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Cardiac hypertrophy induced by active Raf depends on Yorkie-mediated transcription

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Abstract

Organ hypertrophy can result from enlargement of individual cells or from cell proliferation or both. Activating mutations in the serine-threonine kinase Raf cause cardiac hypertrophy and contribute to Noonan syndrome in humans. Cardiac-specific expression of activated Raf also causes hypertrophy in *Drosophila melanogaster*. We found that Yorkie (Yki), a transcriptional coactivator in the Hippo pathway that regulates organ size, is required for Raf-induced cardiac hypertrophy in flies. Although aberrant activation of Yki orthologs stimulates cardiac hyperplasia in mice, cardiac-specific expression of an activated mutant form of Yki in fruit flies caused cardiac hypertrophy without hyperplasia. Knockdown of Yki caused cardiac dilation without loss of cardiomyocytes and prevented Raf-induced cardiac hypertrophy. In flies, Yki-induced cardiac hypertrophy required the TEA domain-containing transcription factor Scalloped, and, in mammalian cells, expression of mouse Raf^{L613V}, an activated form of Raf with a Noonan syndrome mutation, increased Yki-induced Scalloped activity. Furthermore, overexpression of Tgi (a Tondu domain-containing Scalloped-binding corepressor) in the fly heart abrogated Yki- or Raf-induced cardiac hypertrophy. Thus, crosstalk between Raf and Yki occurs in the heart and can influence Raf-mediated cardiac hypertrophy.

INTRODUCTION

Pathophysiologic stimuli, such as pressure overload or heritable mutations in signaling molecules, can cause cardiac hypertrophy, characterized by an increase in cardiomyocyte size. Increased cardiomyocyte size causes the walls of the heart to thicken, decreasing the size of the cardiac chamber, and potentially restricting the heart's ability to pump blood (1). Untreated, cardiac hypertrophy leads to heart failure, which can be fatal.

Activation of receptor tyrosine kinases (RTKs) by extracellular growth factors and downstream signaling mediated by GTPase (guanosine triphosphatase), Ras, and the serine

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SUPPLEMENTARY MATERIALS

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and threonine kinase Raf contribute to cardiac hypertrophy (2). Heritable mutations that activate the Ras-Raf pathway, collectively called rasopathies, are responsible for various syndromes associated with cardiac hypertrophy (3). For example, mutations in human *RAF1* that lead to the substitution of valine for leucine at amino acid 613 are associated with Noonan syndrome, which is characterized in part by cardiac hypertrophy (3).

Knock-in mice for the mutation encoding Raf1^{L613V} have symptoms consistent with Noonan syndrome, including cardiac hypertrophy (4). Additionally, heterozygous Raf1^{L613V} mice have enhanced mitogen-activated protein kinase kinase 1 (MEK1) and extracellular signal-regulated kinase 1 (ERK1) and ERK2 signaling, and treatment of these mice with MEK inhibitors attenuates many phenotypic abnormalities, including cardiac hypertrophy (4).

The adult fly heart is a tubular structure with circumferentially oriented contractile fibers located along the dorsal aspect of the abdomen (5–7). Fifty-two pairs of cardiomyocytes, which can be identified by the expression of the gene *tinC*, comprise the embryonic heart. Although the larval heart undergoes major morphological changes to become the adult heart, the total number of cardiomyocytes remains constant (6, 8). Within each abdominal segment, denoted A1 to A6, the heart has eight *tinC*-expressing myocytes that are laterally paired and comprise the adult fly heart, which has a thickness of single-cell layer. Similar to mammals, cardiac-specific expression of activated human Raf in *Drosophila* decreases the size of cardiac lumens, increases heart wall thicknesses without inducing hyperplasia, and causes abnormalities in cardiomyocyte contractile fibers (9). Similar to pharmacological inhibition of MEK in mice, the cardiac-specific knockdown of MEK or ERK in flies rescues Raf-mediated hypertrophy (9). However, cardiac-specific expression of activated *Drosophila* ERK (ERK^{D334N}), which promotes hyperplasia in noncardiac tissues, does not cause cardiomyocyte hypertrophy (9), suggesting that signals downstream from active Raf and MEK, in addition to ERK, are necessary to drive cardiomyocyte growth.

The evolutionarily conserved Hippo signaling pathway also regulates cardiac growth (10–12). Hippo (MST1 and MST2 in mammals) is a serine and threonine kinase that is activated by low cell density or mechanical strain in epithelial tissues (13–15). In a complex with the scaffolding protein Salvador, Hippo phosphorylates the kinase Warts (LATS1 and LATS2 in mammals) (16, 17). Phosphorylated Warts phosphorylates the transcriptional coactivator Yorkie (Yki; YAP1 and TAZ in mammals), leading to the inhibition of Yorkie-induced transcription due to sequestration of phosphorylated Yorkie in the cytosol through binding to 14-3-3 proteins (18, 19). In the absence of phosphorylation, Yorkie binds to the TEA domain-containing DNA binding transcription factor Scalloped (Sd; TEADs in mammals) to promote the transcription of genes involved in cell growth and proliferation (20–22). In mice, genetic ablation of Salvador or transgenic expression of mutant *YAP1* encoding a serine-to-alanine substitution that prevents phosphorylation by LATS (YAP^{S127A}) in the mouse heart results in cardiomegaly due to increased cardiomyocyte proliferation during development (23–25). The mutation in YAP^{S127A} prevents phosphorylation of this residue by Lats and cytosolic sequestration, thereby resulting in nuclear localization of YAP (24, 25).

