found in Noonan and cardio-facio-cutaneous syndromes. KRAS mutations, unlike mutations of RAF1 and HRAS, are rarely associated with HCM. This has been attributed to the fact that germ-line KRAS mutations cause only a moderate up-regulation of the MAPK pathway. Highly bioactive KRAS mutations have been hypothesized to cause severe cardiomyopathy incompatible with life. The aim of this study was to define the impact of KRAS$^{G12D}$ expression in the heart.

Methods and results
To generate mice with endogenous cardiomyocyte-specific KRAS$^{G12D}$ expression (cKRAS$^{G12D}$ mice), we bred mice with a Cre-inducible allele expressing KRAS$^{G12D}$ from its endogenous promoter (Kras2LSL) to mice expressing Cre under control of the cardiomyocyte-specific $\alpha$-myosin heavy chain promoter ($\alpha$MHC-Cre). cKRAS$^{G12D}$ mice showed high levels of myocardial ERK and AKT signalling. However, surprisingly, cKRAS$^{G12D}$ mice were born in Mendelian ratios, appeared healthy, and had normal function, size, and histology of the heart.

Conclusion
Mice with cardiomyocyte-specific KRAS$^{G12D}$ expression do not develop heart pathology. These results challenge the view that the level of MAPK activation correlates with the severity of HCM in RASopathies and suggests that MAPK-independent strategies may be of interest in the development of new treatments for these syndromes.

Keywords
KRAS • RASopathies • Noonan syndrome • Cardio-facio-cutaneous syndrome • Cardiomyopathy

1. Introduction
Germ-line mutations in KRAS, HRAS, RAF1, BRAF, and PTPN11 increase the activity of the RAS/mitogen-activated protein kinase (MAPK) pathway and lead to developmental syndromes called RASopathies.$^1$ RASopathies, including Noonan, Costello, and cardio-facio-cutaneous syndromes, are characterized by facial dysmorphism, reduced growth, cognitive deficits, skeletal defects, and different types of heart disorders.$^2$ The most common heart disorder is hypertrophic cardiomyopathy (HCM), which is associated with poor prognosis in RASopathy patients.$^3,4$

Although the RASopathy genes encode proteins in the same signalling pathway, some mutations are more often associated with HCM than others. For example, RAF1 mutations cause HCM in patients with Noonan syndrome, and several Raf mutations cause HCM in mice.$^5–10$ Furthermore, mutations in HRAS cause Costello syndrome and lead to HCM in the majority of patients, and mice with germ-line or cardiomyocyte-specific Hras mutations also develop HCM.$^{11–14}$ KRAS mutations, however, found in patients with Noonan and cardio-facio-cutaneous syndromes, are rarely associated with HCM.$^{15–17}$ A potential explanation for why HRAS mutations are associated with HCM to a higher degree than KRAS mutations is that KRAS mutations in RASopathies cause a relatively weak increase in MAPK signalling.$^{15}$ Consistent with this notion, KRAS mutations that cause RASopathies are rarely observed in cancer, whereas RASopathy HRAS mutations are often observed in cancer.$^{11,18}$

Furthermore, cancer-causing KRAS mutations, such as KRAS$^{G12D}$, are believed to be too powerful if they occurred in the germ-line and have been hypothesized to cause severe cardiomyopathy or even heart malignancy.$^{18}$ Indeed, germ-line expression of the KRAS$^{G12D}$
mutation in mice results in cardiomegaly and embryonic lethality.\textsuperscript{19} It is not known, however, whether cardiomegaly is the result of KRAS\textsuperscript{GT12D} expression in cardiomyocytes or in non-cardiac cells. Thus far, the impact of cardiomyocyte-specific KRAS\textsuperscript{GT12D} activation has never been evaluated. To address this issue, we generated mice with cardiomyocyte-specific expression of KRAS\textsuperscript{GT12D} and evaluated MAPK signalling and heart pathology.

