



Drosophila model of HPV18-Induced pathogenesis reveals a role for E6 oncogene in regulation of NF- κ B and Wnt to inhibit apoptosis

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ABSTRACT

Cancers caused by high-risk human papillomavirus (HPV) remain a significant health threat resulting in more than 300,000 deaths, annually. Persistent expression of two HPV oncogenes, E6 and E7, are necessary for cancer development and progression. E6 has several functions contributing to tumorigenesis one of which is blocking programmed cell death, apoptosis. The detailed mechanism of anti-apoptosis function of E6 is not fully understood. Here, using a *Drosophila* model of HPV18E6 and the human UBE3A-induced pathogenesis, we show that anti-apoptotic function of E6 is conserved in *Drosophila*. We demonstrate that the *Drosophila* homologs of human NF- κ B transcription factors, Dorsal and Dif are proapoptotic. They induce the expression of Wingless (Wg, the *Drosophila* homolog of human Wnt), leading to apoptosis. Our results indicate that E6 oncogene inhibits apoptosis by downregulating the expression of Wg, Dorsal, and Dif. Additionally, we find that Dorsal and Dif, not only promote apoptosis but also regulate autophagy and necrosis. Dorsal promotes autophagy while Dif counteracts it, inducing the formation of acidic vacuoles and necrosis. Interestingly, although E6 blocks the proapoptotic function of Dorsal and Dif, it lacks the ability to interfere with their role in apoptosis-independent cell death. Given the high conservation of NF- κ B transcription factors our results provide new insight into potential mechanisms mediated by NF- κ B to intervene with cell immortalization action of E6 oncoprotein in HPV-infected cells.

1. Introduction

High-risk Human Papillomaviruses (HR-HPV) are the leading cause of almost all cervical cancers and one of the most prevalent contributors to anogenital malignancies. HPVs are classified into high and low risk viruses. The high-risk viruses are known for causing several types of cancers including cervical, oropharyngeal, penile, vaginal, vulvar, and anal cancers with the most common types being HPV16 and HPV18. These two types alone account for 70 % of cervical cancers and a significant number of all HPV-induced cancers [1]. HPV infection begins by entry of the virus into the basal cells of the epithelium. Subsequently, HPV replicates its genome using the replication system of the dividing basal cells. However, as the newborn cells migrate to upper layer of the epithelium to become differentiated, HPV delays their differentiation

through cooperative action of its two oncoproteins E6 and E7. E7 induces degradation of cell cycle regulator, Retinoblastoma, promoting S-phase re-entry, whilst E6 targets the tumor suppressor p53 for proteasomal degradation to inhibit apoptosis [2,3]. This function of E6 which requires the assistance of human E3 ubiquitin ligase (hUBE3A) in addition to its role in activation of the enzyme telomerase plays pivotal role in HPV-induced cell survival and immortalization [4,5]. In addition to interference with p53 function, E6 causes inactivation of other apoptotic pathways including intrinsic and extrinsic pathways [6,7] as well as modulation of Hippo and JAK/STAT signaling pathways to promote cell survival and proliferation [8,9]. Despite extensive research, the molecular mechanism underlying cell death evasion function of E6 is not fully understood.

Despite significant advancements in the field and available vaccines

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for HR-HPVs, over 600,000 women are diagnosed with cervical cancer each year, worldwide [10]. Currently, there are no molecularly-targeted drugs that tackle the pre-existing persistent infections. Furthermore, the current treatments for cervical cancer are limited and the disease shows a relatively low survival rate in patients with advanced stages of cancer [11]. This is partly due to the presence of barriers in research studies utilizing mouse and *in vitro* models of the disease [12,13]. While mouse and cell culture models are indispensable tools for cancer studies, mouse models are not practical for large-scale genetic and drug screening. Cell culture system overcomes the limitations of the mouse models, however, it lacks the whole-organism context lacking the tumor microenvironment and systemic effects [14,15]. Therefore, other *in vivo* model organisms that exhibit high conservation of genes and signaling pathways and can overcome the limitations of mouse and cell culture models are highly beneficial. *Drosophila melanogaster* meets these criteria [16]. Despite its limitations in cancer research including morphological and anatomical differences between humans and flies and lack of an adaptive immune system, *Drosophila* offers simplicity, low cost and *in vivo* data with the speed of cell culture model system. Therefore, while *Drosophila* may not be an ideal model system to directly study the HPV infection, it is an excellent model for studying basic cancer biology due to its genetic tractability and conserved signaling pathways that are relevant to HPV-driven cancers including MAPK, PI3K/AKT, Hippo, Wnt, Notch, JAK/STAT, apoptosis and cell cycle regulation [17]. *Drosophila* like mouse is not naturally susceptible to HPV infection and hence transgenic flies that express viral genes are utilized to study viral proteins and their pathogenic effects. Hence, here in this study we utilized a *Drosophila* model of HPV18E6 and the human UBE3A (hUBE3A)-induced pathogenesis to understand the anti-cell death function of E6 [18].