RTK signaling interacts with the Hippo pathway. Epidermal growth factor receptor (EGFR)–mediated signals occur through Raf to activate the Hippo and Yki signaling pathway in *Drosophila* eye discs, wing discs, and brain lobes (26). In mouse and human cells, Raf inhibits MST2 by preventing its dimerization and by recruiting a phosphatase that dephosphorylates key residues involved in activation of MST2. Both of these functions of Raf in mammals require binding to MST2 and are independent of the ERK pathway, including the kinase activity of Raf (28).

We found that Raf stimulates Yki to promote cardiac hypertrophy in flies. Using optical coherence tomography (OCT) of cardiac chambers in awake, adult flies and histological analyses of heart wall thicknesses, we found that cardiac-specific expression of activated Yki caused cardiac hypertrophy, whereas cardiac-specific expression of RNA interference (RNAi) directed against *yki* caused cardiac dilation. Moreover, cardiac-specific knockdown of Yki prevented Raf-mediated cardiac hypertrophy. In mammalian cell culture, activated Raf enhanced the ability of activated Yki to stimulate Sd-dependent transcription, whereas genetic or pharmacological inhibition of the kinase activities of Raf, MEK, or ERK inhibited this effect. Cardiac-specific overexpression of a Tondu domain–containing, Sd-associated corepressor Tgi prevented cardiac hypertrophy in flies induced by expression of activated Raf or activated Yki. In addition, Tgi prevented the ability of activated Raf to augment Yki-induced, Sd-dependent transcription in cell culture.

RESULTS

Yki controls heart size in *Drosophila*

We investigated the role of Yki in the maintenance of organ size in the fly heart. *tinC* is specifically expressed in the heart throughout development and in adult flies (29). The amino acid substitutions S168A, or S111A, S168A, and S250A in Yki are equivalent to S127A in murine YAP and prevent phosphorylation by Warts and thereby lead to constitutive activation of Yki (18). Therefore, we used the Gal4-UAS system to express *yki* transgenes (*UAS-yki^{S168A}* or *UAS-yki^{S111A,S168A,S250A}*) under the control of the enhancer *tinC* (*tinC-GAL4*). We also expressed green fluorescent protein (GFP) from the *tinC* enhancer (*tinC-GFP*) and OCT to visualize the beating heart in awake, adult flies. We measured the size of the ventricular diameter at the end of systole (ESD) and diastole (EDD) and calculated the fractional shortening, which is a measure of systolic function (30, 31). Compared to *tinC-GFP; tinC-Gal4* flies, *tinC-GFP; tinC-Gal4,UAS-yki^{S168A}* (*tinC>yki^{S168A}*) flies had smaller EDDs and ESDs (Fig. 1A). Additionally, the heart walls were thicker in *tinC>yki^{S168A}* and *tinC-GFP; tinC-Gal4,UAS-yki^{S111A,S168A,S250A}* (*tinC>yki^{S111A,S168A,S250A}*) flies compared to *tinC-GFP; tinC-Gal4* flies (Fig. 1, B and C). Thus, aberrant activation of Yki induces cardiac overgrowth.

We also tested if Yki was required for organ size in the adult heart using a double-stranded RNAi transgene targeting *yki* (*UAS-yki^{RNAi}*). Because cardiac tissue is difficult to purify from the ventral longitudinal muscle and surrounding pericardial cells, we tested the ability of *UAS-yki^{RNAi}* to reduce the abundance of *yki* in the eyes of flies expressing Gal4 from an eye-specific driver (*GMR-Gal4*) (32). We found that *yki* expression was reduced by 70% in the eyes of adult *GMR-Gal4;UAS-yki^{RNAi}* flies compared to that in *GMR-Gal4* flies (fig.

S1). Cardiac-specific expression of *ykr^{RNAi}* in *tinC-GFP; tinC-GAL4,UAS-ykr^{RNAi}* (*tinC>ykr^{RNAi}*) flies increased EDDs and ESDs and reduced the percentage of fractional shortening (Fig. 1, A and D), indicative of cardiac dilation. Moreover, the heart wall was thinner in *tinC>ykr^{RNAi}* flies (Fig. 1, B and C), indicating that loss of Yki induces cardiac dilation.

The timing of the expression of *YAP^{S127A}* during the development of the mammalian heart results in different cardiac abnormalities, including abnormal hyperplasia (7–9). Therefore, to determine whether aberrant activation of Yki during development affected heart development in flies, we silenced *ykr^{S168A}* expression in *tinC>ykr^{S168A}* flies using a temperature-sensitive form of the Gal4-binding transcriptional corepressor, Gal80, driven by the tubulin promoter (*tubulin-Gal80^{ts}*), which results in ubiquitous expression of *Gal80^{ts}* (16). *tubulin-Gal80^{ts}; tinC>ykr^{S168A}* flies that were grown at the restrictive temperature (18°C) from egg deposition and then shifted to the permissive temperature (29°C) to induce *ykr^{S168A}* expression at the wandering larvae stage, before morphogenesis of the pupal heart (8), had decreased EDDs compared to *tubulin-Gal80^{ts}; tinC-Gal4* flies temperature-shifted at the same age (fig. S2). In contrast, EDDs in *tubulin-Gal80^{ts}; tinC>ykr^{S168A}* flies shifted to the permissive temperature when adults did not differ from EDDs in *tubulin-Gal80^{ts}; tinC-Gal4* flies (fig. S2), suggesting that the ability of Yki^{S168A} to induce cardiac overgrowth is limited to a developmental stage occurring before or during heart morphogenesis.

