The Hippo pathway acts via p53 and microRNAs to control proliferation and proapoptotic gene expression during tissue growth

Wei Zhang1 and Stephen M. Cohen1,2

1Institute of Molecular Cell Biology, 61 Biopolis Drive, Singapore 138673
2Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543

Authors for correspondence (scohen@imcb.a-star.edu.sg; wzhang@imcb.a-star.edu.sg)

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Summary
The Hippo pathway has a central role in coordinating tissue growth and apoptosis. Mutations that compromise Hippo pathway activity cause tissue overgrowth and have been causally linked to cancer. In Drosophila, the transcriptional coactivator Yorkie mediates Hippo pathway activity to control the expression of cyclin E and Myc to promote cell proliferation, as well as the expression of bantam miRNA and DIAP1 to inhibit cell death. Here we present evidence that the Hippo pathway acts via Yorkie and p53 to control the expression of the proapoptotic gene reaper. Yorkie further mediates reaper levels post-transcriptionally through regulation of members of the miR-2 microRNA family to prevent apoptosis. These findings provide evidence that the Hippo pathway acts via several distinct routes to limit proliferation-induced apoptosis.

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Key words: p53, Hippo pathway, Yorkie/YAP, reaper, MicroRNA, Apoptosis, Proliferation

Introduction
Cell proliferation is intimately linked with cell death. Cues that drive cell growth and division also induce apoptosis (Pelengaris et al., 2002). In an abnormal cell proliferation scenario, such as cancer, cells adopt a variety of strategies to overcome cell death (Hanahan and Weinberg, 2011). Many signaling pathways that drive tissue growth have been found to coordinate cell proliferation and apoptosis during animal development. Defects in these pathways quite often cause tissue overgrowth or cancer.

The Hippo pathway is one such signaling pathway, acting as a negative growth regulator. Mutations in several members of the pathway lead to tumorigenesis, implicating them as tumor suppressors (Cai et al., 2010; Pan, 2010). The core pathway comprises a kinase cascade including the Hippo and Warts kinases with their adaptors Salvador (Sav) and Mats (Moberg et al., 2001; Tapon et al., 2002; Harvey et al., 2003; Udan et al., 2003; Wu et al., 2003). Several proteins have been implicated as upstream regulators of this kinase cascade by genetic studies, including Merlin/NF2, Expanded and the atypical cadherin, Fat (Hamaratoglu et al., 2006; Silva et al., 2006; Yu et al., 2010).

Downstream, the Warts kinase directly phosphorylates and inactivates transcriptional coactivators including YAP, TAZ, and in Drosophila, Yorkie (Yki) (Huang et al., 2005; Zhao et al., 2007; Zhao et al., 2008; Zhao et al., 2010). YAP/TAZ and Yki function to promote cell proliferation and inhibit apoptosis. These proteins possess no DNA binding activity and therefore bind to transcription factors including Scalloped/TEAD to activate their targets.

Genetic studies have identified functional targets of Yki with positive roles in cell proliferation, including cycE and Myc, as well as negative regulators of apoptosis, including the Drosophila Inhibitor of Apoptosis Protein (DIAP1) and the antiapoptotic microRNA bantam (Moberg et al., 2001; Moberg et al., 2004; Huang et al., 2005; Nolo et al., 2006; Thompson and Cohen, 2006; Neto-Silva et al., 2010). These studies have suggested that the Hippo pathway can balance proliferative drive with limitation of proliferation-induced apoptosis. This combination of roles may also explain the potency of mammalian YAP in control of tissue growth and its ability to induce cancer when overexpressed (Dong et al., 2007; Pan, 2010).

The tumor suppressor p53 is another key regulator coordinating cell division and cell death. Activation of p53 by the DNA damage checkpoint or other cell cycle abnormalities, leads to growth arrest, and initiates apoptosis. Activated p53 binds DNA and directs expression of downstream genes including p21, which inhibits the activity of cyclin–CDK complexes and activates cell cycle checkpoints to halt cell division (Gartel and Radhakrishnan, 2005). In addition, p53 promotes transcription of the proapoptotic genes Bax, PUMA and Apaf-1 to induce cell death. Recent studies in human cells have identified the ASPP1 protein (apoptosis-stimulating protein of p53-1) as a key mediator of p53-induced apoptosis (Aylon et al., 2010; Vigneron et al., 2010). The Hippo pathway kinase Lats,
the mammalian homolog of Warts, phosphorylates ASPP1 and forms a complex with ASPP1 and p53 to activate the proapoptotic transcription program. Phosphorylation of ASPP1, however, can be antagonized by another Lats substrate YAP.

