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Hfp inhibits *Drosophila myc* transcription and cell growth in a TFIIH/Hay-dependent manner

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SUMMARY

An unresolved question regarding the RNA-recognition motif (RRM) protein Half pint (Hfp) has been whether its tumour suppressor behaviour occurs by a transcriptional mechanism or via effects on splicing. The data presented here demonstrate that Hfp achieves cell cycle inhibition via an essential role in the repression of *Drosophila myc* (*dmyc*) transcription. We demonstrate that regulation of *dmyc* requires interaction between the transcriptional repressor Hfp and the DNA helicase subunit of TFIIH, Haywire (Hay). In vivo studies show that Hfp binds to the *dmyc* promoter and that repression of *dmyc* transcription requires Hfp. In addition, loss of Hfp results in enhanced cell growth, which depends on the presence of dMyc. This is consistent with Hfp being essential for inhibition of *dmyc* transcription and cell growth. Further support for Hfp controlling *dmyc* transcriptionally comes from the demonstration that Hfp physically and genetically interacts with the XPB helicase component of the TFIIH transcription factor complex, Hay, which is required for normal levels of *dmyc* expression, cell growth and cell cycle progression. Together, these data demonstrate that Hfp is crucial for repression of *dmyc*, suggesting that a transcriptional, rather than splicing, mechanism underlies the regulation of dMyc and the tumour suppressor behaviour of Hfp.

KEY WORDS: *Drosophila*, Cell growth, Transcriptional repression, Half pint (Hfp; pUf68)

INTRODUCTION

Since the identification of the oncogenic potential of *c-myc* in the early 1980s (Vennstrom et al., 1982), the Myc family has been the focus of extensive investigation and key advances have been forged in understanding Myc function (reviewed by Eilers and Eisenman, 2008; Levens, 2002; Levens, 2003). The sole *Drosophila* member of the family, dMyc (Diminutive – FlyBase), is encoded by the *dm* locus and is functionally homologous to the *c-myc* proto-oncogene (Gallant et al., 1996; Johnston et al., 1999). Like c-Myc, dMyc drives ribosome biogenesis and growth and couples this with S-phase progression via upregulation of the genes required for DNA replication (de la Cova et al., 2004; Duman-Scheel et al., 2004; Grewal et al., 2005; Johnston and Gallant, 2002; Maines et al., 2004; Orian et al., 2003; Pierce et al., 2004; Prober and Edgar, 2002). Functional conservation with c-Myc has been demonstrated by the ability of dMyc to transform primary mammalian cells and rescue proliferation defects in *c-myc*-null fibroblasts (Schreiber-Agus et al., 1997). Conversely, the human c-MYC protein can rescue lethal mutations of *dmyc*, demonstrating the biological relevance of this model (Benassayag et al., 2005).

Expression profiling, genomic binding studies and genetic analyses in mammals (Coller et al., 2000; Grandori et al., 2000) and *Drosophila* (Grewal et al., 2005; Orian et al., 2005) have led to an understanding of the expansive function of Myc (Eilers and Eisenman, 2008), which is highlighted by the finding that Myc proteins control transcription of 10-15% of all genes (Grandori et al., 2005; Grewal et al., 2005). Although Myc proteins affect multiple targets (reviewed by Eilers and Eisenman, 2008; Levens, 2002; Levens, 2003), the ability to drive growth (Bouchard et al., 1998; Schmidt, 1999) appears crucial for the oncogenic properties of c-Myc during lymphoma (Barna et al., 2008; Ruggero et al., 2004). Increased *c-MYC* expression occurs in most human cancers (Liao and Dickson, 2000), but despite this our current understanding of the transcriptional regulation of *c-myc* is incomplete.

The RNA-recognition motif (RRM) domain-containing proteins FIR (also known as PUF60) in mammals and its *Drosophila* orthologue Half pint (Hfp; pUf68 – FlyBase) have been ascribed transcriptional (Liu et al., 2006) and splicing (Van Buskirk and Schupbach, 2002) roles. Previous studies have shown that loss of Hfp leads to changes in the relative abundance of the alternative splice variants for the ovary-specific genes *otu* and the eukaryotic initiation factor *eIF4E-I* (Reyes and Izquierdo, 2008; Van Buskirk and Schupbach, 2002). In these studies, reduction of Hfp led to splicing changes; however, further evidence is required to determine whether this effect is due to direct binding of Hfp to the proposed RNA targets. An unresolved question is whether Hfp mediates its tumour suppressor function (Quinn et al., 2004) by a transcriptional mechanism or via effects on splicing. The tumour suppressor behaviour of Hfp, and data that suggest that its closest mammalian homologue, FIR, behaves as a transcriptional repressor of *c-myc* (Liu et al., 2006; Liu and Levens, 2006), led us to investigate whether Hfp normally achieves repression of the cell cycle via repression of *dmyc* transcription.

The in vitro model of FIR as a *c-myc* transcriptional repressor is based on the following lines of investigation. In vitro, RNA polymerase II (Pol II) complex movement within the *c-myc* promoter is controlled by a regulatory sequence known as the far upstream sequence element (FUSE) (Benjamin et al., 2008; Chung and Levens, 2005; Crichlow et al., 2008; Duncan et al., 1994). Interactions between the FUSE, the fuse-interacting repressor (FIR)

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and the XPB helicase (also known as ERCC3) are proposed to regulate Pol II movement along the *c-myc* promoter (Liu et al., 2006; Liu and Levens, 2006). FIR binds both FUSE and the XPB helicase to create a loop upstream of the *c-myc* promoter to tether the TFIIH complex and to disrupt upstream effector elements and transcription factor binding, which results in repression of *c-myc* transcription. FIR is an essential *c-myc* repressor, as reduced FIR expression results in upregulation of *c-myc* transcription (Weber et al., 2005). Thus TFIIH, which is required for basal transcription and DNA repair (Coin et al., 2004; Coin and Egly, 1998; Coin and Egly, 2003; Coin et al., 1998; Coin et al., 2007; Fan et al., 2006), is proposed to have a more specialised role in regulating *c-myc* transcription (Liu et al., 2001; Liu et al., 2000; Liu et al., 2006). TFIIH is a multi-protein complex, but in vitro studies suggest that the active subunit in Pol II escape and transcriptional control of *c-myc* is the DNA helicase XPB (Liu et al., 2001; Liu et al., 2000; Liu and Levens, 2006). Haywire (Hay) is the *Drosophila* orthologue of the mammalian XPB helicase (Merino et al., 2002; Mounkes and Fuller, 1999; Mounkes et al., 1992; Regan and Fuller, 1988).

Consistent with roles in regulating *c-myc* transcription, *FIR* mutations have been correlated with colorectal cancer (Matsushita et al., 2006) and *XPB* has been linked with the human diseases xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (Liu et al., 2001; Liu et al., 2000; Liu et al., 2006). Our previous analysis of weak (hypomorphic) *hfp* mutants suggested that, like FIR (Matsushita et al., 2006), Hfp behaves as a tumour suppressor (Quinn et al., 2004). Here, we use RNA interference (RNAi) to achieve ablation of Hfp in *Drosophila* wing imaginal discs and provide unequivocal evidence that Hfp is essential for repression of *dmyc* transcription in vivo, showing that Hfp most likely achieves cell cycle inhibition via dMyc. These studies show that the effect on *dmyc* transcription is likely to be via interaction between Hfp and the *dmyc* promoter and that repression of *dmyc* transcription requires Hfp. Further support for a transcriptional mechanism is provided by our finding that the increased growth resulting from loss of Hfp is dependent on the XPB helicase Hay. Together, the in vivo data demonstrate that Hfp is essential for keeping a tight check on *dmyc* transcription and suggest that the function of Hfp is conserved between *Drosophila* and mammals, which provides support for a model in which FIR is required to repress *c-myc* transcription in mammalian systems.