Activation of Yki causes hyperplasia in many tissues, including the eye (10, 24, 25). Because a normal adult fly heart is single-cell thick (6, 7), the increased thickness of the heart walls in flies expressing activated Yki could be due to cardiomyocyte proliferation (hyperplasia), increased size of cardiomyocytes (hypertrophy), or a combination of cardiac hyperplasia and hypertrophy (fig. S3). Therefore, we investigated whether cardiac-specific expression of activated Yki increased the abundance of mRNAs associated with cell proliferation, including mRNAs encoding cyclin-dependent kinase 1 (CDK1) and proliferating cell nuclear antigen (PCNA) (33, 34). As a positive control, we examined the effect of Yki activation in the eye using *GMR-Gal4;UAS-ykr^{S168A}* flies. We found that the expression of *CDK1* and *PCNA* was increased in heads dissected from *GMR-Gal4;UAS-ykr^{S168A}* flies relative to *GMR-Gal4* flies (Fig. 2A). In contrast, the expression of *CDK1* and *PCNA* was similar in dissected hearts of *tinC>ykr^{S168A}* and *tinC-Gal4* flies (Fig. 2A), suggesting that aberrant activation of Yki does not promote cell proliferation in the heart.

We directly assessed whether changes in heart wall thickness in *tinC>ykr^{S168A}* and *tinC>ykr^{RNAi}* flies were due to changes in the number of cardiomyocytes. We used a transgene encoding red fluorescent protein (RFP) fused with a nuclear localization sequence (*UAS-RFP^{nuc}*) in *tinC-GFP; tinC-Gal4* flies (*tinC>RFP^{nuc}* flies) to identify cardiomyocyte nuclei in the second and third abdominal segments (A2 and A3). Sixteen cardiomyocytes were present in *tinC>RFP^{nuc}* flies, *tinC>RFP^{nuc}; tinC>ykr^{S111A,S168A,S250A}* flies, and *tinC>RFP^{nuc}; tinC>ykr^{RNAi}* flies (Fig. 2B), indicating that changes in heart wall thickness in flies with cardiac-specific expression of constitutively active Yki or in flies with cardiac-specific loss of Yki occur in the absence of changes in the numbers of cardiomyocytes.

Cardiac hypertrophy in mammals, including humans who have longstanding hypertension, is associated with an increase in myocyte ploidy due to increased endoreplication, a process of genomic DNA synthesis without cytokinesis (35–37). We found that the average ploidy was increased in cardiomyocytes of *tinC>ykr^{S168A}* flies compared to *tinC-GAL4* flies (Fig. 2C), consistent with increased endoreplication. Loss of Yki in *tinC>ykr^{RNAi}* flies did not affect average ploidy (Fig. 2C). Collectively, these results suggest that unlike in mammals, in the fly, Yki controls heart organ size through hypertrophy rather than hyperplasia.

Sd is required for cardiac hypertrophy induced by activated Yki

Several transcription factors interact with Yki, including Sd, Mothers against dpp (Mad), and homothorax (Hth) (20, 38–40). Therefore, we examined the effects of loss of Sd, Mad, or Hth on *ykr^{S168A}*-induced cardiac hypertrophy. Expression of either of two independent RNAi constructs targeting *Sd* in the eye reduced *Sd* abundance by ~50% (fig. S1). Moreover, cardiac-specific expression of either *Sd* RNAi construct increased EDDs and decreased heart wall thickness in *tinC>ykr^{S168A}* flies, but not in *tinC-Gal4* flies (fig. S4). In contrast, RNAi constructs directed against *Mad* or *hth*, which reduced the abundance of *Mad* by ~40% or *hth* by ~70% when expressed in the eye (fig. S1), did not abrogate cardiac abnormalities in adult *tinC>ykr^{S168A}* flies when expressed in the heart (fig. S4). Thus, Sd, but not Mad or Hth, is required for Yki^{S168A}-induced cardiac hypertrophy.

Yki is required for Raf-induced cardiac hypertrophy

Similar to cardiac-specific expression of activated Yki, cardiac-specific expression of a transgene encoding constitutively active human Raf (hRaf^{Act}), which has a deletion of amino acids 2 to 334, causes cardiac hypertrophy in *Drosophila* (9). Therefore, we tested if Yki was required for Raf^{Act}-induced cardiac hypertrophy. Compared to *tinC-GFP*; *tinC-Gal4*; *UAS-hRaf^{Act}* (*tinC>hRaf^{Act}*) flies, *tinC>hRaf^{Act}* flies with *UAS-ykr^{RNAi}* (*tinC>hRaf^{Act} + ykr^{RNAi}* flies) had increased EDDs and ESDs and decreased heart wall thicknesses (Fig. 3, A to C). Moreover, EDDs and heart wall thicknesses in *tinC>hRaf^{Act} + ykr^{RNAi}* flies were similar to those in *tinC-Gal4* flies (Fig. 3, A to C). Cardiac-specific expression of hRaf^{Act} causes abnormal cardiomyocyte fiber morphology (9). *tinC-GFP*-positive cardiomyocytes in hearts dissected from *tinC>hRaf^{Act}* flies lacked well-defined circumferential fibers, whereas cardiomyocytes in *tinC>hRaf^{Act} + ykr^{RNAi}* flies had partially rescued fiber abnormalities (Fig. 3D). In contrast, cardiac-specific expression of *Sd* RNAi did not rescue Raf^{Act}-induced cardiac hypertrophy in *tinC>hRaf^{Act}* flies (fig. S5). Thus, these data suggest that Yki, but not Sd, is at least partially required for cardiac defects induced by aberrant activation of Raf.