2. Methods

2.1 Mouse breeding and genotyping
Mice heterozygous for a conditional oncogenic KRAS allele, Kras2\textsuperscript{LSL} \textsuperscript{19,20}, were bred with mice hemizygous for the α-myosin heavy chain (MHC) Cre transgene,\textsuperscript{21} to produce Kras2\textsuperscript{LSL/αMHC-Cre\textsuperscript{+/-}} mice with the cardiomyocyte-specific expression of KRAS\textsuperscript{GT12D}, here designated cardiomyocyte-KRAS\textsuperscript{GT12D} (cKRAS\textsuperscript{GT12D}) mice. Littermate Kras2\textsuperscript{LSL/αMHC-Cre\textsuperscript{+/-}} mice were used as controls. Mouse experiments were approved by the Research Animal Ethics Committee in Gothenburg (approval number 322-2010) in accordance with the European Parliament directive 2010/63/EU. Mice were killed at 3 or 18 months of age by inhalation with 4% isoflurane followed by excision of the heart. Genotyping was performed by PCR amplification of genomic DNA from tail, heart, liver, and lung tissue of adult mice, and from heart tissue of embryos. The Kras2\textsuperscript{LSL} and the Cre-activated Kras2\textsuperscript{GT12D} alleles were detected as described.\textsuperscript{22} The αMHC-Cre allele was detected with forward primer 5′-ATGACAGACAGATCCCTCCTATCTCC-3′ and reverse primer 5′-CTCATCACTCGTTGCATCATCGAC-3′, yielding a 300 bp fragment.

2.2 Echocardiography
Hair removal gel was applied to the chest of mice 1 day before echocardiography (ECG). ECG examination was performed using a VEVO 770 system with a linear RMV704 transducer (Visualsonics, Inc., Toronto, Canada) on mice anaesthetized with isoflurane inhalation (1.0%); mice were killed at 3 or 18 months of age by inhalation with 4% isoflurane followed by excision of the heart. Genotyping was performed by PCR amplification of genomic DNA from tail, heart, liver, and lung tissue of adult mice, and from heart tissue of embryos. The Kras2\textsuperscript{LSL} and the Cre-activated Kras2\textsuperscript{GT12D} alleles were detected as described.\textsuperscript{22} The αMHC-Cre allele was detected with forward primer 5′-ATGACAGACAGATCCCTCCTATCTCC-3′ and reverse primer 5′-CTCATCACTCGTTGCATCATCGAC-3′, yielding a 300 bp fragment.

2.3 Western blotting
Heart tissue from the left ventricle was homogenized in lysis buffer containing 9 M urea (U0631; Sigma-Aldrich, MO, USA) and complete protease inhibitor cocktail (11836170001; Roche Applied Science, IN, USA), sonicated, and centrifuged for 10 min at 14,000 × g. Protein extracts were size-fractionated on 4–12% polyacrylamide Bis–Tris gels (Nupage, Life Technologies, CA, USA), transferred onto nitrocellulose membranes, and incubated with antibodies to phosphorylated ERK1/2 (4377), total ERK1/2 (9102), phosphorylated AKT (4060), total AKT (9272), Beclin-1 (3495), Atg5 (8540), Atg7 (8558), and LC3A (4599, Cell Signaling Technology, MA, USA). Protein bands were visualized with a secondary antibody IRDye 800CW Goat Anti-Rabbit (Li-Cor, NE, USA) and scanned in the Li-Cor Odyssey Imager with the Odyssey Software, version 3.0 (Li-Cor). Band densities were analysed with ImageJ 1.45S (National Institute of Health, MD, USA).