MATERIALS AND METHODS

Drosophila strains

Except for those detailed below, fly stocks were obtained from the Bloomington Stock Center. *UAS-myc* was a gift from Laura Johnston (Johnston et al., 1999), *Actin<CD2<Gal4 UAS-GFP* from Bruce Edgar (Fred Hutchinson Cancer Research Center, Seattle) and *UAS-p35* from Bruce Hay (Caltech). Transgenic flies containing the *UAS-hay* construct, which contains the full-length *hay* cDNA, were made as described (Quinn et al., 2001). The *dmyc-lacZ* enhancer-trap lines were *P{lacW}l(1)G0354* and *P{lacW}l(1)G0359* (Peter et al., 2002). The *dacapo-lacZ* enhancer-trap line used was *P{lacW}dap^{k07309}*. *UAS-dmyc*-RNAi (v2947), *UAS-hay*-RNAi (v41023) and P{*UAS-Dicer2*, w[+]} (v60008) were obtained from the Vienna *Drosophila* RNAi Centre (VDRC; http://www.vdrc.at) (Dietzl et al., 2007). The *UAS-hfp* RNAi lines (12085R-2 and 12085R-4) were obtained from the National Institute of Genetics Fly Stock Center (NIG fly collection, http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp). The *UAS*-RNAi constructs *UAS-dmyc* RNAi, *UAS-hay* RNAi and *UAS-hfp* RNAi have been predicted to be single hit with no predicted off-target mRNA ablation (VDRC and NIG).

Immunohistochemistry and microscopy

For flip-out clones, larvae were heat shocked for 30 minutes at 37°C 48 hours after egg deposition. Larvae were raised at 25°C for 72 hours to allow development to the third larval instar prior to dissection. Alternatively, larvae were heat shocked 60 hours after egg deposition and raised at 25°C for 60 hours. Antibody staining, BrdU labelling and quantification were carried out as described previously (Mitchell et al., 2008). Antibodies used were: anti-Hay (gift from Mario Zurita, National University of Mexico, Cuernavaca), anti-dMyc (gift from Bob Eisenman, Fred Hutchinson Cancer Research Center, Seattle), anti-bromodeoxyuridine (Becton Dickinson), anti-Fibrillarin (Abcam) and anti-β-gal (Sigma). Anti-Hfp antibody was raised in rats to full-length Hfp-GST fusion protein as described previously (Quinn et al., 2001). Image preparation and analysis were conducted in Adobe Photoshop CS2 v9.0, ImageJ v1.37 and BB Thermometer v1.1 (c/o BenBritten.com). GraphPad Prism was used for statistical analysis and two-way *t*-tests were conducted with a 95% confidence interval.

Quantitative real-time PCR

Total RNA was prepared from imaginal discs from wandering third-instar larvae overexpressing the appropriate RNAi transgenes with *ptc-Gal4* in the *ts-Gal80* background. To deactivate Gal80 function, larvae were raised at 25°C for 72 hours prior to collection. cDNA synthesis was carried out using the SuperScript III First-strand Synthesis System with oligo(dT) primers (Invitrogen). Quantitative real-time PCR (qRT-PCR) was carried out in triplicate and normalised to *Gapdh* using the SYBR Green PCR Master Mix (Applied Biosystems) and the 7900HT Fast Real-Time PCR System (Applied Bioscience). The data analysis was conducted with Sequence Detection Systems v2.3 (Applied Biosystems). The primer sequences (5′ to 3′) were: *dmyc* forward AACGATATGGTGGACGATGG and reverse CGGCAGATTGAAGTTATTGTAGC; *Gapdh* forward AGCCATCACAGTCGATTC and reverse CCGATGCGACCAAATCCAT.

Chromatin immunoprecipitation (ChIP)

ChIP was carried out using the ChIP Assay Kit essentially following the manufacturer's instructions (Upstate Biotech). Specifically, for each sample, 200 larval heads were fixed in 4% paraformaldehyde in PBS for 40 minutes and qRT-PCR was carried out in triplicate as above.

Enrichment was determined by normalising signal to input as follows. Samples of input (i), target gene (tg) and negative control (nc) were all from the same sonication. The i sample was purified, non-immunoprecipitated, sheared chromatin; tg was immunoprecipitated sheared chromatin; and nc was the background chromatin from an immunoprecipitation with non-specific IgG antibody. Average Ct value and s.d. for each were CT.i, CT.tg and CT.nc and SD.i, SD.tg and SD.nc. The ΔCt values for each target region and nc samples relative to the input sample (dCT.tg and dCT.nc) and the propagated error values of these ΔCTs (dSD.tg and dSD.nc) were calculated using the following formulae (where n=3):

dCT.tg = CT.i – CT.tg;
dCT.nc = CT.i – CT.nc;
dSD.tg = sqrt[(SD.i)^2 + (SD.tg)^2] | sqrt(n); and
dSD.nc = sqrt[(SD.i)^2 + (SD.nc)^2] | sqrt(n).

Fold change (FC) and s.d. of fold change (FC.error) over negative control were calculated for each target region as follows:

ddCT = dCT.tg – dCT.nc;
ddSD = sqrt[(dSD.tg)^2 + (dSD.nc)^2];
FC = $2^{(\Delta\Delta CT)}$; and
FC.error = ln(2) × ddSD × FC.

The primer sequences (5′ to 3′) for ChIP were as follows: reverse 1, GTATTTGCGCGGTTTTAAG; forward 1, ACTACTACTAACAACTGTCAC; reverse 2, CAGTCGCTTTCGGCTATATC; forward 2, TCCCCTTCTTTGACGC; reverse 3, TGTGCGGCCATGATCACTG; forward 3, GAATTTCTGGGAAAGGTG; reverse 4, TGCTTTTCCCTTTTCGTA; forward 4, GAAAGACATGTACTGTTA; reverse 5, CTATTAACCATTTGAACCCGAAATC; forward 5, GGTTTTCCTTTTATGCCCTTG. The position of the primers within the *dmyc* 5′UTR are shown in Fig. S6 in the

supplementary material. Enrichment for each primer set was as follows: primer set 1, 22.06±4.19; primer set 2, 22.11±10.86; primer set 3, 8.88±1.98; primer set 4, 1.29±0.11; and primer set 5, 1.69±0.37.

RESULTS

RNAi ablation of Hfp results in wing imaginal disc cell death

Ablation of Hfp protein in larval wing imaginal discs, using the *Actin<CD2<Gal4* flip-out system to overexpress a *UAS-hfp* RNAi, resulted in extensive cell death of the clonal tissue. Analysis of the third-instar wing disc epithelium 72 hours after clone induction revealed the absence of surviving *UAS-hfp* RNAi cells (data not shown). Inspection of the sections under the basal lamina in the wing imaginal disc pouch revealed GFP-marked Hfp loss-of-function cells with pyknotic morphology (Fig. 1D-F, compare with control 1A-C). This loss of cells from the wing disc epithelium and the accumulation of cells under the basal lamina in the wing pouch are consistent with these dead cells being removed to the larval lumen for phagocytosis (Karlsson et al., 2004). Although more extreme, this is consistent with our previous analysis of hypomorphic *hfp* mutant larval wing discs, which revealed that reducing levels of Hfp results in increased apoptosis (Quinn et al., 2004).