We also tested if knockdown of Raf abrogated heart defects in *tinC>ykr^{S168A}* flies. Eye-specific expression of either of two independent RNAi constructs targeting *Raf* decreased the abundance of Raf by ~35% (fig. S1). However, neither *Raf* RNAi construct reduced heart wall thicknesses in *tinC>ykr^{S168A}* flies when expressed in the heart (fig. S6). In contrast, RNAi directed against ERK (41) partially reduced heart wall thicknesses in *tinC>ykr^{S168A}* flies (fig. S6), suggesting that ERK, but not Raf, is required for the ability of activated Yki to induce cardiac hypertrophy.

Raf signaling through MEK and ERK enhances Yki-induced Sd-dependent transcription in mammalian cells

A point mutation in human *RAF* that results in constitutive activation of Raf occurs in patients with Noonan syndrome, who present with cardiac hypertrophy (3, 4). Therefore, we investigated whether Raf and Yki could coactivate Sd-dependent transcription in human cells using cultured human embryonic kidney (HEK) 293T cells. We evaluated Sd-dependent transcription using a luciferase-based reporter comprising three consensus Sd-binding motifs from the *Drosophila* serum response factor enhancer and a minimal promoter (Sd-reporter) (21). Expression of mouse Raf^{L613V} (mRaf^{L613V}), equivalent to the mutant human Raf found in patients with Noonan syndrome, did not activate the Sd-reporter (Fig. 4A). Moreover, expression of mRaf^{L613V} did not enhance the ability of Sd to activate the Sd-reporter (Fig. 4A). However, expression of mRaf^{L613V} increased Sd-reporter activity in cells coexpressing Sd and either wild-type Yki (Yki^{WT}) or Yki^{S168A} (Fig. 4A). Additionally, neither expression of wild-type mRaf (mRaf^{WT}) nor expression of mRaf^{L613V} with a mutation that abolishes catalytic activity (mRaf^{K375M,L613V}) (fig. S7A) (42) enhanced Sd-reporter activity in cells expressing Sd and Yki^{S168A} (Fig. 4B).

We also tested if MEK activity was required for activation of the Sd-reporter by Raf and Yki. The MEK inhibitor PD98059 inhibited phosphorylation of ERK in HEK293T cells expressing mRaf^{L613V} (fig. S7B). Moreover, PD98059 inhibited Sd-reporter activity in cells expressing mRaf^{L613V} and Sd or in cells expressing Sd, mRaf^{L613V}, and Yki^{WT} (Fig. 4C). Inhibition of ERK using FR180204 (43), which partially reduced phosphorylation of the ERK target ELK (fig. S7C), partially inhibited activation of the Sd-reporter in HEK293T cells expressing Sd, mRaf^{L613V}, and Yki^{S168A} (Fig. 4D).

We also determined if the mammalian orthologs of Yki and Sd could be activated by Raf. We coexpressed mRaf^{L613V} with human TEAD3 or TEAD4 and human YAP^{S127A} in HEK293T cells with the Sd-reporter or a connective tissue growth factor (CTGF)-luciferase-based reporter, which can be activated by TEADs and YAP (22). Expression of mRaf^{L613V} increased activation of the Sd- and CTGF-reporter in cells expressing YAP^{S127A} and TEAD3 or TEAD4 (fig. S8).

The Tondu domain-containing repressor Tgi inhibits Raf^{Act}- or Yki^{S168A}-induced cardiac hypertrophy

Expression of activated Raf enhanced Yki-induced activation of Sd-mediated transcription in cultured cells, whereas Yki, but not Sd, was required for Raf-induced cardiac hypertrophy in flies. Because Sd, in combination with Tgi but in the absence of Yki, represses the expression of target genes (12), we tested whether cardiac-specific overexpression of Tgi could inhibit Raf-induced cardiac hypertrophy.

Expression of Tgi inhibited Sd-reporter activity in HEK293T cells expressing Sd and Yki^{S168A} or Sd, Yki^{S168A}, and mRaf^{L613V} (Fig. 5A). Moreover, cardiac-specific expression of Tgi in *tinC-Gal4; UAS-Tgi* flies increased EDDs and decreased heart wall thicknesses in *tinC>yki^{S168A}* flies and *tinC>Raf^{Act}* flies (Fig. 5, B to E). These findings suggest that there is an equilibrium between Yki and Sd complexes and Tgi and Sd complexes that may be

mediated by Raf signaling, and that altering the balance of these complexes produces cardiac hypertrophy (Fig. 5F).

DISCUSSION

We found that the transcriptional coactivator Yki comprises a signal that contributes to Raf^{Act}-induced cardiac hypertrophy in flies. Cardiac-specific expression of activated Yki produced cardiac hypertrophy, whereas cardiac-specific knockdown of Yki inhibited Raf^{Act}-mediated cardiac hypertrophy. Knockdown of Raf did not inhibit hypertrophy induced by aberrant activation of Yki in the heart, suggesting that Yki acts downstream of Raf. Moreover, expression of activated Raf increased Yki-or YAP-induced activation of Sd- or TEAD-dependent transcription in human cultured cells.