2.4 Histology and immunohistochemistry
Hearts were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned horizontally 5 mm from the apex, and stained with Masson’s trichrome. For TUNEL and CD31 staining, ventricular wall tissue was frozen in OCT compound (Sakura Finetek, Sweden). Cryosections were stained using ApoTag® Peroxidase In Situ Apoptosis Detection Kit (Merck-Millipore, Germany) or a CD31/Pecam1 antibody (AF3628, R&D Systems, MN, USA). Histology and immunohistochemistry sections were scanned with a Mirax Scanner (Zeiss, Germany), and quantification

Table 1  ECG analysis

<table>
<thead>
<tr>
<th></th>
<th>Rest (n = 6)</th>
<th>Kras2\textsuperscript{GT12D} (n = 6)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td>423 ± 27</td>
<td>413 ± 33</td>
<td>0.82</td>
</tr>
<tr>
<td>SV, μL</td>
<td>35.4 ± 2.3</td>
<td>35.8 ± 3.9</td>
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<td>CO, mL/min</td>
<td>15.1 ± 1.6</td>
<td>14.4 ± 1.2</td>
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</tr>
<tr>
<td>Cl, mL/min/g</td>
<td>0.45 ± 0.05</td>
<td>0.40 ± 0.03</td>
<td>0.39</td>
</tr>
<tr>
<td>LVEDV, μL</td>
<td>66.7 ± 6.3</td>
<td>59.8 ± 7.1</td>
<td>0.48</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>54.1 ± 2.7</td>
<td>60.2 ± 0.8</td>
<td>0.054</td>
</tr>
<tr>
<td>FS, %</td>
<td>38.0 ± 6.3</td>
<td>44.5 ± 5.2</td>
<td>0.45</td>
</tr>
<tr>
<td>LVAWd, mm</td>
<td>0.914 ± 0.06</td>
<td>0.907 ± 0.02</td>
<td>0.91</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>0.817 ± 0.05</td>
<td>0.731 ± 0.05</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Dobutamin (n = 6)</td>
<td>Kras2\textsuperscript{GT12D} (n = 6)</td>
<td>P-value</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>534 ± 18</td>
<td>498 ± 22</td>
<td>0.24</td>
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<tr>
<td>SV, μL</td>
<td>35.7 ± 2.1</td>
<td>38.5 ± 4.4</td>
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<tr>
<td>CO, mL/min</td>
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<td>Cl, mL/min/g</td>
<td>0.57 ± 0.04</td>
<td>0.52 ± 0.03</td>
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<tr>
<td>LVEDV, μL</td>
<td>46.8 ± 2.6</td>
<td>49.5 ± 6.7</td>
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<tr>
<td>LVEF, %</td>
<td>76.4 ± 1.9</td>
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<tr>
<td>FS, %</td>
<td>54.6 ± 4.6</td>
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<tr>
<td>LVAWd, mm</td>
<td>0.963 ± 0.08</td>
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<tr>
<td>LVPWd, mm</td>
<td>0.773 ± 0.08</td>
<td>0.822 ± 0.05</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. P-values are from Student’s t-test (unpaired, two-tailed).

HR, heart rate; bpm, beats per minute; SV, stroke volume; CO, cardiac output; Cl, cardiac index; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; FS, fractional shortening; LVAWd, left ventricle anterior wall diameter during diastole; LVPWd, left ventricle posterior wall diameter during diastole.
of heart size, cardiomyocyte cross-sectional area, fibrosis and capillary density were done with the BioPix iQ 2.1.8 software (Biopix AB, Sweden).

2.5 Statistical analysis
The values are mean ± SEM. Differences between groups were analysed with Student’s t-test (unpaired, two-tailed) and considered significant when $P < 0.05$.

3. Results
3.1 Cardiomyocyte-specific KRAS$^{G12D}$ expression does not affect heart size and contractility

The $Kras2^{LSL/+}$aMHC-Cre$^{+/+}$ (cKRAS$^{G12D}$) mice were born at Mendelian ratios and developed no visible signs of disease. ECG parameters were similar in 12-month-old $Kras2^{LSL/+}$aMHC-Cre$^{+/+}$ (cKRAS$^{G12D}$) males and littermate $Kras2^{+/+}$aMHC-Cre$^{+/+}$ (control) mice. The response of hearts to dobutamine-induced stress was also similar in the two groups of mice, suggesting that the cardiac reserve was unaffected in cKRAS$^{G12D}$ mice (Table 1).