Ablation of Hfp in the presence of p35 causes abnormal growth of wing imaginal disc cells

In order to promote cell survival and allow further analysis of the *hfp* RNAi cells, we co-expressed the baculoviral caspase inhibitor *p35* (Hay et al., 1994). To confirm that these cells lacked Hfp protein, we stained *hfp* RNAi clones with a polyclonal anti-Hfp antibody. Hfp protein is normally detected in the nucleus of all wing imaginal disc cells (Fig. 1G-I) and, consistent with Hfp knockdown, we could not detect Hfp protein in the *hfp* RNAi clones (Fig. 1J-L, compare with the surrounding non-GFP control cells). By contrast, Hfp protein could still be detected in wing imaginal discs for the *hfp* hypomorph when compared with wild type (see Fig. S1 in the supplementary material), which could account for the increased severity of the cell death phenotype described above, as the RNAi results in more efficient ablation of Hfp. Importantly, the strong reduction of Hfp with the RNAi presents the opportunity to determine the phenotypic consequences of Hfp depletion.

Co-expression of the *UAS-p35* transgene in cells lacking Hfp resulted in large rounded cells 72 hours after clone induction, which were removed from the apical surface of the wing disc epithelium and extruded basally (Fig. 1P-R). Thus, over time, very few Hfp loss-of-function cells were observed apically, with most GFP-positive cells found in the basal sections. This is illustrated by comparing the 60-hour clone in Fig. 1J,K, which is still located in the apical epithelial sections, with the 72-hour clone in Fig. 1P-R that has been largely basally extruded. To allow for better visualisation of this basal extrusion, Fig. 1S-X shows a series of merged 2 μm *z*-sections through the *hfp* RNAi with *p35* clones, starting from the first apical section containing GFP-marked cells (Fig. 1S) and working basally in sequence to the most basal section (Fig. 1X), which includes the space comprising the larval lumen below the basal lamina of the wing imaginal disc. The basal extrusion and accumulation of *UAS-hfp* RNAi plus *UAS-p35* (*hfp* RNAi/*p35*) clones might, therefore, reflect an attempt to remove these abnormal cells from the wing disc epithelium into the larval lumen for elimination, which is disrupted due to the prevention of apoptosis by p35.

Hfp binds the 5′UTR of *dmyc* and is essential for repression of *dmyc* transcription

Given the role of FIR in transcriptional repression of *c-myc*, we were interested to test whether Hfp ablation results in changes to *dmyc* transcription. dMyc is a key mediator of growth and S-phase progression in the pouch of the wing imaginal disc (Johnston et al., 1999). Cell cycle patterning in the wing pouch is based around the dorsal-ventral (D-V) boundary, where developmental signals direct cells to exit the cell cycle and differentiate in late third instar (Becam and Milan, 2008; Duman-Scheel et al., 2004; Herranz et al., 2008; Johnston and Edgar, 1998; Milan, 1998). In line with this, mRNA in situ analysis has shown that *dmyc* transcription is high in the cycling cells of the pouch, but decreased at the D-V boundary (Johnston et al., 1999). In order to follow transcriptional activity of *dmyc* in vivo, we have characterised a *dmyc-lacZ* enhancer-trap line [*P{lacW}l(1)G0354* (Peter et al., 2002)], which reflects the mRNA expression pattern for *dmyc* (Cranna and Quinn, 2009; Siddall et al., 2009). Wing imaginal discs containing control (p35 alone) clones show *dmyc-lacZ* enhancer-trap activity in a pattern reflecting the distribution of *dmyc* transcription in the cycling cells of the wing pouch, with reduced activity within the cell cycle-arrested cells at the D-V boundary (Fig. 2A-D).

Analysis of *dmyc-lacZ* enhancer-trap activity in 60-hour *hfp* RNAi/*p35* clones revealed increased *dmyc* promoter activity throughout the clone (Fig. 2E-H), including *hfp* RNAi cells spanning the D-V boundary, which normally have reduced *dmyc* expression (Fig. 2A-D). Thus, Hfp is required for this developmentally controlled downregulation of *dmyc* transcription.

The few clones co-expressing *p35* and *hfp* RNAi that remain in mid-sections of the wing pouch epithelium after 72 hours showed a clear increase in *dmyc-lacZ* activity (Fig. 2M-P, compare with the mid-section through the wild-type epithelium in 2I-L). Consistent with the observation above (Fig. 1P-X), analysis of the basal sections of wing imaginal discs co-expressing the *hfp* RNAi and *p35* in the *dmyc-lacZ* enhancer-trap background revealed large clones in the basal section of the wing disc epithelium (Fig. 2U-X, compare with the basal sections containing p35 control clones in 2Q-T). Importantly, regardless of the position of the clones, increased *dmyc-lacZ* enhancer-trap activity was observed in all *hfp* RNAi/*p35* cells.

The requirement for Hfp for repression of *dmyc* promoter activity is not restricted to the *dmyc-lacZ* line used above, or confined to the wing imaginal disc pouch. Using an independent *dmyc-lacZ* enhancer trap [*P{lacW}l(1)G0359* (Peter et al., 2002)] we observed increased *dmyc* promoter activity in *hfp* RNAi/*p35* clones throughout the wing imaginal disc, for clones in the hinge and the notum (Fig. 3A-H). In addition, Hfp was also required for repression of the independent *dmyc-lacZ* enhancer trap in other tissues, including the larval brain (Fig. 3I-L), eye and leg imaginal discs (data not shown), which suggests that Hfp is required for *dmyc* repression in a range of larval tissues.

Quantitation of the increase in *dmyc* transcription in larval tissues by qRT-PCR (carried out in triplicate and normalised to *Gapdh*) revealed that knockdown of Hfp resulted in a significant increase (3.9-fold; $P<0.0001$) in *dmyc* mRNA levels compared with the *p35* control (Fig. 2Y). In addition, chromatin immunoprecipitation (ChIP) of the *dmyc* promoter region showed enrichment for Hfp at –1.2 to –1.8 kb (relative to the transcription start site). Hfp complex formation appears to be specific to the upstream sequences, as enrichment was not found further downstream within the intronic sequence (Fig. 2Z). Taken together, these results show that Hfp is enriched within the 5′UTR of the