The evolutionarily conserved Hippo-Yki pathway controls organ size, including the heart, in several species (10, 44). The Yki ortholog YAP was initially identified in lysates of chicken embryonic fibroblasts and is present in various mammalian tissues, including the heart (45, 46). Genetic activation of YAP produces cardiac hyperplasia in mice (23–25). Cardiac-specific expression of activated YAP in mice during heart development causes abnormally thickened myocardia and increased immunostaining for phosphorylated histone-H3 in the heart, indicative of increased proliferation of cardiomyocytes (25). Moreover, the size of hearts in adult mice is not affected by abnormal activation of YAP during development, suggesting that there may be compensatory mechanisms to normalize heart size by reducing the size of cardiomyocytes (25). Expression of activated YAP in the hearts of postnatal mice increases the expression of genes involved in the cell cycle and stimulates proliferation of cardiomyocytes, and these effects require TEAD1 (24). Cardiac-specific genetic ablation of *Yap1* in developing mice causes abnormally thin myocardia (24, 25), associated with fewer ventricular myocytes and embryonic lethality (25). Thus, these data suggest that YAP is important for cardiomyocyte proliferation during development.

In flies, we found that aberrant activation of Yki increased cardiomyocyte cell size rather than cell number. Expression of Yki^{S168A} in the fly eye or wing causes cell proliferation (10, 18, 19). However, cardiac-specific expression of Yki^{S168A} caused hypertrophy without increasing the number of cardiomyocytes or the expression of genes associated with the cell cycle. Moreover, cardiac-specific expression of RNAi directed against *yki* resulted in enlargement of the heart chamber and thinning of the heart walls without changing the number of cardiomyocytes. Thus, Yki appears to control myocyte morphology rather than proliferation in the fly heart. One possible explanation for the difference between cardiomyocyte proliferation in mammals and hypertrophy in flies is that the cardiomyocytes in flies transition from cell division to endoreplication. Although speculative, signals that block in cytokinesis and/or induce endoreplication may exist, and the fly represents a potential model to identify these possibilities.

Using temperature shift experiments to control Yki^{S168A} transgene expression, we found that aberrant activation of Yki in larval flies, but not in adult flies, produced heart defects. One possible explanation for this result is that the cardiac-specific driver used in this study (*tinC-Gal4*) induced the expression of transgenes during a developmental stage after

cardiomyocyte proliferation. Alternatively, fly cardiomyocytes may have different signals that control proliferation during embryonic and adult stages, similar to adult mammalian cardiomyocytes. Comparing the developmental and interspecies differences between the signals that govern cardiomyocyte growth and proliferation may reveal evolutionary changes that resulted in the differences in heart size and morphology across diverse phyla.

Crosstalk between Raf and Yki occurs by several mechanisms (26, 27). The *Drosophila* ortholog of RASSF competes with Salvador for binding to Hippo, thereby reducing Hippo activity in wings and eye discs (47). In human cells in culture, Raf inhibits MST2 in a manner independent of Raf kinase activity and ERK activation by preventing MST2 dimerization and recruiting a phosphatase that removes activating phosphorylations on MST2 (27). We observed that the kinase activities of Raf and MEK were required for the ability of mutant active Raf to enhance Yki-induced activation of an Sd-reporter in cell culture. These findings suggest a mechanism of crosstalk between catalytically active Raf-MEK signaling and the Yki- and Sd-containing transcription complex. Pharmacological inhibition of MEK ameliorates Noonan syndrome-like phenotypes in heterozygous Raf^{L613V} knock-in mice (4). Whether YAP-TEAD signaling is involved downstream of Raf and MEK in causing the phenotypes in this mouse model remains to be investigated.

Pharmacological inhibition of ERK using FR180204 partially reduced the ability of Raf^{L613V} to augment Yki- and Sd-induced Sd-reporter activity in cultured cells. Exposing cells to FR180204 inhibited phosphorylation of the ERK-target ELK, but we cannot rule out the possibility of off-target effects by FR180204 on other kinases. Cardiac-specific expression of RNAi targeting ERK partially rescued Yki^{S168A}-induced cardiac hypertrophy in flies. We previously found that cardiac-specific expression of ERK RNAi completely rescues Raf^{Act}-induced cardiac hypertrophy (9). Thus, ERK may be involved in the crosstalk between Raf and Yki in the heart, but further studies are required to determine the potential involvement of other kinases.

Raf signaling through Yki and Sd in the fly heart is probably more complex than a simple linear model (fig. S9). Flies with cardiac-specific knockdown of Yki, but not Sd, had dilated hearts. However, cardiac-specific knockdown of Sd rescued cardiac hypertrophy induced by expression of Yki^{S168A} in the heart. Cardiac-specific knockdown of Yki, but not Sd, inhibited Raf^{Act}-induced cardiac hypertrophy. Similar observations pertaining to Yki and Sd are reported for phenotypes in the fly eye, ovarian follicle cells, and wing discs, and led to the identification of Tgi, which binds Sd and is required for Sd-mediated transcriptional repression in the absence of coactivators, such as Yki (12). There are four orthologs of Tgi in mammals, including humans, named Vestigial-like (Vgll) proteins (48, 49). Vgll4 decreases the activity of TEAD1 and counteracts activation of gene expression in cardiac myocytes by activation of α 1-adrenergic receptors (50). In other tissues, including lung and gastric tissues, Vgll proteins, including Vgll4, function as tumor suppressors by inhibiting YAP- and TEAD-based transcriptional activation (51, 52). We observed that cardiac-specific overexpression of Tgi inhibited the ability of Raf^{L613V} to enhance Yki-induced activation of an Sd-reporter in cell culture and the ability of cardiac-specific expression of Raf^{Act} or Yki^{S168A} to induce cardiac hypertrophy in flies. Thus, Tgi represses Yki-induced Sd activity and inhibits crosstalk between Raf and Yki and Sd (Fig. 5F). In addition, Tgi may act