3.2 Expression of KRAS$^{G12D}$ is heart specific and starts late in embryonic development

To confirm the tissue specificity of KRAS$^{G12D}$ expression, DNA was extracted from heart, lung, and liver tissue of cKRAS$^{G12D}$ and control mice. As expected, the unrecombined $Kras2^{LSL}$ allele was found in all tissues of cKRAS$^{G12D}$ mice, but in none of control mouse tissues. The recombined $Kras2^{G12D}$ allele in adult cKRAS$^{G12D}$ mice was readily detected in DNA from heart tissue, but was undetectable in the lung and liver (Figure 2A). Analyses of DNA from hearts of developing embryos revealed that the recombined $Kras2^{G12D}$ allele was detected at embryonic day 20, but not at embryonic days 12–18 (Figure 2B). Thus, KRAS$^{G12D}$ expression in cKRAS$^{G12D}$ mice is heart specific and begins at embryonic day 19 or 20.

To confirm bioactivity of the inducible $Kras2^{LSL}$ allele in this strain of mice, we administered a Cre-adenovirus by inhalation to lungs of $Kras2^{LSL}$ mice as described earlier. As expected, 8 weeks after Cre-adenovirus administration, $Kras2^{LSL}$ mice exhibited advanced lung tumour lesions, demonstrating that the allele produces bioactive KRAS$^{G12D}$ (see Supplementary material online, Figure S2).
3.3 Hyperactivation of MAPK signalling pathways in the myocardium of cKRAS<sup>G12D</sup> mice

To test whether signalling downstream of KRAS was affected in hearts of cKRAS<sup>G12D</sup> mice, we performed western blots of lysates of left ventricular tissue. The levels of phospho-ERK1/2 and phospho-AKT were higher in cKRAS<sup>G12D</sup> hearts than in control hearts at 18 months of age (Figure 2C and D), indicating a significant hyperactivity of two important pathways downstream of KRAS. Similar results were observed in hearts of 3-month-old mice (Figure 2C and D), suggesting that cKRAS<sup>G12D</sup> mice exhibit life-long hyperactivation of MAPK signalling in the myocardium.

3.4 Myocardial KRAS<sup>G12D</sup> expression does not induce heart fibrosis and cardiomyocyte hypertrophy, and does not affect capillary density and protein degradation pathways

Although heart function in cKRAS<sup>G12D</sup> mice was unaffected, we argued that the increased MAPK signalling might induce subclinical levels of interstitial fibrosis, cardiomyocyte hypertrophy, or apoptosis—well-known signs of myocardial pathology in humans and mice. However, this was not the case. Analyses of Masson’s trichrome-stained left ventricle sections revealed similar low levels of fibrosis in cKRAS<sup>G12D</sup> and control mice (Figure 3A). Cardiomyocyte diameter was
also unaffected in cKRASG12D mice, ruling out cell hypertrophy, and levels
of apoptosis, evaluated with TUNEL staining, were similar in the two
groups of mice (Figure 3B and C). Based on previous studies,24 we hypothe-
sized that the increased phospho-AKT levels in hearts of cKRASG12D mice
might reduce myocardial capillary density. However, capillary density was
similar in cKRASG12D and control hearts at 18 months of age, as assessed
by quantification of CD31 immunostaining (Figure 3D).

A potential explanation for the absence of hypertrophy in
cKRASG12D hearts is that activation of protein degradation pathways,
such as ubiquitination or autophagy, counteracts the hypertrophic
signalling. However, mRNA levels of the two main cardiomyocyte ubi-
quitin ligases Atrogin1 and MuRF1 and protein levels of the well-
established autophagy markers Beclin-1, Atg5, Atg7, and LC3A were
similar in cKRASG12D and control hearts (Figure 4A and B).