The upstream control of the apoptosis program is conserved in Drosophila, with p53 serving as a mediator of the DNA damage checkpoint. However, the effector program involves a set of insect-specific proapoptotic genes: reaper, head involution defective (hid), grim and sickle (skl) (Steller, 2008). The proapoptotic activity of these four proteins results from their ability to bind and inactivate DIAP, which in turn inhibits caspases. In mammals the corresponding functions are provided by Apaf-1 to cleave and activate caspases instead of derepression of caspases (Pop et al., 2006). Previous studies in Drosophila have shown that the bantam microRNA acts to repress hid to limit proliferation induced apoptosis (Brennecce et al., 2003). bantam mediates interaction between the EGFR and Hippo growth control pathways (Herranz et al., 2012). microRNAs of the miR-2 seed family have also been shown to regulate the expression of the proapoptotic genes reaper, grim and skl (Stark et al., 2003; Brennecce et al., 2005; Leaman et al., 2005; Thermann and Hentze, 2007) and to limit apoptosis in the developing nervous system (Ge et al., 2012).

In view of the importance of the Hippo pathway in regulating proliferation-induced apoptosis, we have examined other modes of action for Yki. Here we provide evidence for additional parallel pathways involving Yki, p53 and the miR-2 family of microRNAs in controlling the expression of reaper another key proapoptotic gene. Yki acts via regulation of p53 on reaper transcription. In some tissues, Yki acts independently via members of the miR-2 family to regulate expression of reaper post-transcriptionally. Our findings place Yki at the center of a network of regulatory relationships balancing cell proliferation, p53-dependent checkpoints, proapoptotic genes and miRNAs in control of tissue growth.

Results
Hippo pathway controls apoptosis by limiting reaper expression
The transcription coactivator Yorkie mediates Hippo pathway activity to control gene expression in Drosophila. We used RNAi to deplete yorkie (yki) mRNA from S2 cells, to assess the contribution of the Hippo pathway to expression of genes involved in regulation of apoptosis. Depletion of yki mRNA was effective, and resulted in increased expression of reaper mRNA and a smaller increase in hid mRNA (Fig. 1A, **P<0.01). To test this relationship in a growing tissue, yki mRNA was overexpressed in the wing imaginal disc under control of mubbin-Gal4. yki overexpression decreased the level of reaper mRNA (Fig. 1B, P<0.01; control for yki mRNA level in supplementary material Fig. S1A). Thus Yki appears to negatively regulate expression of reaper.

Does regulation of reaper contribute to the growth regulatory activity of the Hippo pathway in vivo? We made use of patched-Gal4 (ptc-Gal4) to direct depletion of yki in a defined region of the wing (shaded in Fig. 1C). Expression of a UAS-ykiRNAi transgene under ptc-Gal4 control reduced the area of the relevant region of the wing (Fig. 1C). This effect was quantified by measuring the ratio of the width of the vein 3–4 region to that of the vein 4–5 region (indicated by solid and dashed red lines, upper left panel of Fig. 1C). Depletion of yki reduced the relative size of the region where the Gal4 driver was expressed (Fig. 1D).

This effect was partially offset by concurrently limiting reaper expression using a chromosomal deletion, Df(3L)XR38, which removes reaper and skl, but not the adjacent grim and hid genes (Peterson et al., 2002). Df(3L)XR38 on its own showed no effect on growth, but limited the undergrowth caused by yki depletion (Fig. 1C,D; P<0.001). These observations suggest that increased expression of reaper contributes to the effects of yki depletion in vivo.