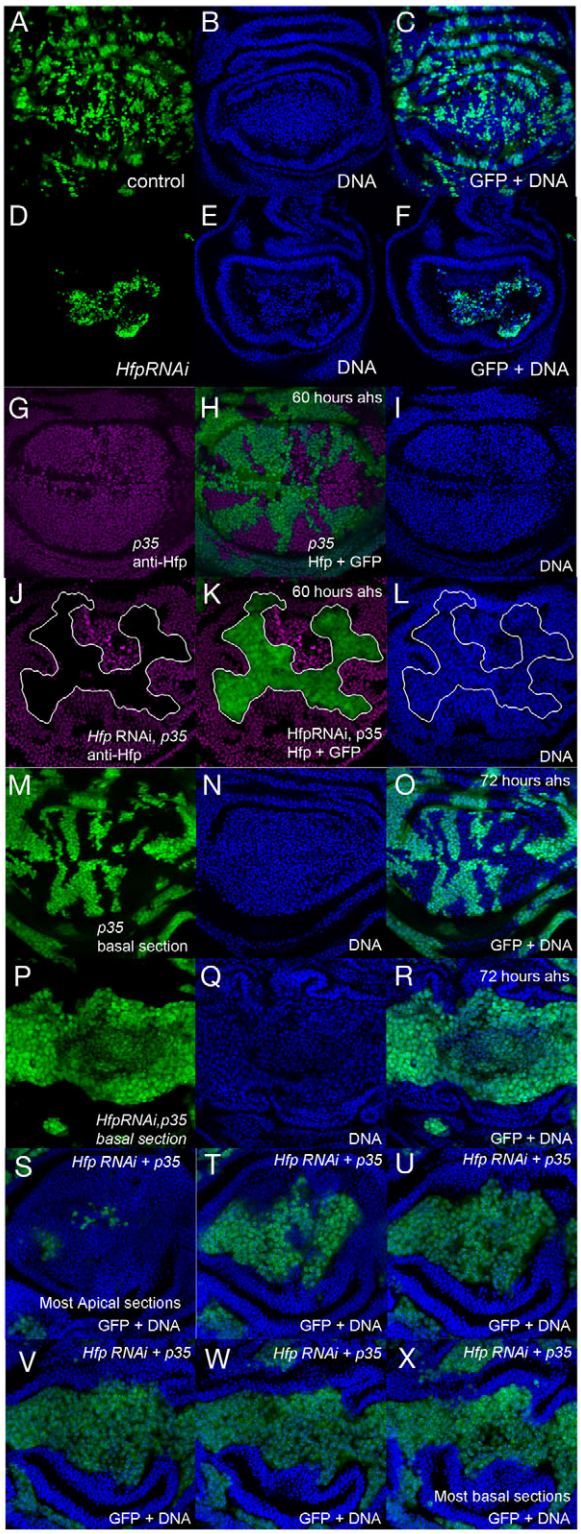

Fig. 1. Hfp knockdown causes cell death. (**A-F**) Basal section of *Drosophila* wing imaginal disc 72 hours after heat shock (ahs) containing clones for (A-C) *UAS-GFP* and (D-F) *hfp RNAi*. (A,D) Clones marked with GFP; (B,E) DNA stain (blue); (C,F) merge of GFP and DNA stain. (**G-L**) Ablation of Hfp in clones co-expressing *UAS-hfp* RNAi and *UAS-p35*. Apical section of wing imaginal disc 60 hours ahs. (G-I) *UAS-p35* control or (J-L) *UAS-hfp* RNAi + *UAS-p35* detected with anti-Hfp antibody (G,J, purple), anti-Hfp and GFP (H,K) or DNA stain (I,L, blue). Ablation of Hfp in the presence of p35 gives abnormal cell growth. (**M-R**) Basal section of the wing disc epithelium containing clones 72 hours ahs. (M-O) Control *UAS-p35* and (P-R) clones co-expressing *UAS-hfp* RNAi and *UAS-p35*. (M,P) GFP-marked clones (green); (N,Q) DNA stain (blue); (O,R) merge of GFP and DNA. (**S-X**) Confocal series of merged 2 μm *z*-sections through *UAS-hfp* RNAi + *UAS-p35* clones, starting with the most apical section containing GFP-marked cells in S and working basally in sequence to the most basal section in X. The confocal 2 μm *z*-sections were merged as follows: S, 1-4; T, 5-8; U, 9-12; V, 13-16; W, 18-21; and X, 22-26.

dmyc promoter and that knockdown of Hfp induces ectopic *dmyc* expression, consistent with Hfp acting as a repressor of *dmyc* transcription.

Given the increased levels of *dmyc* transcription in the *hfp* RNAi/*p35* cells, we tested whether the resulting phenotype was similar to that resulting from co-expression of *p35* and a previously characterised *UAS-dmyc* line (de la Cova et al., 2004; Johnston et al., 1999). As shown in Fig. 4I,J, *hfp* RNAi/*p35* caused increased nucleolar size, as reported for *dmyc*-overexpressing cells (Grewal et al., 2005). In the presence of p35, *dmyc*-overexpressing clones were similar to the Hfp loss-of-function clones with regard to cell overgrowth, cell aggregation and extrusion from the epithelium (Fig. 4A-F). There are, however, differences between *dmyc*-overexpression and Hfp loss-of function clones in the absence of p35, the main difference being that all *hfp* RNAi cells die, whereas apoptosis is observed in some, but not all, cells overexpressing *dmyc* in the wing imaginal disc (de la Cova et al., 2004). These differences between the apoptotic phenotypes from *dmyc*-overexpressing and Hfp loss-of-function cells suggest that although Hfp is required for repression of *dmyc* transcription, there are most likely other targets of Hfp that are important for cell survival.

Owing to the increased nucleolar size/ribosome biogenesis in the clones, we tested whether the dramatic increase in *dmyc-lacZ* activity might be due to global increases in β-galactosidase (β-gal) translation. For this experiment, we used an enhancer trap for a cell cycle-inhibitory protein, the cyclin-dependent kinase inhibitor Dacapo, that we predicted would normally be unlikely to be transcriptionally regulated by Hfp. Loss-of-function Hfp clones in the eye imaginal disc did not show increased levels of β-gal protein for the *dacapo-lacZ* enhancer trap (see Fig. S2 in the supplementary material). Together with the qRT-PCR data (Fig. 2Y), this suggests that the increases in β-gal levels in the Hfp loss-of-function clones are primarily due to increased *dmyc* promoter activity rather than to global increases in the synthesis of β-gal protein.

Overgrowth caused by Hfp loss is dependent on dMyc

Consistent with the predicted role of FIR in *c-myc* repression, FIR mutants lacking the N-terminal *c-myc* repression domain are found frequently in human primary colorectal cancer tissues, which suggests that inactive FIR might contribute to tumour progression by enabling higher levels of *c-myc* expression (Matsushita et al., 2006). However, it is unclear from these studies whether the FIR loss of function is: (1) the cause of the increased *c-myc* expression in the tumour or if this is a secondary event; and (2) whether overgrowth and tumour progression in these cancers are c-Myc dependent. Accordingly, we tested whether loss of Hfp is sufficient to drive cell growth and whether this growth occurs in a dMyc-