Figure 3 Cardiomyocyte-specific expression of KRASG12D does not affect myocardial fibrosis, cardiomyocyte diameter, cardiomyocyte apoptosis, or
myocardial capillary density in mice. (A–D) Representative photographs (left) and quantification (right, n = 6/genotype) of myocardial sections from
control and cKRASG12D mice sacrificed at 18 months of age. (A) Masson's trichrome (collagen) staining showing low levels of myocardial fibrosis in
control and cKRASG12D mice. (B) Masson's trichrome staining showing similar cardiomyocyte size in control and cKRASG12D mice (scale bar = 20 μm). (C) TUNEL staining showing low levels of apoptosis control and cKRASG12D mice. Negative and positive controls are lung and thymus
from a wild-type mouse sacrificed at 3 weeks of age. (D) CD31 staining (brown) showing similar capillary density in the myocardium of control and
cKRASG12D mice (scale bar = 30 μm).
two studies. Firstly, KRASG12D expression was turned on early during late embryonic development and throughout life. Secondly, KRASG12D was expressed in all tissues of the embryos in Tuveson’s study and only in cardiomyocytes in the current study. Consequently, we cannot rule out the possibility that the cardiomegaly in embryos with widespread KRASG12D expression is caused by KRASG12D expression in cell types other than cardiomyocytes, such as heart fibroblasts or endothelial cells.

The basal levels of phospho-ERK1/2 were increased in the myocardium of cKRASG12D mice. Previously published RASopathy mouse models have not exhibited increased myocardial phospho-ERK1/2 levels under basal conditions. There is uncertainty in the literature as to whether increased MAPK activation causes HCM in RASopathies or if it is mediated by other mutation-specific mechanisms. Our data clearly demonstrate that life-long hyperactivation of the MAPK pathway in cardiomyocytes does not cause HCM in RASopathy patients, which has been suggested earlier. An alternative explanation is that highly bioactive mutations, such as KRASG12D, stimulate myocardial defence mechanisms, making them less harmful than moderately activating mutations. Two of the most important defence mechanisms that suppress pathological cardiomyocyte hypertrophy are ubiquitination and autophagy, leading to protein degradation. Markers of ubiquitination and autophagy were unaffected in cKRASG12D hearts, suggesting that the expression of KRASG12D does not activate known hypertrophy defence mechanisms in the heart.

The cKRASG12D mice also exhibited increased levels of myocardial phospho-AKT. AKT signalling in myocardial biology is complex. On one hand, increased AKT signalling promotes survival of cardiomyocytes and is thereby cardioprotective in mouse models of pressure-overload-induced heart failure and myocardial infarction. On the other hand, overexpressing AKT in cardiomyocytes results in hypertrophy and heart failure in mice. Our data demonstrate that life-long activation of AKT signalling by expression of physiological levels of KRASG12D is harmless.

Expression of KRASG12D from the endogenous promoter in mouse embryonic fibroblasts leads to reduced levels of phospho-ERK and -AKT compared with wild-type MEFs. Here, we found significantly increased levels of phospho-ERK and -AKT in KRASG12D-expressing cardiomyocytes, demonstrating that the impact of endogenous KRASG12D on downstream signalling is cell type-specific.

There were no signs of heart malignancy in the cKRASG12D mice, as assessed by macroscopic examination, histology, and ECG. KRAS mutations have been found in patients with cardiac rhabdomyosarcoma, a rare form of heart malignancy. The likeliest explanation for the absence of malignancy in cKRASG12D mice is that whereas endogenous KRASG12D is sufficient to initiate tumours in many different organs in the mouse, additional genetic events are required for heart tumour formation.

We conclude that mouse hearts are resistant to cardiomyocyte-specific expression of KRASG12D despite hyperactivation of downstream ERK and AKT signalling. A clinical implication of this surprising result is that treatment of HCM in RASopathies should not only focus on targeting MAPK signalling, but also on identifying other mutation-specific downstream effects.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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## Conflict of interest

None declared.
Resistance of mice hearts to KRASG12D expression

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