through transcription factors other than Sd to attenuate Raf-induced cardiac hypertrophy. Moreover, Vgll proteins may be involved in Raf-induced cardiac hypertrophy in mammals, including humans with Noonan syndrome.

MATERIALS AND METHODS

Fly stocks

The *p{tinC-GFP}; p{tinC-Gal4}* stocks were derived from *p{tinC-GFP}* and *p{tinC-Gal4}* stocks as previously described (30, 53). *p{tinC-GFP}; p{UAS-RFP^{NUC}}; p{tinC-Gal4}* was homologously recombined using *p{tinC-GFP}; p{tinC-Gal4}* and *p{UAS-RFP^{NUC}}* transgenic stocks. *p{UAS-Tgi}* was obtained from D. Pan (12). All other fly stocks, including *p{UAS-yki^{S168A}.V5}* (stock #28818), *p{UAS-yki^{S111A,S168A,S250A}.V5}* (stock #28817), *p{UAS-yki^{RNAi}}* (stock #31965), *p{UAS-hRaf^{Act}}* (stock #2074), *p{UAS-sd^{RNAi}}* (stocks #29352 and #35481), *p{UAS-Raf^{RNAi}}* (stocks #31038 and #31596), *p{UAS-MAD^{RNAi}}* (stocks #31315, #31316, and #35648), *p{UAS-Hth^{RNAi}}* (stocks #27655 and #34637), and *p{UAS-ERK^{RNAi}}* (stock #34855), were obtained from the Bloomington Stock Center or the Transgenic RNAi Project (TRiP) at Harvard Medical School (www.flyrnai.org). The *p{UAS-Raf^{Act}}* stock corresponds to the truncated version of human Raf-1 (D2–334) (54). *P{tubP-Gal80^{ts}}; p{tinC-Gal4}* was generated as previously described (55). All fly stocks were maintained on standard cornmeal-yeast protein medium at room temperature (56).

Plasmids

The Sd luciferase reporter (3xSd2-Luc) was provided by J. Jiang (University of Texas Southwestern) (21). The CTGF-luciferase reporter was generated using *CTGF* promoter sequence described by Zhao *et al.* (22) cloned into pGL3-Basic Vector. cDNAs (complementary DNAs) corresponding to *yki* (clone LD21311) and *Sd* (clone IP16090) were obtained from the *Drosophila* Genomics Resource Center. cDNAs corresponding to *YAP^{S127A}*, *TEAD3*, and *TEAD4* were obtained from Addgene (15, 22). *TEAD3* and *TEAD4* were tagged on the C terminus with the hemagglutinin (HA) epitope, wild-type *yki* and *yki^{S168A}* were tagged on the N terminus with a V5 epitope, and *Sd* was tagged on the C terminus with an HA epitope by PCR and subcloned into pCDNA3.1. cDNA encoding mouse wild-type *Raf* was amplified by reverse transcription PCR (RT-PCR) from C57B6 mouse heart RNA, tagged on a C-terminal Flag epitope, and sub-cloned into pCDNA3.1. Mouse *Raf^{L613V}* and *Raf^{K375M,L613V}* were generated by PCR mutagenesis and subcloned into pCDNA3.1. Fly *Tgi* was amplified by RT-PCR from total RNA from *w¹¹¹⁸* flies, tagged on the N terminus with a Myc epitope, and subcloned into pCDNA3.1. All plasmids were sequenced across the entire open reading frame for validation.

OCT measurement of cardiac function in adult *Drosophila*

Cardiac function in *Drosophila* was measured using a custom-built OCT microscopy system (Bioptigen Inc.) as previously described (9, 30, 53). EDD and ESD were determined from three consecutive heartbeats. Fractional shortening was calculated as $[\text{EDD} - \text{ESD}] / \text{EDD} \times 100$.

Histological analysis

Fly heart wall thicknesses were measured as previously described (31, 53). Briefly, adult female flies of 2 to 7 days after eclosion were collected and fixed in Telly's fixation buffer (60% ethanol, 3.33% formalin, 4% glacial acetic acid) for at least 1 week at 4°C. Flies were embedded in paraffin, serially sectioned at 8-μm thicknesses, and stained with hematoxylin and eosin. Hearts were imaged using a Leica DM2500 microscope equipped with a Leica DFC310 FX digital camera. Wall thickness was calculated by measuring the cardiac chamber wall width along the middorsal, midventral, left lateral, and right lateral wall in three serial sections per fly.

Evaluation of adult cardiac morphology and ploidy

Adult *Drosophila* corresponding to the F1 offspring of *p{tinC-GFP}; p{tinC-Gal4}* stocks crossed to specific *p{UAS-transgenes}* or *w¹¹¹⁸* (controls) were collected at 2 to 3 days of age, post-eclosion, to examine adult cardiac morphology as previously described (9, 57). Dissected specimens were stained with an antibody against GFP (1:500) (Invitrogen) and a secondary antibody conjugated to Alexa Fluor 488 (1:500) (Invitrogen). Labeled heart preparations were imaged using a Zeiss LSM 510 confocal microscope, and 0.4-μm Z-stack images were collected.

Cardiomyocyte ploidy was measured as previously described (9, 58). Briefly, adult *Drosophila* corresponding to the F1 offspring of *p{tinC-GFP}; p{tinC-Gal4}* stocks crossed to specific *p{UAS-transgenes}* or *w¹¹¹⁸* (controls) were collected at 2 to 3 days after eclosion, and hearts and testis were dissected. Testis are haploid and therefore were used as internal controls. Chromosome number was determined by quantification of TO-PRO-3 staining of cardiac nuclei compared to haploid testis nuclei. Ploidy was expressed as the *C* value, where a *C* value of 1 refers to the amount of DNA contained within a haploid nucleus.

Quantitative RT-PCR

Total RNA samples from 20 to 30 dissected adult fly hearts or 15 to 25 fly heads per experiment were extracted using RNA-Bee (Tel-Test "B"). Two micrograms of RNA was used for generation of cDNA using SuperScript II reverse transcriptase (Invitrogen Inc.). Applied Biosystems TaqMan Gene Expression Assays were used to perform quantitative (real-time) qRT-PCR (table S1) with ribosomal protein L32 (Rpl32): Dm 02151827-g1 as an endogenous control. The following reaction components were used for each probe: 2 μl of cDNA, 12.5 μl of 2X TaqMan Universal PCR Master Mix without AmpErase (Applied Biosystems Inc.), 1.25 μl of probe, and 9.25 μl of water in a 25-μl total volume. Reactions were amplified and analyzed in triplicate using a Bio-Rad CFX96 Real-Time System. PCR conditions were as follows: step 1: 95°C for 10 min; step 2: 40 cycles of 95°C for 15 s followed by 60°C for 1 min. Expression relative to Rpl32 was calculated using $2^{-\Delta C_t}$, and levels were normalized to baseline.

Cell culture, luciferase assays, and Western blots

HEK293T cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were plated in triplicate wells in 24-well plates and

transfected with FuGENE HD (Promega Inc.) and the following amounts of plasmid DNA: 100 ng of Sd-reporter or CTGF-reporter; 20 ng of *Renilla* luciferase (*pBIND*, Promega); 200 ng of Yki^{WT}, Yki^{S168A}, or YAP^{S127A}; 200 ng of Sd, hTEAD3, or hTEAD4; 200 ng of Tgi; and 800 ng of mRaf^{WT}, mRaf^{L613V}, or mRaf^{K375M,L613V}, or pcDNA3.1 to make the total amounts of transfected DNA the same for each experiment. Cells were harvested after 48 hours of transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's protocol using a BMG Labtech NOVOstar plate reader. DMSO, PD98059 (10 mM; Santa Cruz Biotechnology), FR180204 (2, 5, or 10 μ M; Santa Cruz Biotechnology), or DMSO was added to the transfected cells 24 hours before harvest.

For Western blots, HEK293T cells were seeded in six-well plates and transfected with 2 μ g of mRaf^{WT}, mRaf^{L613V}, mRaf^{K375M,L613V}, or pcDNA3.1 (2 μ g each) and incubated for 48 hours. Cells were harvested and lysed in modified radioimmunoprecipitation assay (RIPA) buffer [137 mM NaCl, 50 mM tris-HCl (pH 8.0), 5 mM EDTA, 1% NP-40, 0.5% (w/v) Na deoxycholate, 10% glycerol] containing phosphatase inhibitors (Roche, #04906837001) and protease inhibitors (Roche, #11836170001) for 3 hours at 4°C. Samples containing 100 μ g of total protein were resolved on SDS–polyacrylamide gels before transfer to Immobilon PVDF (polyvinylidene difluoride) Transfer Membranes (Millipore). The following antibodies were used: rabbit phosphorylated ERK (Thr²⁰² and Tyr²⁰⁴) (Cell Signaling Technology, #9101) (1:1000), rabbit ERK (Millipore, # 06-182) (1:3000), rabbit ELK (Cell Signaling Technology, # 9182S) (1:1000), rabbit phosphorylated ELK (Ser³⁸³) (Cell Signaling Technology, # 9181S) (1:1000), and rabbit Flag (Sigma, #F7425) (1:1000).

Statistical analysis

GraphPad Prism (GraphPad Software Inc.) software was used for all statistical analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Expression of activated Yki causes cardiac hypertrophy, and knockdown of Yki causes cardiac dilation

(A) Representative longitudinal and transverse B- and M-mode OCT images of hearts of flies with the indicated genotypes. Arrows denote cardiac chambers, and arrowheads denote EDDs and ESDs. (B) Representative transverse sections of hearts in the A1 segment from flies with the indicated genotypes. Scale bar, 50 μm. (C) Quantification of heart wall thicknesses from images similar to those shown in (B). Data are means ± SEM for $n = 4$ to 7 flies per genotype. * $P < 0.05$ compared to control. Analysis of variance (ANOVA) with Tukey's post hoc test. (D) Quantification of EDDs, ESDs, and fractional shortening from images similar to those shown in (A). Data are means ± SEM for $n = 8$ flies per genotype. * $P < 0.05$ for indicated cardiac parameter for *yki^{RNAi}* compared to control. Two-sample t test.

Fig. 2. Expression of activated Yki induces cardiac hypertrophy not hyperplasia

(A) Graph of the relative abundance of *CDK1* and *PCNA* mRNA measured by quantitative polymerase chain reaction (qPCR) in dissected hearts of flies with cardiac-specific (*tinC*) or in dissected heads of flies with eye-specific (*GMR*) expression of the indicated transgenes. Data are means $\pm$ SEM for $n = 3$ to 5 experiments per genotype normalized to the negative control genotype. * $P < 0.05$ for *GMR>yki^{S168A}* compared to *GMR-Gal4*. Two-sample *t* test. (B) Images of cardiomyocytes in the A2 and A3 segments for *tinC-GFP; tinC>RFP^{nuc}* flies of the indicated genotypes. All transgenes were heterozygous. Images are representative of 10 flies per genotype. Scale bar, 200 μ m. (C) Distribution of cardiomyocyte ploidy (*C* value) of hearts of flies with the indicated genotypes. The median *C* value is shown for each genotype in parenthesis. $n = 18$ to 60 cardiomyocytes per genotype. * $P < 0.0001$ by Wilcoxon signed rank test.

Fig. 3. Knockdown of Yki inhibits Raf^{Act}-induced cardiac hypertrophy
(A and B) Quantification of EDDs (A) or ESDs (B) from M-mode OCT of hearts of flies with the indicated genotypes. Data are means $\pm$ SEM for $n = 16$ to 29 flies per genotype. * $P < 0.05$ compared to control. # $P < 0.05$ compared to *tinC>hRaf^{Act}*. ANOVA with Tukey's post hoc test. **(C)** Quantification of heart wall thicknesses of hearts of flies with the indicated genotypes ($n = 4$ to 7 per group). * $P < 0.05$ compared to control. # $P < 0.05$ compared to *tinC>hRaf^{Act}*. ANOVA with Tukey's post hoc test. **(D)** Representative confocal microscopy with Z-stack reconstruction showing cardiac fiber morphology of hearts of flies with the indicated genotypes. All flies were examined in a *tinC-GFP* heterozygous background. *tinC-GFP*-positive cardiomyocytes were immunolabeled for GFP, din. Arrow denotes cardiomyocyte fibers. Images are representative of three to five flies.

Fig. 4. Activated Raf enhances Yki-induced Sd activity in a manner that depends on the kinase activities of Raf, MEK, and ERK

(A to D) Quantification of luciferase activity from an Sd-reporter in HEK293T cells transfected with the indicated plasmids. Data are means $\pm$ SEM for $n = 3$ experiments per group. (A) * $P < 0.05$ compared to control. # $P < 0.05$ compared to Sd + yki^{WT}. $P < 0.05$ compared to Sd + yki^{S168A}. ANOVA with Tukey's post hoc test. (B) * $P < 0.05$ compared to control. # $P < 0.05$ compared to Sd + Yki^{S168A}. ANOVA with Tukey's post hoc test. (C) Transfected cells were exposed to vehicle [dimethyl sulfoxide (DMSO)] or the MEK inhibitor PD98059 (10 mM) for 24 hours before lysis. * $P < 0.05$ compared to control. # $P < 0.05$ compared to Sd + Yki^{WT}. $P < 0.05$ compared to Sd + Yki^{WT} + mRaf^{L613V} exposed to vehicle. ANOVA with Tukey's post hoc test. (D) Transfected cells were exposed to DMSO or FR1800204 for 24 hours before lysis. Data are means $\pm$ SEM for $n = 3$ experiments per group. * $P < 0.05$ compared to control. # $P < 0.05$ compared to Sd + Yki^{S168A}. $P < 0.05$ compared to Sd + Yki^{S168A} + mRaf^{L613V} + DMSO. ANOVA with Tukey's post hoc test.

Fig. 5. Overexpression of Tgi inhibits the ability of activated Raf to enhance Yki^{S186A}-induced Sd activity in cultured cells and Yki^{S186A}- or Raf^{Act}-induced cardiac hypertrophy in flies
(A) Quantification of luciferase activity from an Sd-reporter in HEK293T cells transfected with the indicated plasmids. Data are means $\pm$ SEM for $n = 3$ experiments per group. $*P < 0.05$ compared to control. $\#P < 0.05$ compared to Sd + Yki^{S186A}. $P < 0.05$ compared to Sd + Yki^{S186A} + mRaf^{L613V}. ANOVA with Tukey's post hoc test. **(B and C)** Representative M-mode OCT (B) and quantification (C) of EDDs of hearts of flies with the indicated genotypes. Data are means $\pm$ SEM for $n = 15$ to 32 per group. $*P < 0.05$ compared to control. $\#P < 0.05$ compared to *tinC>yki^{S186A}*. $P < 0.05$ compared to *tinC>hRaf^{Act}*. ANOVA with Tukey's post hoc test. **(D and E)** Representative histology (D) and quantification (E) of heart wall thicknesses of hearts of flies with the indicated genotypes. $n = 4$ to 6 per genotype. $*P < 0.05$ compared to control. $\#P < 0.05$ compared to *tinC>yki^{S186A}*. $P < 0.05$ compared to *tinC>hRaf^{Act}*. Scale bars, 50 μ m. **(F)** Model of Raf^{Act}- and Yki-induced cardiac hypertrophy.