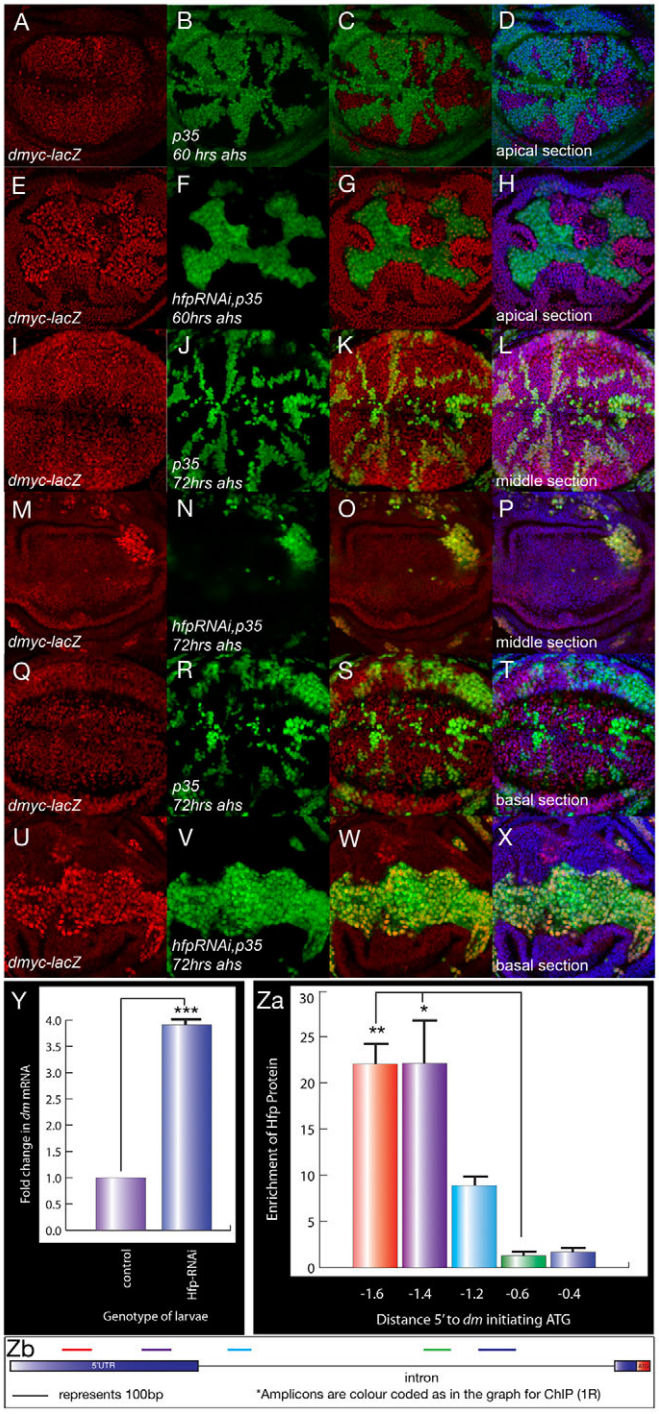

Fig. 2. Hfp binds the 5′UTR of *dmyc* and is required for repression of *dmyc* transcription. (**A-H**) Apical section of wing disc epithelium containing GFP-marked clones at 60 hours ahs in the *dmyc-lacZ* background. (A-D) *UAS-p35* control; (E-H) clones co-expressing *hfp* RNAi and *p35*; (A,E) β-gal (red); (B,F) GFP; (C,G) β-gal and GFP; (D,H) β-gal, GFP and DNA (blue). (**I-P**) Mid-section of wing imaginal disc containing GFP-marked clones at 72 hours ahs. (I-L) *UAS-p35* control; (M-P) most apical section of *hfp* RNAi/*p35* clones. (I,M) β-gal stain (red); (J,N) GFP; (K,O) merge of β-gal and GFP; (L,P) merge of β-gal, GFP and DNA (blue). (**Q-X**) Basal section with 72 hour ahs clones. (Q-T) *p35* control; (U-X) *hfp* RNAi/*p35*. (Q,U) β-gal (red); (R,V) GFP; (S,W) β-gal and GFP; (T,X) β-gal, GFP and DNA (blue). (**Y**) qRT-PCR for *dmyc*. Control, 1.00±0.17; *hfp* RNAi, 3.91±0.19. Hfp knockdown results in a significant increase ($P<0.0001$) in *dmyc* mRNA. (**Za**) ChIP followed by qRT-PCR shows significant enrichment for Hfp in the *dmyc* 5′UTR at 1.6 kb and 1.4 kb upstream of the *dmyc* initiating ATG. Significantly less Hfp protein was detected 1.2 kb ($P<0.0079$), 600 bp ($P<0.0010$) and 400 bp ($P=0.0011$) upstream of the ATG compared with 1.6 kb upstream of the ATG. (**Zb**) Schematic showing the position of the amplicons within the *dmyc* promoter.

dependent manner. As shown in Fig. 4I,J, Hfp knockdown using RNAi led to increased nucleolar size, as measured using a Fibrillarin antibody, an indirect measure of ribosome biogenesis/growth (Grewal et al., 2005; Poortinga et al., 2004). In line with a previous analysis of *dmyc* mutant clones in the wing (Grewal et al., 2005), dMyc knockdown via RNAi resulted in reduced nucleolar size (Fig. 4K,L; see Fig. S3 in the supplementary material for confirmation of dMyc knockdown). To test whether the increased cell growth resulting from loss of Hfp is dependent on dMyc, we ablated dMyc in the Hfp loss-of-function cells and observed a reduction in nucleolar size (Fig. 4M,N), suggesting that the cell overgrowth is dependent on dMyc.

Although the increased ribosome biogenesis, cell and tissue growth resulting from loss of Hfp are suppressed by the *dmyc* RNAi (Fig. 4), the double-knockdown clones are not wild type as they still have a rounded morphology (Fig. 5D-F). This suggests that although loss of dMyc can reduce overgrowth resulting from loss of Hfp, dMyc is unlikely to be the only target of Hfp. Indeed, our previous study provided genetic evidence that Hfp negatively regulates the G2-M cell cycle regulator String (Quinn et al., 2004), which might also contribute to the tumour suppressor behaviour of Hfp. Thus, although Hfp is required for repression of *dmyc* transcription, *dmyc* is unlikely to be the only target of Hfp.

Importantly, dMyc protein can still be detected in the double-knockdown cells (Fig. 5D-I), which suggests that the increased growth of the *hfp* RNAi/*p35* clones is dependent on the increased level of dMyc, rather than the suppression of nucleolar size being due to the general requirement for dMyc in growth. It is important to note that the level of dMyc protein in the double-knockdown cells was generally lower than that in the immediate neighbours of the clone (Fig. 5D-I), which might be due to the effect of ‘undead’ cells increasing dMyc protein in cells near the clonal boundary (as discussed below). Thus, we conclude that the increased growth/hyperplasia upon Hfp depletion is dependent on increased dMyc and that the suppression of nucleolar size is unlikely to be due to a fundamental role for Myc in ribosome biogenesis.

dmyc promoter activity is not increased non-cell-autonomously by undead cell signalling effects

In *Drosophila*, stress events, such as irradiation, give rise to apoptosis in imaginal discs. However, the surviving neighbouring cells undergo compensatory proliferation to produce relatively normal adult tissues. The signals that drive this proliferation are proposed to come from the dying cells (reviewed by Martin et al., 2009). Activation of cell death signalling in the presence of p35 to prevent caspase activity produces undead cells, which can drive increased proliferation non-cell-autonomously via ectopic expression of the secreted signalling proteins Wingless (Wg) and Dpp (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004; Wells et al., 2006).

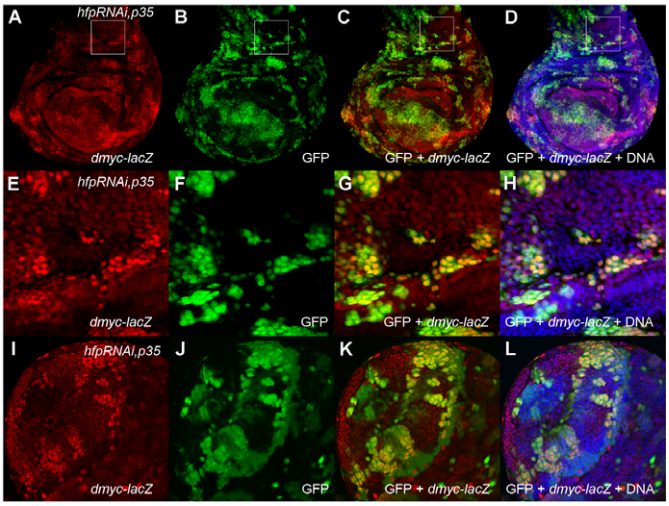

Fig. 3. Hfp is required for *dmyc* repression in a range of *Drosophila* larval tissues. *hfp* RNAi/*p35* clones in the *dmyc-lacZ* enhancer-trap background. (**A-D**) Wing imaginal disc, showing the hinge and notum. (**E-H**) Higher magnification of the notum, from the boxed regions in A-D. (**I-L**) Third-instar larval brain lobe. (A,E,I) β-gal (red); (B,F,J) GFP; (C,G,K) β-gal and GFP; (D,H,L) β-gal, GFP and DNA (blue).

Although it is known that the undead cells secrete these growth factors, the mechanism for driving proliferation in the adjacent cells is unknown. As Dpp has previously been reported to positively regulate dMyc expression in the wing imaginal disc (Prober and Edgar, 2002), it has been speculated that the effects of Dpp might be mediated by dMyc (Gallant, 2005).

The *hfp* RNAi/*p35* clones could potentially drive non-autonomous proliferation due to undead effects, which could account for the abnormal morphology of the surrounding tissue (Figs 1, 2 and 5). Like undead cells, *hfp* RNAi/*p35* cells move towards the basal membrane of the wing disc epithelia and exhibit shape alterations, such as rounding. Importantly, the increased *dmyc-lacZ* enhancer activity and nucleolar size in the *hfp* RNAi/*p35* clones is cell-autonomous, whereas compensatory proliferation would produce non-autonomous growth.

Thus, the non-autonomous induction of proliferation in neighbouring cells is unlikely to occur via increased *dmyc* promoter activity. By contrast, analysis of *hfp* RNAi/*p35* clones using the dMyc antibody (Fig. 5A-C) revealed increased dMyc protein both within the clonal tissue and in cells neighbouring the *hfp* RNAi/*p35* clones. The increased level of dMyc protein was also observed in the cells neighbouring the *hfp/dmyc* double-knockdown clones (Fig. 5D-I). This non-autonomous increase in dMyc protein suggests that the *hfp* RNAi/*p35* cells might have properties of undead cells. We also tested whether the Hfp loss-of-function cells acquire other features characteristic of undead cells, such as increased production of the Wg signal. In the *hfp* RNAi/*p35* clones, we observed an increase in Wg protein (see Fig. S4 in the supplementary material), which suggests that increased Wg secretion by the *hfp* RNAi/*p35* cells might non-autonomously affect dMyc protein levels, but does not affect *dmyc-lacZ* promoter activity in neighbouring cells. These data therefore provide the first evidence that undead cells may induce increased dMyc in their neighbours via a post-transcriptional mechanism.

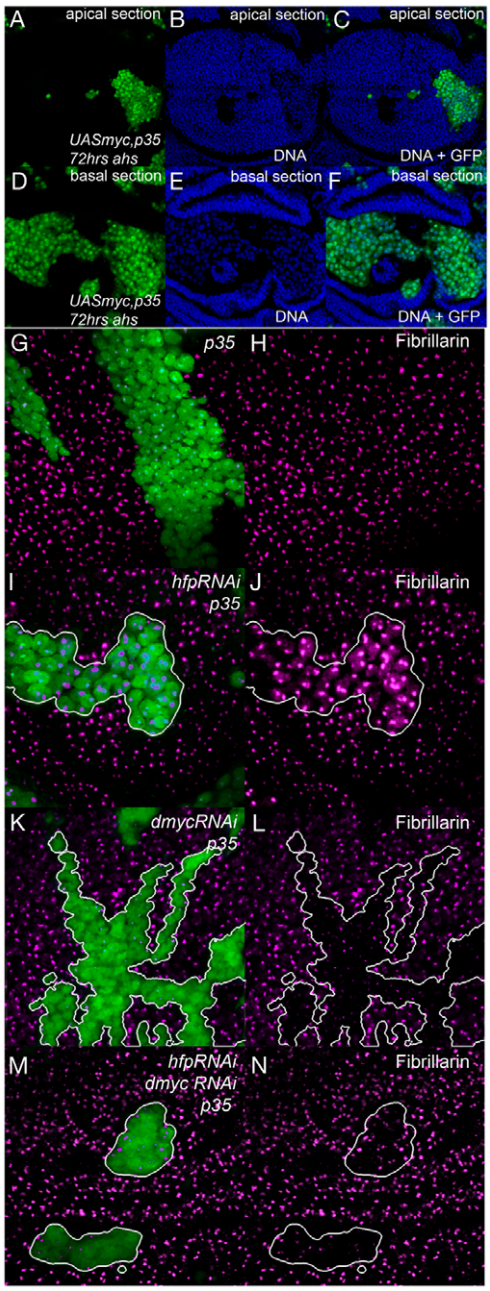

Fig. 4. Cell overgrowth resulting from *hfp* knockdown is dependent on dMyc. (**A-F**) Clones at 72 hours ahs co-expressing *UAS-p35* and *UAS-myc*. (A-C) Apical section; (D-F) basal section. (A,D) GFP; (B,D) DNA stain (blue); (C,F) merge of DNA and GFP. (**G-N**) Clones at 72 hours ahs for (G,H) *p35*, (I,J) *hfp* RNAi and *p35*, (K,L) *dmyc* RNAi, (M,N) *hfp* RNAi, *p35* and *dmyc* RNAi. (G,I,K, M) Fibrillarin (purple) with GFP-marked clones; (H,J,L,N) Fibrillarin alone.

Hay physically interacts with Hfp, is expressed in the wing disc and is necessary for normal levels of *dmyc* expression and S-phase progression

Inappropriate interactions between FIR and XPB have been hypothesised to contribute to cancer predisposition in patients with *XPB* mutations via altered *c-MYC* transcription (Liu et al., 2001; Liu et al., 2000), but the growth and proliferation phenotypes that are expected to precede malignancy have not been investigated for XPB or FIR. We first examined whether the *Drosophila* XPB

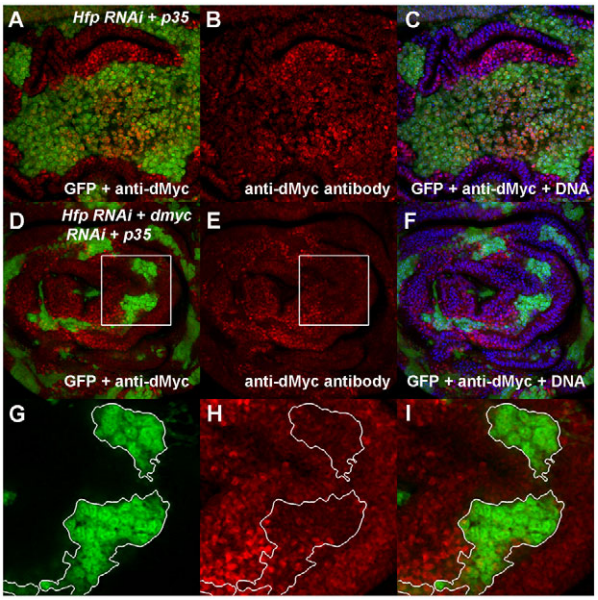

Fig. 5. dMyc protein levels in and around *hfp* knockdown clones and *hfp/myc* double-knockdown clones suggest p35-related 'undead' effects. (**A-I**) Wing imaginal disc clones 72 hours ahs (A-C) co-expressing *hfp* RNAi and *p35*, (D-I) *hfp* RNAi, *p35* and *dmyc* RNAi. (A,D,I) GFP-marked clones merged with anti-dMyc antibody (red); (B,E,H) anti-dMyc; (G) GFP only; (C,F) merge of anti-dMyc, DNA and GFP. (G-I) Higher magnification of the boxed region in D,E with the GFP clones from G outlined in white; I is a merge of G and H.

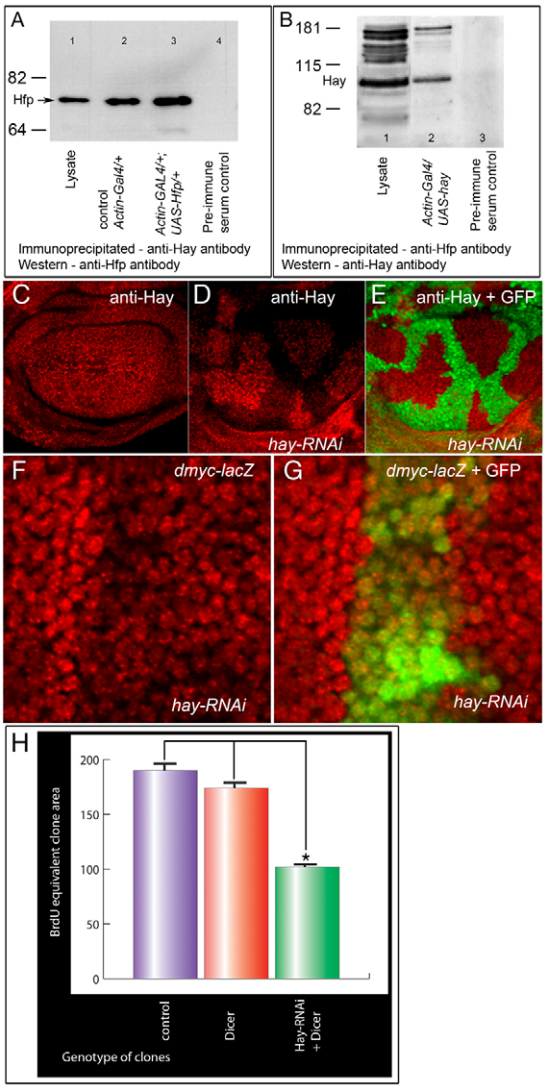

Fig. 6. Hay physically interacts with Hfp in vivo, and Hay is expressed in the wing disc and is necessary for endogenous levels of *dmyc* expression and S-phase progression. (**A**) Lysates from larval imaginal discs precipitated with anti-Hay antibody and probed with anti-Hfp antibody. (**B**) Lysates precipitated with anti-Hfp antibody and probed with anti-Hay antibody. (**C**) Control wing disc showing endogenous Hay protein detected with anti-Hay antibody (red). (**D**,**E**) Expression of the *hay* RNAi construct and *Dicer2* effectively reduces Hay protein levels. (D) Hay antibody (red); (E) merge with GFP. (**F**,**G**) *hay* RNAi and *UAS-Dicer2* transgene in the *dmyc-lacZ* enhancer-trap background. (F) β-gal (red); (G) merge of β-gal and GFP. (**H**) Quantification of S-phase progression measured via BrdU for an equivalent clonal area. Control (*GFP* alone), 190.89±12.14; *Dicer2* alone, 174.23±7.0; clones with *hay* RNAi and *Dicer2*, 102.07±3.11. Ablation of Hay results in a significant reduction ($P<0.0002$) in S-phase cells compared with control clones. $n \geq 10$ sets of 70,000 pixels.

homologue, Hay, interacts physically with Hfp in co-immunoprecipitation experiments (Co-IP). The expected 75 kDa Hfp protein (Van Buskirk and Schupbach, 2002) was immunoprecipitated with the anti-Hay antibody (Fig. 6A). Immunoprecipitation with Hfp antibody followed by an anti-Hay western detected the predominant 94 kDa Hay protein isoform (Fig. 6B) reported previously, with additional bands that are likely to reflect regulation of Hay protein by ubiquitin-mediated proteolysis (Mounkes et al., 1992). We were unable to detect endogenous Hay with the available antibodies, most likely because endogenous Hay is rapidly turned over (Mounkes et al., 1992); overexpression of the *UAS-hay* construct was required to detect a protein of 94 kDa. This demonstrates that Hay can form a complex with Hfp in larval imaginal tissues, which suggests a physical interaction between the Hay and Hfp proteins in vivo.

We next tested whether Hay was either necessary or sufficient for *dmyc* expression and/or S-phase progression. Hay protein is ubiquitously expressed in the wing and localised to the nucleus, as we would predict for the helicase component of TFIIH (Fig. 6C). In order to efficiently ablate Hay, we generated flip-out clones co-expressing *UAS-hay* RNAi with *UAS-Dicer2* (Fig. 6D,E). We detected a reduced *dmyc-lacZ* enhancer-trap activity in cells co-expressing *hay* RNAi and *Dicer2* (Fig. 6F,G) and, consistent with this, there was a significant reduction in the number of S-phase cells (Fig. 6H). Thus, Hay is required to maintain endogenous levels of *dmyc* transcription and for S-phase progression.

In order to test whether an increase in Hay levels was sufficient to increase *dmyc* expression and drive S-phase progression, we generated *UAS-hay* transgenic lines. Hay protein was strongly upregulated in *UAS-hay* clones; however, we observed neither increased *dmyc-lacZ* enhancer-trap activity nor any change to S-phase progression (see Fig. S5 in the supplementary material). These data suggest that increasing the level of Hay protein alone is not sufficient to drive increased *dmyc* transcription. We postulate that this is because endogenous levels of Hfp protein are sufficient

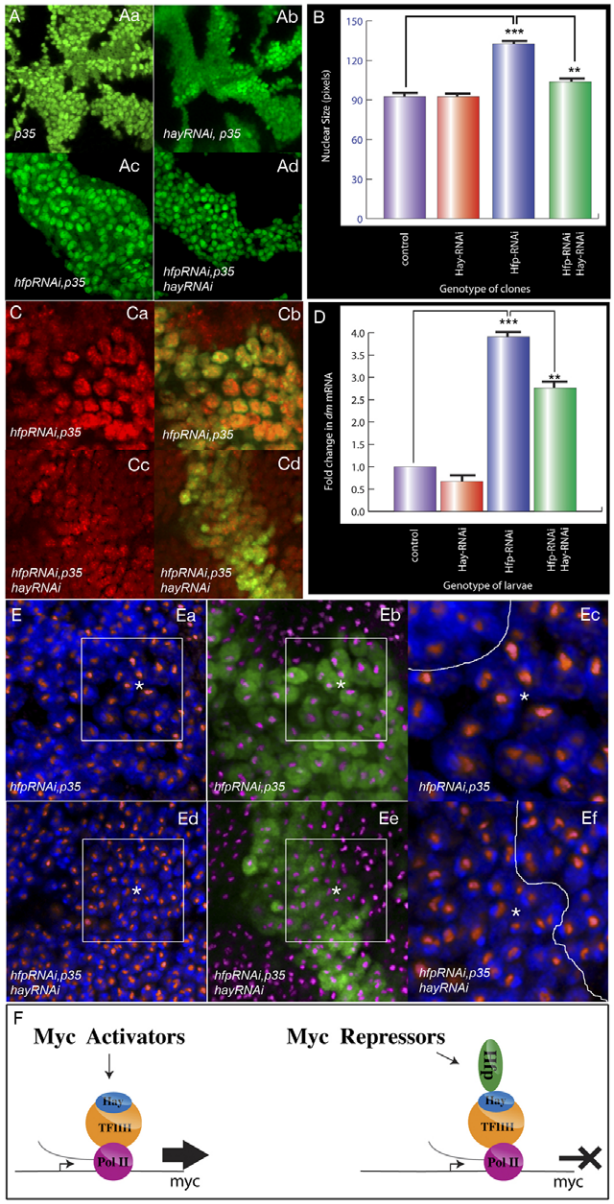

Fig. 7. *dmyc* transcription and cell growth caused by loss of Hfp are dependent on Hay. (**A**) Nuclear GFP in 72 hour ahs clones. (**a**) *p35*; (**b**) *hay* RNAi and *p35*; (**c**) *hfp* RNAi, *p35* and an additional copy of *UAS-GFP*; (**d**) *hfp* RNAi, *hay* RNAi and *p35*. (**B**) Quantification of nuclear area in pixels. Control, 92.5±1.44; *hay* RNAi, 91.2±1.25; *hfp* RNAi, 132.5±2.5; *hfp* RNAi + *hay* RNAi, 103.7±2.39. The nuclei in *hfp* RNAi cells are significantly larger than in the control (P<0.0001) and the nuclei in *hfp* RNAi + *hay* RNAi cells are significantly smaller than in the *hfp* RNAi alone (P<0.0002). (**C**) *dmyc-lacZ* enhancer-trap activity. (**a**) β-gal (red) on *hfp* RNAi, *p35* and *UAS-GFP*. (**b**) Merge with GFP. (**c**) β-gal on *hfp* RNAi, *p35* and *hay* RNAi. (**d**) Merge with GFP. (**D**) qRT-PCR for *dmyc* mRNA for the genotypes described in Ca-d. *p35*, 1.00±0.17; *hay* RNAi, 0.67±0.24; *hfp* RNAi, 3.91±0.19; *hfp* RNAi + *hay* RNAi, 2.76±0.20. Knockdown of Hfp results in a significant increase (P<0.0001) in *dmyc* mRNA, and significantly less *dmyc* mRNA was detected in the *hfp* RNAi + *hay* RNAi compared with the *hfp* RNAi alone (P<0.0020). (**E**) Nucleolar size. (**a-c**) *hfp* RNAi and *p35* clones with (a) Fibrillarin (purple) and DNA (blue), (b) merge with GFP, and (c) 2-fold magnification of Fibrillarin and DNA. The asterisk marks the same Hfp loss-of-function cells in each panel. Control cells are to the top, left of the white line. (**d-f**) *hfp* RNAi, *p35*, *hay* RNAi with (d) Fibrillarin and DNA (blue), (e) merge with GFP, and (f) 2-fold magnification of Fibrillarin and DNA. The asterisk marks the same cells in each panel, for comparison with the Hfp loss-of-function cells in a-c. Control cells are to the right of the white line. (**F**) Working model for role of Hay and Hfp in control of *c-myc* expression. In response to mitogenic signals, *myc* transcription is activated, whereas growth inhibitors can lead to inhibition of *myc* transcription via Hfp.

for maintaining inhibition of *dmyc* transcription. Thus, although Hay is required for *dmyc* transcription it is not sufficient when Hfp is present, suggesting that Hfp is the rate-limiting factor in controlling *dmyc* expression.

Hfp regulates *dmyc* transcription and cell growth in a Hay-dependent manner

As Hfp and Hay interact physically and genetically, we aimed to determine whether the changes in *dmyc* transcription resulting from ablation of Hfp protein were sensitive to the level of Hay. As mentioned above, Hfp loss of function leads to increased *dmyc* promoter activity and increased cell growth (Figs 2-4). As observed for *dmyc* overexpression (Grewal et al., 2005), a consistent feature of the cell growth resulting from loss of Hfp was the increased size of the nucleus, which can be visualised and quantified using the nuclear-localised GFP that marks the clones (Fig. 7A). Indeed, quantification of nuclear size revealed that this was significantly larger in *hfp* RNAi cells than in controls (P<0.0001). The increased nuclear size was sensitive to the level of Hay, as the *hfp* RNAi and *hay* RNAi cells were significantly smaller than the *hfp* RNAi nuclei (P<0.0002) (Fig. 7B).

We then tested whether ablation of Hay in the Hfp loss-of-function cells altered *dmyc* promoter activity or levels of *dmyc* mRNA expression. As expected, *hfp* RNAi/*p35* cells showed increased *dmyc-lacZ* activity and, in line with the decrease in nuclear size, co-ablation of Hay resulted in reduced *dmyc* enhancer-trap activity in the clones (Fig. 7C, compare the *hfp* RNAi cells in Ca,b with those co-expressing *hay* RNAi in Cc,d). Consistent with the observations for the *dmyc* enhancer trap, qRT-PCR revealed a significant reduction in *dmyc* mRNA when Hfp and Hay were co-ablated, compared with the *hfp* RNAi alone (P<0.0020) (Fig. 7D). In line with this, the increased nucleolar size associated with Hfp loss of function was suppressed by loss of Hay (Fig. 7E, compare the nucleolar size in the *hfp* RNAi cells in Ea-c with those co-expressing *hay* RNAi in Ed-f).

Together, these data demonstrate that Hfp and Hay interact physically and that the increase in *dmyc* promoter activity, mRNA expression and cell growth in Hfp loss-of-function cells is dependent on Hay.

DISCUSSION

Tight control of *c-myc* transcription is essential as upregulation of *c-MYC* expression is associated with most human cancers (Liao and Dickson, 2000). In vitro mammalian studies have suggested that one mechanism for *c-myc* promoter regulation involves the presence of a paused, but transcriptionally engaged, Pol II at the *c-myc* start site (Bentley and Groudine, 1986; Kim et al., 2005; Marcu et al., 1992; Spencer and Groudine, 1990; Strobl and Eick, 1992). The paused polymerase can allow a rapid response to developmental/mitogenic signals and protect the *c-myc* promoter

from unwanted activation. Here, we provide strong evidence that the FIR homologue Hfp is crucial for transcriptional repression of *dmyc* and cell growth, suggesting that a transcriptional, rather than a splicing, mechanism underlies the tumour suppressor behaviour of Hfp. In addition, these data show that the mechanism proposed for repression of *c-myc* transcription by the mammalian RRM protein FIR is conserved in *Drosophila*.

First, FIR negatively regulates *c-myc* transcription (Liu et al., 2006), and we have shown that Hfp can bind the *dmyc* promoter and is essential for repression of *dmyc* transcription. Although *FIR* mutations correlate with colorectal cancer incidence (Matsushita et al., 2006), whether dysregulated FIR is the cause of the increased *c-myc* expression and/or the overgrowth phenotypes associated with these cancers is unknown. We have demonstrated that loss of Hfp results in a cell growth phenotype, which occurs in a dMyc-dependent manner. These data strongly suggest that dysregulated FIR in the human context might be causative in cancer initiation and progression. Further support for conservation of the proposed FIR and XPB mechanism for *c-myc* control is provided by our finding that the repression of *dmyc* by Hfp occurs in a manner dependent on the XPB helicase homologue Hay, as the increases in *dmyc* transcription and cell growth associated with loss of Hfp are dependent on the presence of Hay. Thus, these studies provide novel insights into the molecular mechanisms required for controlling *c-myc* transcription, which are likely to be important for understanding FIR- and XPB-related cancers.

Although in vitro mammalian studies have shown that the response of *c-myc* to serum is defective in FIR loss-of-function and XPB-related cancer cells (Liu et al., 2006), the upstream factors in the pathway by which serum mediates *c-myc* repression via XPB and FIR have not been identified. In *Drosophila*, we have shown that Hfp protein levels are regulated, in part, by Wg (Quinn et al., 2004). As Hfp is responsive to the Wg pathway, and promoter occupancy by FIR responds to factors in serum, we hypothesise that Hfp levels and/or activity will be controlled by developmental/growth signals. We predict that cross-talk between a specific complement of growth signals, including Wg, will tightly regulate *dmyc* transcription and growth via Hfp and Hay, which are likely to be relevant to the processes involved in the dysregulation of c-MYC during human malignancy. Thus, we have developed the current working model for repression of *dmyc* by Hfp (Fig. 7F). In response to negative growth signals Hfp binds to inhibit *dmyc* transcription, but upon mitogenic stimulation *dmyc* transcription results from the prevention of promoter occupancy by Hfp. We cannot, however, rule out the possibility that Hfp might in some instances provide a repressive effect that must be overcome by the presence of activators. Thus, the mechanism(s) regulating Hfp levels and/or occupancy of the *dmyc* promoter is the subject of ongoing studies.

In conclusion, our work suggests analogous systems are required for transcriptional regulation of the *c-myc* oncogene and *dmyc*. The knowledge gained from future studies on the developmental regulation of these proteins in *Drosophila* will be informative in understanding the regulation of *c-myc* by the homologous proteins in mammals.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
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