Yki acts via induction of p53 activity
Previous reports have shown that p53 can directly regulate reaper expression in Drosophila (Brodsky et al., 2000; Peterson et al., 2002; Zhou and Steller, 2003). This raised the possibility that Yki might act via p53 to control reaper during tissue growth in vivo. To test this we used the ptc-Gal4 UAS-ykiRNAi undergrowth assay. Coexpression of a dominant negative form of p53 (p53DN) partially suppressed the tissue undergrowth caused by depletion of yki (Fig. 2A; P<0.001). Expression of p53DN on its own had no effect on growth. Similarly, reducing p53 activity by introducing a null allele of the p53 gene also partially suppressed the effects of engrailed-Gal4 UAS-ykiRNAi on tissue growth (Fig. 2B; P<0.05). The p53 mutant had no effect on its own.

p53 can also be activated through the caspase Drong (Nedd2-like caspase, Nc (Wells et al., 2006; Shlevkov and Morata, 2012)). This raised the possibility that depletion of Yki by RNAi could lead to reduced DIAP1 expression and thereby trigger Drong-mediated activation of p53. To address this possibility, we depleted both Yki and Drong from S2 cells and found that the increase in reaper mRNA levels was not reduced compared to cells depleted of Yki only, as might have been expected if the effects of Yki depletion were mediated through this feedback loop (Fig. 2C). Furthermore, reaper mRNA levels were increased in wing discs coexpressing UAS-DIAP1 and UAS-YkiRNAi compared to UAS-YkiRNAi alone (Fig. 2D; P<0.05; DIAP1 overexpression quantified in supplementary material Fig. S1B). The increase in reaper levels may reflect improved survival of Yki-depleted cells when expressing DIAP1. Caspase activation due to low DIAP1 levels also seems unlikely to explain the effects of Yki depletion on reaper mRNA levels.

In mammalian cells expressing the oncogenic form of H-Ras, Lats, a component of the Hippo pathway, has been shown to phosphorylate ASPP1 and form a complex with ASPP1 and p53 to direct expression of pro-apoptotic genes (Aylon et al., 2010; Vigneron et al., 2010). To ask whether Drosophila ASPP (CG18375) might also be involved in the context of Yki regulation of p53 activity in normal tissue growth, we assessed the effects of removing one copy of the ASPP gene on reaper mRNA levels in wing discs. Quantitative RT-PCR showed that reaper mRNA was reduced by ~25%, when ASPP mRNA was reduced to ~50% in these discs (Fig. 2E; ***P<0.01). Next, we assessed the effects of depleting ASPP by RNAi and the effects of removing one copy of the ASPP gene in the ptc-Gal4 UAS-ykiRNAi undergrowth assay. In both scenarios reduced ASPP activity partially restored growth of the yki-depleted tissue (Fig. 2F,G; ***P<0.001).

Taken together, these observations suggest that the Hippo pathway acts through Yki and p53 to control reaper expression. The involvement of ASPP, suggests that this regulation is likely to be mediated through Yki binding to Lats/Wts and competing for ASPP1 phosphorylation, as described in mammalian cell culture models (Aylon et al., 2010; Vigneron et al., 2010).
we present evidence that limiting reaper levels by manipulating p53-ASPP1 activity contributes to suppressing the tissue growth effects of the Hippo pathway. This observation is consistent with a model in which the Hippo pathway regulates p53 activity to control proliferation-induced apoptosis.

Yki regulates miRNA expression to control reaper level

Previous reports have shown that microRNAs of the miR-2 seed family (Fig. 3A) can regulate reaper, grim and skl (Stark et al., 2003; Leaman et al., 2005; Brennecke et al., 2005; Thermann and Hentze, 2007). This prompted us to ask whether there might be a miRNA-based mechanism by which the Hippo pathway controls reaper expression. As a first step we asked which of the miR-2 family miRNAs is subject to regulation by the Hippo pathway in S2 cells. Depletion of yki in S2 cells by RNAi led to a significant reduction in the levels of expression of miR-2a and b (P<0.05, Fig. 3B; miR-13a/b were on average lower, but the effect was variable and so not statistically significant). miR-11 was not significantly changed. miR-6 is expressed at very low levels in S2 cells.

As a first step to address how Yki might regulate miR-2 expression, we sought to identify cis-regulatory control elements that direct expression of miR-2 loci in S2 cells. miR-2a-1, miR-2a-2 and miR-2b-2 are expressed as a cluster of 3 miRNAs located in an intron of the spitz gene (Fig. 3C). A 1.9 Kb DNA fragment covering the intronic sequences upstream of the miRNA cluster and spanning the next upstream exon proved sufficient to direct expression of a luciferase reporter gene in S2 cells (Fig. 3C; supplementary material Fig. S2A). We then used this luciferase reporter to assess the effects of depleting yki by RNAi. Expression of the miR-2a cluster reporter decreased significantly in yki-depleted cells (Fig. 3D), suggesting that Yorkie regulates transcription of the miR-2a cluster.