The *Drosophila* compound eye serves as an excellent model for investigating cell death including apoptosis as cell death plays an essential role in *Drosophila* eye patterning [19]. The programmed cell death, apoptosis, consists of compound sequences of protease pathway activation intended to eliminate cells in a programmed manner for both inducing proper tissue patterning in development and removing unhealthy and dysfunctional cells later in life [20]. An example of this patterning can be seen in the *Drosophila* eye, which is formed of approximately 750 eye units called ommatidia. For these ommatidia to serve their function, they must arrange in a hexagonal shape, where every single ommatidium is composed of eight photoreceptor cells, four cone cells and two primary pigment cells, which are surrounded by the inter-ommatidial secondary, tertiary and bristle cells. During eye patterning apoptosis eliminates excess cells and incomplete ommatidia from the eye periphery [21]. In the present study we investigated the E6-mediated cell death evasion in the *Drosophila* eye. We found that like its function in the human cell, E6 blocks apoptotic cell death in the *Drosophila* eye epithelia. Our study reveals that the *Drosophila* homologs of human NF- κ B transcription factors, Dorsal and Dif induce the expression of Wingless (Wg), leading to apoptosis. E6 inhibits apoptosis by downregulating the expression of Wg, Dorsal, and Dif. We further show that Dorsal and Dif not only are proapoptotic but also regulate autophagy and necrosis. Dorsal promotes autophagy while Dif counteracts it, inducing the formation of acidic vacuoles and necrosis. Our results indicate that although E6 oncogene blocks the proapoptotic function of Dorsal and Dif it fails to inhibit the Dif-induced necrotic cell death. Collectively, our results suggest a role for NF- κ B signaling in Wg activation, induction of apoptosis, autophagy, and necrosis in *Drosophila* eye. Furthermore, the failure of E6 to block Dif-induced necrosis provides new insight into potential mechanisms mediated by NF- κ B to intervene with the cell death evasion action of E6 oncoprotein in infected cells.

2. Materials and methods

2.1. *Drosophila* strains and genetics

All fly strains were reared at room temperature and the genetic crosses were performed at 25 °C to avoid variability due to temperature fluctuation. The following *Drosophila* strains were used: GMR-Gal4, UAS-Dorsal (BL9319, y[1] w[*]; P{w[+mC] = UAS-dl.H}2), UAS-Dorsal (BL9320, P{w[+mC] = UAS-dl.H}1, y[1] w[*]; sna[ScO]/CyO, y[+]), UAS-Dif (BL22201, y[1] w[*]; P{y[+7.7] = Mae-UAS.6.11}Dif [LA00958]), UAS-HPV18E6 [18], UAS-hUBE3A [22], UAS-Relish (BL36547, P{w[+mC] = UAS-Rel.HA-VP16}9F, w[*]), UAS-Atg8a-GFP (BL51656, P{ry[+7.2] = hsFLP}1, y[1] w[1118]; P{w[+mC] = UAS-Atg8a.GFP}3).

2.2. Immunohistochemistry

Pupal eyes were dissected in 1X ice-cold PBS, fixed in 4 % paraformaldehyde for 20 min followed by several washes with 0.1 % PBT. The samples were blocked in NGS and incubated with primary antibodies overnight at 4 °C. The following day samples were washed with 0.1 % PBT three times each for 10 min and subsequently incubated in secondary antibodies for 2 h at room temperature. Next, the samples were washed with 0.1 % PBT three times each for 10 min and then mounted in Vectashield (VectorLabs, Inc.) The primary antibodies used were Rabbit anti-cleaved Dcp-1 (Cell signaling cat# 9578) 1:300, mouse anti-Wingless (Developmental Studies Hybridoma Bank 4D4) 1:100, and mouse anti-GFP (Santa Cruz SC-9996) 1:50. The primary antibodies were detected by anti-rabbit and anti-mouse Alexa 488 or Alexa 594 secondary antibodies from Invitrogen. Images were acquired using a SP8 Leica confocal microscope and processed using Image J and Adobe Photoshop.

2.3. Propidium iodide staining

For Propidium Iodide (PI) staining, the pupal eyes were dissected in 1X ice-cold PBS and immediately incubated with 1.5 μ M of PI (Sigma) for 10 min at room temperature protected from light. Next the samples were washed with PBS several times and fixed in 4 % paraformaldehyde for 20 min. Samples were subsequently washed with 0.1 % PBT and mounted on microscope slide in Vectashield.

2.4. LysoTracker staining

Pupal eyes were dissected in 1X ice-cold PBS and immediately incubated with LysoTracker Red DND-99 (Thermo Fisher L7528) 1:100 in PBS for 8 min at room temperature protected from light. The samples were washed with PBS several times and subsequently fixed in 4 % paraformaldehyde for 20 min. After fixation the samples were washed in 0.1 % PBT three times each 10 min and proceeded with immunofluorescence staining.

2.5. RT-PCR and data analysis

Fifty to sixty pairs of pupal eyes from individuals staged at 41–42 h after puparium formation were dissected in 1X ice-cold PBS and their RNA was extracted using the QIAGEN RNeasy Mini Kit. After isolation of RNA the concentration and purity of RNA was measured using a nanodrop spectrophotometer and then equal amounts of RNA across the samples were used to synthesize cDNA. cDNA synthesis was done using the QIAGEN QuantiTect Reverse Transcription Kit and the protocol provided with the kit. PCR was then performed, and PCR products were detected on an electrophoresis gel and imaged. The results were quantified using Image J. The following primers were used:

RP49F: 5' - CCGCTTCAAGGGACAGTATC-3'

RP49R: 5' - GACAATCTCCTTGCCTTCT-3'

DIFF: 5'-TGGAACGCAATTCGCTTCG-3'
 DIFR: 5'-TCATCTGCTGGAGGTTGCTG-3'
 WgF: 5'-CCTGCAGTGAAGGAACGATAG-3'
 WgR: 5'-GGGAGAACTGAACCCGAAT-3'
 DLF: 5'-GGAGGATAGCTTCGATGACG-3'
 DLR: 5'-GCCTGAGAGTTACCTCGAC-3'

The quantification results summarized the analysis of 3 replicates for each experiment. All 3 replicates were quantified using the same method. To quantify first the threshold was adjusted to show the intensity of each band. The plugged-in function for gel analysis allowed the convenient analysis of each band by measuring the area of intensity. Each sample area was then compared to the control, RP49, generating a ratio for that comparison, which was used to compare the significant changes between samples. A two-sample *t*-test assuming unequal variances was performed in Excel to compare the intensity ratio between different samples. Results with a *p* value of 0.05 or lower were considered statistically significant.

2.6. Quantification of cell death

To quantify the apoptotic cell death count in the fly eyes, we used Image J. For each genotype 5 eyes were analyzed. The threshold was adjusted to capture all dying cells and the particles were analyzed to obtain the count of apoptotic cells in each eye. The cell death count was then averaged for the 5 eyes of each genotype and the average was used to compare the cell death count among genotypes. A two-sample *t*-test assuming unequal variances was performed in Excel to compare the apoptotic cell counts between different genotypes. Comparisons that resulted in a *p* value of 0.05 or lower were considered statistically significant.

2.7. In situ hybridization

Developing pupal brains and eyes of *Canton S* pupae were dissected at 42 h after puparium formation (hAPF), in cold PBS, and fixed in 4 % PFA in PBT overnight. The samples were washed with 100 % methanol and 100 % ethanol and stored in -20 °C freezer for later use. *Dorsal* FI20003 (DGRC Stock 1645088; <https://dgrc.bio.indiana.edu/stoc/k/1645088>; RRID:DGRC_1645088) and *Dif* FI01004 (DGRC Stock 1623040; <https://dgrc.bio.indiana.edu/stock/1623040>; RRID:DGRC_1623040) plasmids obtained from the *Drosophila* Genomics Resource Center at Indiana University were used to generate sense and antisense DIG-labeled probes using the DIG-RNA labeling mix (Roche applied science cat #11277073910) with 4 h of incubation at 37 °C. In situ hybridization was performed using a modified version of the method previously described in Refs. [23,24]. Briefly, fixed developing pupal brains and eyes were placed in a mixture of 50 % xylenes/50 % ethanol solution for 30 min with rocking. Samples went through washes of ethanol, methanol, and PBT and were treated with 1:25000 Proteinase K in PBT. Samples were washed with PBT and rehydrated using a buffer of 50 % formamide and 5X SSC pH 5.5. Samples were then incubated in the hybridization buffer with the appropriate DIG probe overnight (<16 h) at 65 °C. Subsequently, samples were washed with hybridization buffer and PBT. They were then moved to a spot dish and incubated overnight with 1:6000 anti-digoxigenin antibody at 4 °C with rocking. Unbound antibody wash was removed with several PBT washes, and alkaline phosphatase staining was developed for 20–40 min in NBT/BCIP at 37 °C.

3. Results

3.1. Coexpression of HPV18 E6 and human UBE3A blocks apoptosis of peripheral ommatidia in *Drosophila* eye

Since genetic modifications to the *Drosophila* eye epithelia often have no effect on the animal's viability, the eye tissue serves as an excellent

model for studying molecular and signaling pathways underlying epithelial-derived human diseases including cancer [25]. E6 oncoprotein is known to have several functions contributing to the tumorigenesis, one of which is resisting cell death. The cell death evasion function of E6 is mediated by inhibition of p53-dependent and independent pro-apoptotic mechanisms [2]. Our previous studies revealed that E6 does not target *Drosophila* p53 due to evolutionary differences in E6-binding regions [18]. While *Drosophila* lacks p53-E6 interaction it has demonstrated conserved mechanism in targeting several other E6 targets including PDZ domain-containing proteins, polarity and junctional regulators [18,26]. Hence, it is a valuable system for investigating highly conserved intrinsic and extrinsic apoptotic pathways that are altered by E6 oncoprotein. To do this we first examined the eye tissues that coexpressed E6+hUBE3A for alterations in apoptotic cell death. We have previously shown that cooperative action of E6 and hUBE3A is necessary for alteration in cell morphology and function as expression of E6 or hUBE3A does not result in any cellular abnormalities [18]. To assess cell death, the studies were concentrated on the 41–42 h after puparium formation (APF) as E6+hUBE3A-induced cellular aberrations are most severe at this developmental stage compared to prior stages. During this stage, the fly eye contains a ring of apoptotic cells to eliminate often incomplete ommatidia from the eye perimeter [27]. To quantify the number of apoptotic cells we performed immunofluorescent staining with an antibody against cleaved form of a critical executioner caspase in *Drosophila*, the death caspase 1 (Dcp-1) [28,29] which is the homolog of human Caspase 3 and subsequently counted the number of Dcp-1 positive cells. We found that in control eye expressing GMR-Gal4, a full ring of cleaved Dcp-1 expressing ommatidia was present (Fig. 1A). Similarly, eyes expressing either E6 or hUBE3A contained a ring of apoptotic cells with no significant changes in the amount of cleaved Dcp-1 expressing cells (Fig. 1C and D). In contrast, experimental eyes that express E6+hUBE3A under the control of GMR-Gal4 showed very few cleaved Dcp-1 expressing cells (Fig. 1B). This phenotype was 100 % penetrant (*n* = 20) and suggested that the anti-apoptotic function of E6 is conserved in *Drosophila*.

3.2. HPV E6 oncogene blocks apoptosis by reducing the level of Wingless

Conserved anti-apoptotic function of E6 in *Drosophila* prompted us to investigate the underlying mechanism. This function of E6 appears to be independent of p53 as it is not targeted by E6 oncoprotein in *Drosophila* tissues expressing E6+hUBE3A [18]. Hence the anti-apoptotic activity of E6 is mediated by mechanisms that act independent of p53. Previous studies found that the expression of Wingless protein (Wg) occurs simultaneously with apoptosis of peripheral ommatidia at the eye perimeter and is necessary for elimination of those ommatidia [27]. Thus, we asked whether E6-mediated inhibition of apoptosis is due to alterations in Wg expression. To address this question, we examined the eye tissues coexpressing E6+hUBE3A for the level and localization of Wg. Immunofluorescent analysis using an antibody against Wg, revealed that whilst in the control eyes expressing GMR-Gal4 a full ring of Wg expression was evident in the perimeter (Fig. 2A), the Wg ring was nearly absent in the eyes expressing E6+hUBE3A (Fig. 2B). This result was 100 % penetrant (*n* = 15) and indicated that E6-mediated inhibition of apoptosis is due to alteration in Wg signaling. We next investigated whether alteration in Wg protein level was due to reduced transcript level of Wg. Hence, an RT-PCR analysis was performed on RNA extracts of eye tissues coexpressing E6+hUBE3A as well as controls expressing the GMR-Gal4 or wild type eyes. