Krueppel-like factor 15 regulates Wnt/β-catenin transcription and controls cardiac progenitor cell fate in the postnatal heart

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Wnt/β-catenin signalling controls adult heart remodelling in part via regulation of cardiac progenitor cell (CPC) differentiation. An enhanced understanding of mechanisms controlling CPC biology might facilitate the development of new therapeutic strategies in heart failure. We identified and characterized a novel cardiac interaction between Krueppel-like factor 15 and components of the Wnt/β-catenin pathway leading to inhibition of transcription. In vitro mutation, reporter assays and co-localization analyses revealed that KLF15 requires both the C-terminus, necessary for nuclear localization, and a minimal N-terminal regulatory region to inhibit transcription. In line with this, functional Klf15 knockout mice exhibited cardiac β-catenin transcriptional activation along with functional cardiac deterioration in normal homeostasis and upon hypertrophy. We further provide in vivo and in vitro evidences for preferential endothelial lineage differentiation of CPCs upon KLF15 deletion. Via inhibition of β-catenin transcription, KLF15 controls CPC homeostasis in the adult heart similar to embryonic cardiogenesis. This knowledge may provide a tool for reactivation of this apparently dormant CPC population in the adult heart and thus be an attractive approach to enhance endogenous cardiac repair.

INTRODUCTION

A couple of studies have shown the capacity of adult mammalian heart cells to proliferate especially after injury (Beltrami et al., 2001; Bergmann et al., 2009; Hsieh et al., 2007; Kajstura et al., 2004). Specific groups of putative cardiac progenitor cell (CPC) populations have been identified and characterized including c-kit (Beltrami et al., 2003), Islet-1 (Laugwitz et al., 2005; Moretti et al., 2006; Wang et al., 2006), Sca1 (Oh et al., 2003), side population cells (Pfister et al., 2005, 2010) and cardiac neural crest-derived cells (Tomita et al., 2005). However, some studies showed insignificant rate of cardiac proliferation (Rubart & Field, 2006). Nonetheless, the extent to which these cells contribute to tissue homeostasis and/or repair as well as the signalling pathways controlling their proliferation and differentiation remains unclear. This scenario leads to a lack of robust endogenous cardiac regeneration to replace the infarcted myocardium. Therefore, understanding the mechanisms that control progenitor cells during normal cardiac homeostasis and repair is the basis for developing new therapeutic strategies to activate these cells for effective regeneration.

The notion that genes involved in early cardiogenesis may be re-employed to protect or regenerate heart muscle has motivated efforts to understand early developmental pathways (Epstein & Parmacek, 2005; Oka et al., 2007). One important factor regulating progenitor cell biology during early cardiogenesis is the canonical Wnt/β-catenin pathway. Active Wnt/β-catenin...
signalling induces the stabilization of cytosolic β-catenin, which then associates with lymphoid enhancer factor and T-cell factor, such as transcription factor 7-like 2 (TCF7L2 or TCF4) to form a transcription complex that activates Wnt target genes. This cascade is subject to multiple levels of negative control where, among others, the Nemo-like kinase (NLK) play an important role by interacting with and phosphorylating TCF to repress β-catenin/TCF-mediated transcription (Ishitani et al, 2003). Wnt/β-catenin signalling controls maintenance, proliferation and differentiation of embryonic cardiovascular progenitors in a multi-phasic fashion. Initially, Wnt/β-catenin pathway has a positive effect on embryonic mesodermal cell activation and proliferation. Subsequent inactivation is required for proper differentiation into functional cardiomyocytes while default activation leads the cells to a haemangioblast lineage (Gessert & Kuhl, 2010; Naito et al, 2006; Ueno, 2007). Interestingly, gene expression profiling analysis indicated a participation of Wnt signalling also in pathological myocardial remodelling (LaFramboise et al, 2005; Toischer et al, 2010). We previously demonstrated that cardiac β-catenin downregulation is required for adaptive hypertrophy and enhances endogenous CPC cardiomyogenic differentiation after infarction (Baurand et al, 2007; Zelarayan et al, 2008). Collectively, the available data argues strongly for a pivotal role of Wnt/β-catenin signalling in embryogenic and postnatal heart.

Krueppel-like factors (KLF) are a large family of zinc finger-containing transcription factors involved in diverse arrays of cellular processes including regulation of cell differentiation, cardiac remodelling, haematopoiesis, angiogenesis and stem cell fate determination by interacting with co-activators and corepressors (Kaczynski et al, 2003). Recent studies revealed the important role of KLF15 as a central regulator of stress response and repressor of pathological cardiac hypertrophy and fibrosis by controlling factors such as GATA binding protein 4 (GATA4), myocyte enhancer factor-2 (MEF2), myocardin (Fisch et al, 2007; Leenders et al, 2010) and the transforming growth factor β (TGFβ) (Wang et al, 2008), respectively. However, the role of KLF15 in stem cell biology of the adult heart remains unexplored.

Here, we report a novel interaction between KLF15 and components of the Wnt pathway resulting in an inhibition of β-catenin/TCF-transcriptional activity in cardiac cells. The biological role of this interaction was investigated in vitro and in vivo in the postnatal hearts of mice with a global KLF15 functional deletion. These mice exhibited a cardiac β-catenin/TCF-transcriptional de-repression and cardiac dysfunction. Deletion of KLF15 results in a constitutive β-catenin transcriptional activation that directs the CPCs to an endothelial phenotype. Collectively, our data underscore the relevance of KLF15 and the Wnt/β-catenin pathway for cardiac cellular homeostasis.

RESULTS

KLF15 interacts with β-catenin, NLK and TCF4 in cardiac cells

Based on previous observations concerning β-catenin and its beneficial role in cardiac remodelling, we sought to identify new β-catenin interaction partners by means of a yeast-two-hybrid screen. We identified and characterized a specific interaction between β-catenin and a member of the Krueppel-like transcription factor family, KLF15. To validate this interaction, we initially co-transfected the human embryonic cells HEK293 with cmyc-β-catenin and Flag-KLF15-full-length plasmids. β-catenin was immunoprecipitated with an anti-c-myc antibody and KLF15 detection was performed with an anti-Flag antibody. A clear band in the KLF15 expected size was detected, thus, confirming the interaction between KLF15 and β-catenin (Fig 1A, lane 3). Moreover, we verified the endogenous interaction of these proteins using antibodies against β-catenin and KLF15 in adult mouse cardiac tissue (Fig 1A, lane 8). To identify the binding domain, HEK293 were co-transfected with cmyc-β-catenin and N-terminal mutants lacking the first 45, 152 and 260 amino acids (ΔN45, ΔN152, ΔN260) or a C-terminal truncated KLF15 (-ΔC) plasmid. Co-immunoprecipitation revealed that deletion of the 260 N-terminal amino acids in the KLF15 sequence abrogated the β-catenin binding (Fig 1A, lane 4–6), while lack of the KLF15 C-terminus did not play a role (Fig 1A, lane 7).

Next, we investigated the potential interaction of additional factors among the Wnt/β-catenin components. By co-immunoprecipitation we found that the NLK, a known inhibitor of the β-catenin-LEF/TCF complex (Ishitani et al, 1999; Yamada et al, 2006), interacted with KLF15. HEK293 cell lysates transfected with Flag-KLF15-full-length and haemagglutinin (HA)-NLK-full-length were immunoprecipitated with an anti-HA antibody and KLF15 detection was performed with an anti-Flag antibody. The endogenous interaction was further confirmed in adult mouse cardiac cells (Fig 1B, lane 3 and 8). Flag-KLF15-ΔN152 and HA-NLK-full-length were transfected in HEK293 cells to identify binding domains. Co-immunoprecipitation revealed that KLF15-ΔN45 and ΔC but not the KLF15-ΔN152 and ΔN260 interacted with NLK (Fig 1B, line 4–7).

Since NLK interacts with and phosphorylates TCF to repress β-catenin/TCF-mediated transcription (Ishitani et al, 2003), we tested KLF15 and TCF4 interaction. HEK293 cells were co-transfected with a HA-TCF4 and Flag-KLF15-full-length. TCF4 was immunoprecipitated with an anti-HA antibody and KLF15 was detected using an anti-Flag antibody. This analysis showed interaction of KLF15 with TCF4, which was further confirmed endogenously in adult mouse cardiac cells (Fig 1C, line 3 and 8). TCF4 interaction with KLF15-ΔN45, -ΔN152, -ΔN260 and -ΔC mutants was tested in HEK293 cells. Co-immunoprecipitation showed interaction of TCF4 with all the N-terminal KLF15 mutants but not with the KLF15-ΔC (Fig 1C, line 4–7). As positive controls β-catenin/TCF4 and β-catenin/NLK known interactions were confirmed in HEK293 cells (Supporting Information Fig S1A). The corresponding controls for the KLF15 antibody used for endogenous detection are provided in Supporting Information Fig S1B.

Altogether these data show the association of KLF15 to the β-catenin/TCF complex, where the first 45 amino acids of KLF15 are essential for binding β-catenin, amino acids 45–152 for binding NLK and the C-terminal region for TCF4.
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KLF15 requires the C-terminal domain for nuclear localization

To localize the KLF15/β-catenin and KLF15/NLK interactions, we detected the endogenous expression of these proteins in neonatal rat cardiomyocytes (NRC) by immunofluorescence. We observed a co-localization of β-catenin and KLF15 as well as NLK and KLF15 in the nucleus (Fig 2A) suggesting the role of KLF15 as transcriptional regulator. NRC were co-transfected with both β-catenin and either Flag-KLF15-full-length or the indicated mutants. Immunofluorescence analysis showed co-localization of the KLF15-full-length as well as the N-terminal mutants of KLF15 with β-catenin in the nucleus, while the KLF15 construct lacking the C-terminal domain was exclusively found in the cytoplasm (Fig 2A). Immunoblot of enriched-cell fractions confirmed the nuclear localization of KLF15-ΔN mutants and the cytosolic localization of KLF15-ΔC (Fig 2B) suggesting that the C-terminal domain responsible for TCF binding is important for nuclear translocation.

The N-terminal KLF15 domain mediates inhibition of β-catenin/TCF4 transcriptional activity

We further investigated the role of these interactions in β-catenin signalling in NRC and HEK293 employing the pTOPflash reporter plasmid, which is activated when β-catenin binds to LEF/TCF elements driving firefly luciferase expression. KLF15 expression suppressed endogenous β-catenin-induced luciferase activity in NRC (p < 0.001, Fig 3A) and in HEK293 cells (Supporting Information Fig S1B). Furthermore, co-transfection of a stabilized form of β-catenin (β-cat-ΔN) was used to increase reporter activity. KLF15 expression was able to suppress the β-cat-ΔN-induced luciferase in NRC (p < 0.001, Fig 3A) and in HEK293 cells (Supporting Information Fig S1B) in a concentration-dependent manner. Next, we tested the effect of KLF15 on TCF-mediated signalling in SW480 cells, a tumour cell line that has constitutive active transcriptional β-catenin/TCF activity. Likewise, KLF15 repressed the endogenous and TCF4-induced luciferase expression in SW480 cells (p < 0.001, Fig 3B).

Figure 1. KLF15 interacts with β-catenin, NLK and TCF4. The transfected constructs in A, B and C were detected in the protein lysates. IgG isotype (IP-CT) (lane 1) and empty vector (EV) (lane 2) were used as controls; GAPDH served as loading control. (A, B and C) Endogenous validation of the identified interactions between KLF15 and β-catenin, NLK as well as TCF4 in adult cardiac tissue are depicted (lane 8). Lane 9 shows the IgG isotype control for IP. Endogenous detection of KLF15, β-catenin, NLK as well as TCF4 in the different lysates is shown. (*) IgG heavy chain (50 kDa).

A. Co-immunoprecipitation (IP) analysis showing interaction of KLF15 and β-catenin in HEK293 cells overexpressing cmyc-β-catenin and Flag-KLF15-full-length (lane 3). Co-transfection of truncated (ΔN45, ΔN152, ΔN260)-KLF15 (lane 4–6) or a ΔC-KLF15 (lane 7) and cmyc-β-catenin plasmids in HEK293 cells showing the requirement of the first 45 amino acids of KLF15 for binding β-catenin.

B. Interaction of NLK with KLF15-full-length, -ΔN45 (lane 3 and 4) and -ΔC (lane 7) were shown by co-IP assays in HEK293.

C. TCF4 interacted with KLF15-full-length and all N-terminal mutants (lane 3–6), but not with the -ΔC construct (lane 7) in HEK293.
B. Immunoblots of enriched cell fractions confirming the subcellular localization of KLF15-degradation.

These findings show that KLF15 promotes TCF4 proteasomal ubiquitination in cells expressing TCF4 alone was comparable to KLF15/TCF4 and NLK/TCF4 expressing cells. In contrast, we observed comparable increased TCF4 ubiquitination in both lysates and detected with an anti-ubiquitin antibody. We hypothesized that NLK and KLF15 affects TCF stability. We tested ubiquitination of TCF4 upon KLF15 overexpression in SW480 and HEK293 cells. NLK co-expression was used as a positive control (Ishitani et al, 1999). HA-TCF4 was immunoprecipitated from cytosolic lysates and detected with an anti-ubiquitin antibody. We observed increased TCF4 ubiquitination upon KLF15 overexpression in HEK293 cells. NLK co-expression was used as a positive control since NLK was shown to target TCF4 for ubiquitination (Ishitani et al, 1999). HA-TCF4 was immunoprecipitated from cytosolic lysates and detected with an anti-ubiquitin antibody. We observed increased TCF4 ubiquitination upon KLF15 overexpression in HEK293 cells. NLK co-expression was used as a positive control since NLK was shown to target TCF4 for ubiquitination (Ishitani et al, 1999).

Analysis of the different KLF15 mutants on β-catenin/TCF transcription showed that only KLF15-ΔN45 was able to repress β-catenin/TCF-induced luciferase in contrast to mutants lacking longer N-terminal regions as well as the C-terminus in NRC, SW480 and HEK293 (Fig 3A and B and Supporting Information Fig S1C). Thus, the required domain for β-catenin/TCF-transcriptional repression seems to be localized in the N-terminal fragment excluding the first 45 amino acids of the KLF15 protein. Our observations reveal that KLF15 requires both, a minimal N-terminal domain for binding β-catenin and NLK as well as a C-terminal domain for TCF binding and nuclear translocation to achieve β-catenin/TCF transcriptional repression (summarized in Fig 3E).

KLF15 inhibition on β-catenin-LEF/TCF-transcription did not affect β-catenin localization or protein levels (Fig 3C), therefore, we hypothesized that NLK and KLF15 affects TCF stability. We tested ubiquitination of TCF4 upon KLF15 overexpression in HEK293 cells. NLK co-expression was used as a positive control since NLK was shown to target TCF4 for ubiquitination (Ishitani et al, 1999). HA-TCF4 was immunoprecipitated from cytosolic lysates and detected with an anti-ubiquitin antibody. We observed comparable increased TCF4 ubiquitination in both KLF15/TCF4 and NLK/TCF4 expressing cells. In contrast, ubiquitination in cells expressing TCF4 alone was comparable with cells transfected with the empty vector (EV; Fig 3D). These findings show that KLF15 promotes TCF4 proteasomal degradation.

KLF15 regulates Wnt/β-catenin activation in cardiac tissue in vivo

Since we found that KLF15 regulates β-catenin-dependent transcription in vitro, we tested the hypothesis that this regulation takes place in the adult heart. Therefore, we analyzed a Klf15 functional knock-out mouse model (Klf15 KO), carrying an out-of-frame insertion of a partial lacZ cassette replacing exon 2 of the KLF15 coding region (Supporting Information Fig S2A). Klf15 KO mice were viable and fertile and showed no apparent defects at baseline. Quantitative real time (qRT)-PCR analysis of cardiac tissue demonstrated no Klf15 mRNA expression in Klf15 KO mice (Supporting Information Fig S2B). Histological analysis of liver, kidney, and lung up to 6 months of age showed no apparent morphological defects (Supporting Information Fig S2C). KLF15 exerted inhibition of β-catenin transcription, thus an opposite effect was expected upon KLF15 deletion. Indeed, analysis of cardiac tissue of 16-week-old mice revealed that β-catenin expression remained unchanged, but its target genes Tcf4 and cMyc were significantly increased at the mRNA (p < 0.05; Fig 4A) and protein level (Fig 4B) in Klf15 KO versus wild-type (WT) mice. Endogenous ubiquitination of TCF4 was significantly decreased in Klf15 KO (Supporting information Fig S3) complementing the in vitro finding showing increased TCF4 ubiquitination upon KLF15 overexpression. These observations show an in vivo cardiac de-repression of β-catenin/TCF-transcriptional activity in absence of KLF15.
Analysis of cardiac function assessed by ejection fraction (EF) and fractional shortening (FS) showed significant reduction in left ventricular (LV) function at 16 weeks of age in Klf15 KO compared to WT at baseline (p < 0.05; Fig 4C). Neither wall thickness nor end-diastolic dimension of the LV (LVEDd) or cardiac mass showed significant differences between Klf15 KO and WT mice (Supporting Information Table S1A). Since β-catenin/TCF-transcriptional activity was increased in the adult heart of Klf15 KO mice, we were interested in the cardiac phenotype of mice exhibiting a direct cardiac β-catenin upregulation. We previously described a mouse model with cardiac inducible β-catenin stabilization (β-cat<sup>lox<sup>3</sup></sup>) showing worse cardiac function compared with controls after AngiotensinII (AngII)-induced hypertrophy (Baurand et al., 2007). Now, we analyzed cardiac function of β-cat<sup>lox<sup>3</sup></sup> mice 16 weeks following induction of β-catenin stabilization. Similar to the functional phenotype observed in Klf15 KO mice, β-cat<sup>lox<sup>3</sup></sup> mice showed a significant decrease in EF and FS (Fig 4D). No
differences were observed in wall thickness and/or cardiac mass between β-cateninKO and control mice (Supporting Information Table S1B). These observations suggest that KLF15-dependent regulation of β-catenin-dependent transcription is important to maintain normal cardiac homeostasis.

To discern the cause of the observed phenotype we investigated the expression of foetal genes A-type natriuretic peptide (ANP), B-type natriuretic peptide (BNP), GATA4 and connective tissue growth factor (CTGF) by qRT-PCR since it was shown that KLF15 regulates hypertrophic signalling and fibrosis (Fisch et al, 2007; Wang et al, 2008). No significant differences were observed between KO and WT mice with respect to cardiac hypertrophy, fibrosis as well as apoptosis (Supporting Information Fig S4A-D). Our data strongly suggest that regulation of hypertrophy and fibrosis via KLF15 occurs exclusively upon cardiac stress and are not the explanation for baseline cardiac hypertrophy and fibrosis.

KLF15 controls cardiac progenitor cell fate in the adult murine heart

Since we previously demonstrated that downregulation of β-catenin enhanced differentiation of Sca1-derived cardiomyogenic progenitor cells post-infarction (Zelarayan et al, 2008), we tested the hypothesis that KLF15-dependent regulation of β-catenin transcription affects CPC homeostasis. Expression of KLF15 was confirmed in the total CPCs as well as in the Sca1POS sub-population as demonstrated by qRT-PCR and Western blot (Fig 5A and B). Whole heart lysate was used as control. The non-cardiomyocyte cell fraction containing CPCs isolated from 16-week-old KLF15 KO and WT were analyzed by flow cytometry. The cell-cycle analysis showed a decreased cell number in G0/G1 resting phase and an increased cell number of cells in G2/M phase indicative of an increased number of dividing progenitors in KLF15 KO mice (p < 0.05; Fig 5C). Furthermore, we investigated specific CPCs sub-populations previously described in the adult heart. The total number of Sca1POS and c-kitPOS sub-populations were significantly increased in KO mice (p < 0.001 and <0.05; Fig 5D), although it is important to mention that the c-kit population was very low in both WT and KLF15 KO mice. Next, we analyzed these CPC sub-populations in combination with endothelial and cardiomyogenic markers to identify progenitor cells in transition to different lineages (see Supporting Information Fig S5 for controls). Cells co-expressing Sca1 or c-kit and the endothelial cell marker CD31 were significantly increased in KLF15 KO mice (p < 0.001 and <0.05; Fig 5D). In contrast, we observed a significant reduction of Sca1POS/αMHCPOS as well as the Tbx5POS/cTnTPOS cardiomyogenic populations in KLF15 KO in comparison to WT mice (p < 0.001 and <0.05, respectively). No significant changes were detected in the double populations labelled by c-kit and the cardiomyogenic markers αMHC and Hand1 (Fig 5D).

While the total (Ki67POS) and endothelial proliferating cell fraction (Ki67POS/CD31POS) was significantly increased in KLF15 KO mice (p < 0.001 and <0.05; Fig 5C/D), we observed a significant reduction of Sca1POS/αMHCPOS and Tbx5POS/cTnTPOS cardiomyogenic populations in KLF15 KO in comparison to WT mice. This suggests that KLF15 is important for the maintenance of CPC homeostasis and that its downregulation leads to an increased cell number of cells in G2/M phase indicative of an increased number of dividing progenitors in KLF15 KO mice. Furthermore, we investigated specific CPCs sub-populations previously described in the adult heart. The total number of Sca1POS and c-kitPOS sub-populations were significantly increased in KO mice (p < 0.001 and <0.05; Fig 5D), although it is important to mention that the c-kit population was very low in both WT and KLF15 KO mice. Next, we analyzed these CPC sub-populations in combination with endothelial and cardiomyogenic markers to identify progenitor cells in transition to different lineages (see Supporting Information Fig S5 for controls). Cells co-expressing Sca1 or c-kit and the endothelial cell marker CD31 were significantly increased in KLF15 KO mice (p < 0.001 and <0.05; Fig 5D). In contrast, we observed a significant reduction of Sca1POS/αMHCPOS as well as the Tbx5POS/cTnTPOS cardiomyogenic populations in KLF15 KO in comparison to WT mice (p < 0.001 and <0.05, respectively). No significant changes were detected in the double populations labelled by c-kit and the cardiomyogenic markers αMHC and Hand1 (Fig 5D).

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**Figure 5.**

**A**
- RT heart CPC Sca1pos
- Klf15
- Gapdh

**B**
- total kDa heart CPC Sca1pos
- KLF15
- GAPDH

**C**
- G0/G1 phase (%)
- G2/M phase (%)

**D**

**E**
- vWF
- CD105
- CD133

**F**
- Tcf4
- vWF
- CD105

**G**
- Tbx5pos/cTnTneg (%)

* 0.05
** 0.10
*** 0.15
**** 0.20

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KO mice (p < 0.05), the cardiomyogenic proliferative cells, Ki67pos/αMHCpos and Ki67pos/Hand1pos, were unchanged (Fig 5D). Moreover, we detected increased mRNA expression of endothelial markers von Willebrand Factor (vWF) (p < 0.001) and Endoglin/CD105 (p < 0.01). Expression of CD133, which is known to be lost upon endothelial cell differentiation, was significantly reduced (p < 0.01) in cardiac tissue of Klf15 KO versus WT mice (Fig 5E). Together these data show an increased progenitor cell fraction, which exhibits an endothelial phenotype in the Klf15 KO mice.

Next, we were interested in the effect of KLF15 depletion in CPCs upon cardiac remodelling. Therefore, we applied a mild stimulus to induce hypertrophy by AngII and a more stringent stimulus by transverse aortic constriction (TAC) in 12-week-old Klf15 KO and WT mice. In line with previous functional studies, 2 weeks following AngII treatment, Klf15 KO showed accentuated cavity dilation as shown by LV diameter measurement (p < 0.01; Supporting Information Fig S6A and Table S2A). FACs analysis revealed that the Sca1pos/αMHCpos as well as the Tbx5pos/cTnTneg populations were significantly decreased in Klf15 KO versus WT mice (p < 0.01) upon AngII infusion (Supporting Information Fig S6B). Two weeks following TAC, stronger dilation of the LV and accentuated cardiac dysfunction along with increased mortality was observed in Klf15 KO in comparison to WT mice (p < 0.01; Supporting Information Fig S6B). Uprolization of TCF4 mRNA expression (p < 0.01; Fig 5F), reduction of the cardiomyogenic Tbx5pos/cTnTneg population (p < 0.05; Fig 5G) and significant upregulation of vWF and CD105 expression (p < 0.001 and <0.05, respectively; Fig 5F) were confirmed in Klf15 KO mice upon TAC. Altogether these data indicate that loss of KLF15 induces an activation of β-catenin-dependent transcription and this reduces the cardiomyogenic progenitor cell pool both in the normal and stressed adult heart.

Endothelial identity of Sca1pos CPCs in Klf15 KO mice

Since we found expression of KLF15 in Sca1pos cells, we used this marker to assess gene regulation analysis in a purified cardiac progenitor population. The cardiac Sca1pos cells were purified from Klf15 KO and WT mice by magnetic cell sorting (MACS). 96.4% purity of sorted Sca1pos cells was confirmed by flow cytometry. Analysis of the Wnt/β-catenin pathway by qRT-PCR showed upregulation of the β-catenin target genes cMyc and TCF4 in the purified Sca1 cell population. The early mesodermal haemangioblast marker Flk1 as well as the endothelial markers vWF, CD105 were upregulated while the cardiomyogenic markers αMHC and Hand1 were lower expressed in Klf15 KO mice in comparison to WT Sca1pos cells (Fig 6A). These data further support the role of KLF15 in CPC cell fate determination in the adult heart.

KLF15 deletion directs CPCs to acquire an endothelial phenotype via activation of β-catenin dependent transcription

Loss of KLF15 results in upregulation of β-catenin transcription and promotes an endothelial phenotype of CPCs. In contrast, we previously described that cardiac inducible deletion of β-catenin (β-catex2–6) enhanced cardiomyogenic CPC differentiation (Zelarayan et al, 2008). Now, we reasoned that β-catenin transcriptional inactivation would lead to the opposite regulation of endothelial fate of CPCs in comparison to KLF15. Therefore, we analyzed the CPC fraction in β-catex2–6 by FACS and found indeed a significant reduction of CD31pos as well as Ki67pos/CD31pos CPCs in comparison to control mice (Fig 6B). This observation was confirmed by a semi-quantitative immunohistochemistry analysis, which shows enhanced CD31 positive areas in Klf15 KO in comparison to control mice and moreover, a significant reduction of in β-catex2–6 (Fig 6C and D).

To further elucidate the mechanism of KLF15 to drive CPC cell fate determination we established a co-culture system with adult cardiac fibroblasts as feeder layer for the enriched adult CPCs. The same cell number of cardiovascular progenitors from Klf15 WT and KO mice were plated and cultured on arrested adult cardiac fibroblasts. Following 10 days co-culture an augmented cell population was detected in the Klf15 KO cultured cells in comparison to WT cells when analyzed by flow cytometry (Gate G1; Fig 7A). Similar to our in vivo finding, we found a highly proliferative Ki67pos cell fraction (p < 0.05) along with a decrease in αMHCpos (p < 0.01) and Hand1pos expressing cells. Interestingly, cells expressing Flk1 were augmented (p < 0.01;
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Fig 7A). Along with this finding, mRNA expression of Flk1 and CD31 was increased, whereas expression of Hand1 was decreased following 10 days culture in Klf15 KO versus WT CPCs (p < 0.05; Fig 7B). An increased endothelial-like cell formation expressing CD31 was observed in Klf15 KO cells in comparison to WT (Fig 7C).

To verify that the preferentially endothelial fate of Klf15 KO CPCs is mediated by activation of β-catenin transcription, we performed a co-culture rescue experiment where CPCs were treated with 5 μmol/L Quercetin, a known β-catenin transcriptional inhibitor (Park et al, 2005). Quercetin was able to reverse the β-catenin transcriptional activation in vitro as demonstrated by luciferase gene reporter assay in HEK293 cells and attenuation of β-catenin target gene activation in co-cultured CPCs (Supporting Information Fig S7A and B). FACS analysis after 10 days co-culture showed that the significant increase of Flk1 in Klf15 KO CPCs was partially reversed upon Quercetin treatment in comparison to the DMSO-treated Klf15 KO CPCs. Moreover, the increase in cells expressing Ki67 as well as the reduction of αMHCpos and Hand1pos CPCs observed in Klf15 KO CPCs was completely abrogated (Fig 7A and D). The significantly increased cell number in Klf15 KO CPCs as well as the significantly decreased proliferation in Quercetin treated Klf15 KO CPCs was confirmed via semi-quantification analysis following of 10 days culture (Fig 7D). Quercetin treatment did not affect WT CPC cell number.

Collectively, these data show that regulation of β-catenin transcriptional activity mediates regulation of cardiovascular cell fate downstream KLF15 in the postnatal heart.

DISCUSSION

To the best of our knowledge, we provide the first evidence that KLF15 specifically interacts with components of the Wnt/β-catenin pathway and inhibits β-catenin/TCF-dependent transcriptional activation in cardiac cells. Moreover, our findings show that KLF15 controls CPCs via regulation of β-catenin transcription, which contributes to normal cardiac homeostasis.
Figure 7.
Cardiac interaction and regulation of the Wnt/β-catenin pathway by KLF15
In this study, we found KLF15 to specifically interact with β-catenin and TCF4 and to inhibit β-catenin/TCF-dependent transcription in cardiac cells. In line with the fact that the KLF proteins recruit co-factors to regulate transcription (Kaczynski et al, 2003) we found KLF15 to interact with NLK, an inhibitor of the β-catenin-LEF/TCF complex. We showed that a minimal KLF15 N-terminal domain is essential for both protein interaction and inhibition, which is consistent with the structure of KLF proteins containing a transcriptional activation or repression domain at the N-terminal region (Kaczynski et al, 2003). We showed that the first 45 amino acids are essential for KLF15-β-catenin interaction. Moreover, the binding domain for NLK as well as the repression domain is located between amino acids 45 and 152 of the KLF15 N-terminus indicating an important role of this interaction for transcriptional repression. Thus, different KLF15 N-terminal domains are required for binding both β-catenin and NLK suggesting that two different interaction domains are present at the N-terminus probably allowing for simultaneous binding to both β-catenin and NLK.

We also demonstrated that KLF15 C-terminus is essential for binding TCF4 and for transcriptional repression, which may be explained by the requirement of the C-terminal domain for nuclear localization. This is in line with the identification of a putative nuclear localization signal for KLF15 at amino acid 369 (Gray et al, 2002). Mechanistically, we found that KLF15 targets TCF4 to ubiquitination, which was previously shown for NLK (Ishitani et al, 2003). Our data suggest that KLF15 may recruit NLK to the β-catenin/TCF-complex to prevent transcriptional activation by targeting TCF4 for proteasomal degradation.

We demonstrate that several N-terminal mutants of KLF15 were able to translocate into the nucleus but were insufficient to inhibit β-catenin/TCF4 transcription. These data show that KLF15 requires both the C-terminus for nuclear translocation and a minimal N-terminal regulatory region to exert inhibition on β-catenin/TCF transcriptional activation. Similarly, another KLF member, KLF4, was shown to inhibit β-catenin function by preventing β-catenin binding to transcriptional mediators (Zhang et al, 2006). These findings suggest specific members of the KLF family proteins to allow tissue-specific inhibition of β-catenin-LEF/TCF transcription.

KLF15 regulates cardiac β-catenin transcription and is important for cardiac homeostasis
Our in vitro finding showing KLF15-dependent β-catenin/TCF transcriptional regulation prompted us to investigate this regulation in vivo using a Klf15 KO mouse. Deletion of KLF15 resulted in an initial mild cardiac functional deterioration, which was aggravated with aging. Cardiac activation of β-catenin/TCF-dependent transcription was observed in Klf15 KO mouse. It is consistent with our in vitro results showing inhibition of β-catenin transcription by KLF15, indicating that no compensation for KLF15 concerning Wnt transcriptional regulation takes place in the heart. Previous studies demonstrated a role of KLF15 in regulating hypertrophic signalling and fibrosis of the adult heart (Fisch et al, 2007; Haldar et al, 2010; Leenders et al, 2010; Wang et al, 2008). Similar to these functional observations, we confirmed exacerbated reduced LV function in response to TAC along with cardiac hypertrophy and fibrosis as well as accentuated LV dilation after AngII infusion in Klf15 KO mice. However, at baseline we did not find neither activation of hypertrophic pathways, nor increased fibrosis in Klf15 KO mice. Our data show that the previously identified KLF15-dependent mechanisms regulating hypertrophy and fibrosis upon induced cardiac hypertrophy are not activated in absence of any stimuli, suggesting an alternative mechanism through which KLF15 controls normal cardiac homeostasis.

KLF15 regulates CPC fate in the postnatal heart
We previously described increased cardiomyogenic differentiation of the sub-population of Sca1pos cells upon β-catenin depletion in the postnatal heart (Zelarayan et al, 2008). In this study, we demonstrated expression of KLF15 not only in total CPCs but also specifically in the Sca1pos sub-population. Therefore, we reasoned that KLF15-dependent regulation of β-catenin transcription affects CPC biology. CPCs from Klf15 KO were augmented in the proliferative cell cycle phase G2 and specifically the Sca1pos and c-kitpos CPC sub-populations showed increased proliferation in Klf15 KO versus WT. This might be explained by an enhanced β-catenin transcriptional activation as described in several studies showing that Wnt/β-catenin signalling is required for proliferation and expansion of specified cardiovascular progenitors during development (Gessert & Kuhl, 2010). Due to the lack of specific markers for different CPC populations, we combined stem cell markers with markers of early and mature lineages to identify stem cells in...
transition to cardiomyogenic (Hand1, Tbx5, αMHC) and mesodermal/endothelial cells (Flk1, CD31). Klf15 KO mice showed a significant reduction of the cardiomyogenic progenitors in contrast to an increase of endothelial progenitors in the Sca1<sup>pos</sup> CPC pool in vivo and in vitro. Moreover, an early upregulation of cells expressing the mesodermal/haemangioblastic marker Flk1 followed by an increase in CD31 expression was detected in vitro. These finding suggest an endothelial preference of Klf15 KO CPCs further supported by an increased expression of a combination of endothelial markers including vWF and CD105, the latter commonly used to trace endothelial differentiation. In parallel we observed a reduction of CD133, which is expressed in undifferentiated progenitors of the endothelial lineages and becomes lower during its differentiation (Shmelkov et al, 2005).

Further substantiating these data, the isolated Sca1<sup>pos</sup> subpopulation showed upregulation of endothelial markers in Klf15 KO mice suggesting an activation of an endothelial differentiation gene program. Recently, the Sca1<sup>pos</sup>/CD31<sup>pos</sup> cardiac side population cells were described as progenitors of cardiac endothelial cells (Liang et al, 2011). Similar to our observations, they showed vascular tube-like network formation along with upregulation of CD133 in undifferentiated state and upregulation of vWF during differentiation.

Our analysis showed an increase in the c-kit<sup>pos</sup> CPC as well as the c-kit<sup>pos</sup> endothelial fraction upon KLF15 deletion, although the number of cells was very low and no changes in the cardiomyogenic pool were observed. These observations might be explained by the fact that the adult c-kit population was shown not to play a role in generating cardiomyogenic cells in the adult heart (Zaruba et al, 2010).

Imbalance of the cardiomyogenic and endothelial CPCs in Klf15 KO mice was also observed upon mild and stringent induced stress, which indicates that KLF15-dependent control of CPCs plays also a role during stress induced remodelling. In conclusion, KLF15 loss-of-function favours the expansion and commitment of CPCs to endothelial cells thus regulating the compartment of CPCs in the postnatal heart, which has critical implications in the control of cardiac homeostasis. Similarly, pathways involved in early CPC specification were shown to control homeostasis of the normal and injured heart (Boni et al, 2008).

**KLF15 regulates CPC fate via regulation of β-catenin transcription**

Our study clearly shows a β-catenin/TCF activated transcription upon KLF15 deletion in vivo, which results in a preferentially endothelial CPC phenotype. In contrast, we previously showed that cardiac β-catenin deletion promoted CPC differentiation towards cardiomyogenic cells (Zelarayan et al, 2008). Here, we further showed that mice with cardiac β-catenin deletion exhibit the opposite phenotype as observed in Klf15 KO, namely a reduction of endothelial-like CPCs in vivo. As expected, CPCs from mice with a cardiac stabilization of β-catenin showed a decreased cardiomyogenic CPC differentiation in vitro (Zelarayan et al, 2008) similarly to Klf15 KO CPCs. These findings strongly indicate that KLF15 controls CPCs homeostasis in the adult heart via regulating β-catenin/TCF transcription.

To further test the hypothesis that β-catenin transcriptional activation mediates CPC regulation downstream KLF15, we blocked β-catenin transcription in CPCs using a pharmacological approach via Quercetin. Confirming our hypothesis, this treatment reversed the increase in CPC proliferation, Flk1 cells and endothelial markers. Moreover, the reduction of cardiomyogenic cells was observed specifically in Quercetin treated-Klf15 KO versus DMSO-Klf15 KO. Quercetin treatment of WT CPCs did not affect endothelial or cardiomyogenic CPCs, while the slight reduction observed in proliferation can be explained by the fact that Wnt/β-catenin transcription is also attenuated in the WT CPCs. Together these data indicate that β-catenin transcription downstream KLF15 is critical for CPC homeostasis. This is supported by the fact that similar to the cardiac phenotype observed in Klf15 KO, mice with a long term induction of cardiac β-catenin stabilization exhibited reduced LV function. Other studies showed that stimulation of β-catenin transcription leads to cardiac impairment and accelerated myocardial remodelling (Malekar et al, 2010).

It is important to mention that during early development, two mesodermal Flk1<sup>pos</sup> cell populations are defined. An early mesodermal haemangioblastic cell population expressing Flk1 but not the multi-potent cardiac marker Mesp1 develops into endothelial cells and the blood lineage. A later mesodermal Flk1 population co-expressing Mesp1 results in the all the cardiovascular lineage (Gessert & Kuhl, 2010; Kouskoff et al, 2005). Expression of Flk1 is sequentially lost in the cardiomyogenic population while it expression persists in the endothelial lineages. Active Wnt/β-catenin signalling activates these mesodermal progenitors to become haemangio-blasts and inactivation of Wnt/β-catenin through Notch will allow them to become cardiomyogenic cells (Chen et al, 2008; Gessert & Kuhl, 2010; Naito et al, 2006; Ueno, 2007). Similarly, our in vivo and in vitro data showed that KLF15 deletion leads to an early upregulation of mesodermal haemangioblastic cell marker Flk1, which leads to a final upregulation in the endothelial CD31 population by regulating activation of the Wnt/β-catenin signalling. Likewise, β-catenin maintains normal homeostasis of progenitor pools in the gut (Pinto & Clevers, 2005) and nervous system (Zechnet et al, 2003).

We conclude that β-catenin transcriptional activation in the absence of KLF15 favours an increased endothelial cell while repressing cardiac myogenic differentiation. KLF15 is essential to maintain the balance of early differentiating CPCs, which may have critical implications in the control of heart homeostasis and its adaptation to pathologic states (Fig 8). These observations allow us to conclude that an altered CPC homeostasis might be another cellular mechanism aside cardiac hypertrophy that contributes to enhanced cardiac deterioration upon deletion of KLF15. Both scenarios are not mutually exclusive, and may contribute to heart failure development. Importantly, KLF15 is expressed in human adult cardiac tissue and similar to the finding in mouse, is known to be downregulated in the failing heart (Fisch et al, 2007; Haldar et al, 2010). Our study reveals an interesting regulation that could beexploited to activate human CPCs upon cardiac remodelling.
For isolation of cardiac fibroblast, adult mice were anesthetized and hearts were dissected, minced and digested with collagenase I/trypsin and cultivated in DMEM/F12 supplemented with 10% FCS, L-glutamine/antibiotics/100 μM ascorbic acid. For isolation of a non-cardiomyocyte cell fraction, adult mice were anesthetized and hearts were dissected, minced and filtered through 40 and 30 μm mesh. Cells were used directly for FACS staining or seeded on Mitomycin C (Sigma-Aldrich)-arrested adult cardiac fibroblasts and cultivated for 10 days at 37 °C, 5% CO2 in DMEM/F12 supplemented with 2% FCS, L-glutamine/antibiotics/100 μM ascorbic acid. For rescue experiments co-cultures were treated with 5 μmol/L Quercetin hydrate (Acros Organics) for 10 days; control cells were treated with the solvent DMSO.

**Co-immunoprecipitation and ubiquitination assays**

HEK293 cells and NRC were co-transfected with expression plasmids for cmyc-β-catenin, HA-TCF4, HA-NLK, Flag-KLF15 (full length, -ΔN152, -ΔN260, -ΔC) and pcDNA3.1. Cells were harvested 48 h after transfection, lysed with radio-immunoprecipitation assay (RIPA) buffer with protease inhibitors, and immunoprecipitated with antibodies against cmyc-(Santa Cruz), HA-, or Flag-tag (Sigma–Aldrich). Interacting proteins were detected by immunoblotting using the appropriate anti-tag-HRP antibodies (1:2000; Sigma–Aldrich). As controls, protein lysates transfected with EV and precipitation with IgG isotype control were used. Whole cell lysates were immunoblotted with respective antibodies to detected protein expression. Endogenous co-immunoprecipitation was performed with an antibody against KLF15 (Santa Cruz). Interacting proteins were detected by immunoblotting using the appropriate anti-β-catenin, -NLK and -TCF4 antibodies (1:1000). IgG isotype control was used as a co-immunoprecipitation control. For ubiquitination assay RIPA buffer was supplemented with 10 μmol/L N-ethylmaleimide (NEM; Sigma–Aldrich). TCF4 was immunoprecipitated with anti-HA antibody (Sigma–Aldrich). Ubiquitination of TCF4 was analyzed by immunoblotting using anti-ubiquitin antibody (1:1000; Cell Signaling) and anti-rabbit-HRP secondary antibody (1:10,000; Dako).

**Luciferase reporter assay**

HEK293, SW480 cells, and NRC were co-transfected with plasmids expressing non-degradable β-catenin (β-catenin-ΔN) or TCF4 and various amounts of full-length and truncated constructs of KLF15 (250 pg–250 ng) along with the pTOPFlash luciferase reporter (750 ng) and Renilla luciferase expressing plasmid (5 ng) as a normalizing control. pTOPFlash containing mutated TCF binding sites was used as negative reporter control. Luciferase activity was determined using Dual-Luciferase Reporter Assay (Promega) 48 h after transfection, according to manufacturer’s instructions.

**Flow cytometry analysis**

The non-cardiomyocyte population was prepared as mentioned above. Cells were fixed in 1% formaldehyde/PBS, permeabilized in FACs buffer containing 0.5% Saponin (Sigma–Aldrich) and stained with anti-CD31-FITC, -Flk1-PE, -Sca1-FITC, -c-kit-APC (1:200; ebioscience) anti-αMHC, -Tbx5, -cTnT (1:500; Abcam); -Ki67-PE, and -Hand1 (1:200; Santa Cruz). The cells were subsequently stained with anti-rabbit IgG-APC or anti-mouse F(ab)2-FITC (1:500; Jackson ImmunoResearch). Respective isotype controls were used. For cell cycle analysis the
isolated cells were fixed in cold 70% ethanol, treated with 100 μg/ml RNase and incubated with 50 μg/ml propidium iodide. Fluorescence signals were detected with a FACS-Calibur flow cytometer (Becton Dickinson).

**Magnetic activated cell sorting**
The non-cardiomyocyte population was prepared by mincing and 1 mg/ml collagenase II digestion, stained with anti-Sca1-FITC (1:10; Miltenyi Biotec) and labelled with anti-FITC-MicroBeads (1:5; Miltenyi Biotec). Sca1<sup>pos</sup> cells were enriched by two rounds of MS Columns (Miltenyi Biotec) separation. Purity was checked by FACS analysis. Sca1<sup>pos</sup> cells were directly applied to mRNA extraction.

**RNA isolation, reverse transcription and quantitative real-time PCR analysis**
Total RNA was isolated from cardiac mouse tissue using the NucleoSpin RNA II kit (Macherey-Nagel), according to manufacturer's instruction and treated with DNase. cDNA was synthesized with Oligo(dT) 20 primer and M-MLV reverse transcriptase (Promega). Quantitative real-time PCR analyses were performed with SYBR Green (Qiagen) on an iCycler instrument (BioRad). Copy numbers were calculated using the iCycler software with a relative standard curve obtained using the log dilutions of cDNA of gene of interest. All reactions were run in triplicates and normalized to both reference control genes Gapdh and β-actin applying the geometric mean calculation. Primers are listed in Supporting Information Table S3.

**Immunocytochemistry**
HEK293 and NRC were grown on poly-γ-lysine or 1% gelatine coated coverslips, respectively; fixed in 4% PFA, permeabilized in 0.1% TritonX-100 and blocked in 1% BSA for 1 h at RT. Cells were incubated with respective antibodies against β-catenin (BD Bioscience), NLK (Bethyl Laboratories), KLF15 (Abcam), HA-tag (Sigma–Aldrich) and Flag-tag (Sigma–Aldrich) at 1:200 dilutions O/N at 4°C. Cardiac progenitor cells were grown on fibroblast on gelatine-coated coverslips. Cells were blocked in 2% FCS, surface markers were detected with anti-Flk1 (1:200; eBioscience), anti-CD31-FITC (1:200; eBioscience) for 1 h at 4°C. Next, cells were fixed in 1% PFA, permeabilized in 0.3% TritonX-100/0.2% BSA and blocked in 0.1% TritonX-100/5% BSA for 1 h at RT. Intracellular staining was performed with antibodies against Hand1 (1:100; Santa Cruz) and O/N at 4°C. AlexaFluor488 and AlexaFluor594 conjugated secondary antibodies (1:200; Invitrogen) were used for immunofluorescence labelling. For nuclei visualization cells were incubated with Hoechst33342 (Sigma–Aldrich) for 5 min and mounted with ProlongGold (Invitrogen). Microscopic images were captured with a confocal microscope (710, Zeiss).

**The paper explained**

**PROBLEM:**
Heart failure is characterized by an imbalanced myocardial damage and repair. Unfortunately, the therapeutic modalities to prevent heart failure are currently limited. It is now well accepted that regeneration events occur in the adult mammalian hearts during normal aging and disease. Although low levels of cardiomyocyte renewal are detectable in the adult heart, promising pharmaceutical or genetic therapy approaches could be developed to enhance this effect. Therefore, the elucidation of cardiac progenitor cell contribution to cardiac homeostasis, aging and injury along with the molecular signalling directing these events is essential to help to promote postnatal cardiac regeneration. Thus, we need to focus our efforts to explore these processes to benefit from endogenous mechanisms and to be able to exploit them therapeutically. Wnt/β-catenin pathway is essential for embryonic cardiogenesis and for normal cardiac homeostasis and remodelling. Therefore, this pathway is a good candidate to be investigated in endogenous CPC biology.

**RESULTS:**
The present study provides a detailed biochemical and functional analysis of a novel interaction between a member of the KLF family (KLF15) and Wnt-pathway components, which leads to repression of the Wnt transcriptional activity. We clearly show the presence of two important domains within the KLF15 protein sequence, a minimal N-terminal region and the C-terminus, which are essential for β-catenin/TCF4 transcriptional repression in cardiac cells. Moreover, using a genetic model we report the role of KLF15 in the regulation of the CPC biology in the postnatal heart in vivo and in vitro. Klf15 knockout mice exhibit a derepression of β-catenin/TCF4 transcription. This shifts the cell fate of cardiovascular progenitors towards an endothelial lineage antagonizing cardiogenic cell formation in the healthy and stressed heart, similar to what has been observed in embryonic development. Moreover, the endothelial cell fate shift can be reversed upon inhibition of the Wnt activation in vitro, which demonstrated that KLF15 controls CPCs cell fate via regulation of β-catenin transcription.

**IMPACT:**
This work may have identified a novel molecular switch to therapeutically modulate CPC fate and unlock the regenerative potential of the adult heart. It might help to prevent massive cardiac loss upon injury. Specifically, our observations highlight the role of KLF15 in controlling the early specification of the progenitor pool to specifically generate different cardiovascular cellular subtypes in the postnatal heart. This observation is of relevance given the fact that KLF15 is expressed in the human heart and is known to be dysregulated upon prohypertrophic stimuli. The present data help us to understand the biological processes leading to formation of cardiovascular cells from resident precursors, which may have critical implications in the control of heart homeostasis and its adaptation to pathologic states.
Histological analysis
Heart, liver, lung and kidney were rinsed in PBS, fixed in 4% PFA, embedded in paraffin and sectioned at 3 μm. Sections were deparaffinized and used for the different stainings. Liver, lung and kidney were stained with Weigert’s haematoxylin/Eosin. For visualization of cardiac fibrosis sections were stained with Weigert’s iron haematoxylin/Eosin. Heart, liver, lung and kidney were rinsed in PBS, fixed in 4% PFA, embedded in paraffin and sectioned at 3 μm. Sections were deparaffinized and used for the different stainings. Liver, lung and kidney were stained with Weigert’s haematoxylin/Eosin. For visualization of cardiac fibrosis sections were stained with Weigert’s iron haematoxylin/Eosin. 

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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