To further assess this regulation in vivo, we first asked whether overexpressing members of the miR-2 family could rescue the ptc-Gal4 UAS-ykiRNAi undergrowth assay. Coexpression of a miR-2a-2b cluster transgene or a miR-11 transgene suppressed the undergrowth of yki-depleted tissue caused by elevated reaper mRNA (Fig. 4A,B; P<0.001). Expression of miR-2a/2b or miR-11 on their own had no effect on growth. Next we introduced a
miR-2a sensor into the ptc-Gal4 UAS-ykiRNAi assay to report miR-2a activity in vivo. The sensor transgene expresses GFP under control of the ubiquitously-expressed tubulin promoter and carries two miR-2a sites in its 3' UTR (as described (Brennecke et al., 2005)). However, depletion of yki had no effect on the expression of the miR-2a reporter in wing imaginal discs (supplementary material Fig. S3). Although ectopically expressing members of miR-2 family could suppress undergrowth of yki RNAi tissue, the negative result with the miR-2a reporter suggests that the effects of Yki on reaper are not mediated by regulation of miR-2a expression in the wing discs. To ask whether this regulation occurred in other tissues in vivo, in addition to S2 cells, we expressed UAS-ykiRNAi ubiquitously under tubulin-Gal4 control and found a significant reduction of miR-2a and b in the whole 3rd instar larvae (Fig. 4C; P<0.05). These findings suggest that the Hippo pathway contributes to control of apoptosis through regulation of miR-2 expression in some but not all tissues.

Discussion
Studies conducted in mammals and Drosophila have suggested that the downstream effectors of the Hippo pathway, YAP/TAZ and Yki direct expression of multiple targets linking cell division and cell death. Identified targets include the cell cycle regulator cycE and the cellular growth effector Myc (Huang et al., 2005; Neto-Silva et al., 2010). When the level of Hippo pathway

Fig. 2. p53 mediates the effects of Yorkie. (A) Photomicrographs of adult wings of the indicated genotype. Left panels: ptc-Gal4 control flies. Right panels ptc-Gal4 driving expression of a UAS-ykiRNAi transgene to reduce yki mRNA levels in the ptc-Gal4 expression domain. Upper panels: control flies without expressing any other transgene. Lower panels: flies expressing a dominant negative form of p53, UAS-p53DN transgene. Histogram at right shows quantification of the effects on growth of the ptc-Gal4 expression domain. Error bars represent standard deviation from measurement of at least 8 wings for each genotype. *** indicates statistically significant increase in the width of the ptc-Gal4 expression domain when p53 activity was reduced (P<0.001). (B) Photomicrographs of adult wings of the indicated genotype, as in panel A, except that en-Gal4 was used to drive transgene expression in the posterior compartment (shaded), and the ratio of anterior (A) to posterior (P) was measured. Lower panels: flies carrying two copies of a null allele of p53(AA)1-4. Histogram shows quantification of the effects on growth of the P compartment. Error bars represent standard deviation from at least 4 wings for each genotype. * indicates statistically significant increase in the width of the ptc-Gal4 expression domain when p53 activity was reduced (P<0.05). (C) Histogram showing the levels of rpr, yki and Dronc (Nc) mRNAs. S2 cells were treated with dsRNA to deplete yki (grey bars) or both yki and Nc (black bars) or GFP as a control (white bars). Total RNA was extracted and normalized for cDNA synthesis. RNA levels were normalized to kinesin mRNA. Error bars represent standard deviation from 3 independent experiments. (*) Student’s t-test for rpr vs GAPDH: P<0.05; (**) Student’s t-test for rpr vs GAPDH: P<0.01. (D) Histogram showing the levels of rpr and yki mRNAs measured by quantitative RT-PCR. RNA was extracted from wing imaginal discs expressing ub-Gal4 alone (white bars) or ub-Gal4 with UAS-ykiRNAi and UAS-Diap1 transgenes. RNA levels were normalized to kinesin mRNA. Error bars represent standard deviation from 6 independent experiments. (**) Student’s t-test for rpr vs yki: P<0.05; (**) Student’s t-test for rpr vs yki: P<0.01. (E) Histogram showing the levels of rpr, GAPDH and ASPP mRNAs. RNA was extracted from wing imaginal discs of 3rd instar control larvae (+/+ or Df(2R)AA21/+) larvae (grey bars). RNA levels were normalized to rp49 mRNA. Error bars represent standard deviation from 3 independent experiments. (**) Student’s t-test for rpr vs GAPDH: P<0.01. (F) Photomicrographs of adult wings of the indicated genotype, as in panel A. Lower panels: flies expressing a UAS-ASPPRNAi transgene to reduce ASPP mRNA levels in the ptc-Gal4 domain. Histogram shows quantification of the effects of the UAS-ASPPRNAi transgene alone (left) and together with UAS-ykiRNAi (right). Left and right pairs were from separate experiments. The ratio of the L3–4 to L4–5 width is constant at 1.2:1 in all experiments. Error bars represent standard deviation from at least 7 wings for each genotype. *** indicates statistically significant increase in the width of the ptc-Gal4 expression domain when ASPP activity was reduced (P<0.001). (G) Photomicrographs of adult wings of the indicated genotype, as in panel A. Lower panels: flies carrying one copy of Df(2R)AA21, which removes the ASPP gene. Error bars indicate standard deviation from measurement of at least 8 wings for each genotype. *** indicates statistically significant increase in the width of the ptc-Gal4 expression domain when one copy of ASPP was removed (P<0.001).
activity of having adequate checkpoints to limit proliferation. Bypassing apoptosis and negative growth regulatory signals are important steps along the path to cancer (Hanahan and Weinberg, 2011).

Materials and Methods

Fly strains

Df(3L)XR38, which removes rpr and skl, but not hid and grim, is described by Peterson et al. (Peterson et al., 2002) and was provided by Kristin White. UAS-p53DN is described by Brodsky et al. (Brodsky et al., 2000). GUS:p53DN (p53.Cb), pS3A−/−, Df(2R)AA21 (flying), and UAS-miR-2a/2b lines were from the Vienna Drosophila RNAi Center. UAS-miR-2a/yki (transformant ID: 40497 and 104523) and UAS-RNAi-ASPP lines were from the Vienna Drosophila RNAi center. UAS-miR-2a/2b and miR-2a GFP sensor flies were described by Stark et al. (Stark et al., 2003). UAS-miR-11 transgene was described by Szulwewski et al. (Szulwewski et al., 2012).

Cell culture and treatments

S2 cells were grown at 25°C in SFM (Gibco) supplemented with L-glutamine. dsRNA was prepared using MegascriptT7 (Ambion) with the following templates: yki, nucleotides 331–875 of yki 215AA isoform coding sequence; Dronc, nt649–1122 of the ORF; CDG, nt 61–633 of EGFP2. S2 cells were treated with 37 nM dsRNA. The primers used to clone the 1.9 Kb DNA fragment before miR-2b-1 using the same method were: forward, 5′-GGATCCGGCTGCTAGCCCGGGCTCGAGAAACTTTGGTTTTGGAATATA-9′; reverse, 5′-AAGCTTACTTAGATCGCAGATC-9′. The primers used to clone the 1.5 Kb DNA fragment before miR-2b-1 using the same method were: forward, 5′-GGATCCGGCTGCTAGCCCGGGCTCGAGAAACTTTGGTTTTGGAATATA-9′; reverse, 5′-AAGCTTACTTGATCGCAGATC-9′.

Quantitative RT-PCR

Total RNA was extracted from S2 cells or wing imaginal discs and treated with DNase-1 to eliminate genomic DNA contamination. Reverse transcription to synthesize the first strand used oligo-dT primers and Superscript RT-III (Invitrogen). PCR was performed and analyzed on Applied Biosystems 7500 fast real-time PCR system. The following primers were used: yki-4,
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Student's t-test for miR-2 and normalized for cDNA synthesis. RNA levels were normalized to small RNA ubiquitous tubulin-Gal4 indicate inhibitory interactions.

Competing Interests

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Anti-GFP and Anti-Gal4 were incubated at 4 ˚C overnight. Secondary antibodies PBS with 4% paraformaldehyde at room temperature for 20 min, then rinsed and Wandering 3rd instar larvae were collected and dissected. Tissues were fixed in

Immunostaining and microscopy

Wandering 3rd instar larvae were expressed UAS-ykiRNAi together with UAS-ykiRasU. Error bars represent standard deviation from at least 6 wings for each genotype.

** indicates statistically significant increase in the width of the ptc-Gal4 expression domain.

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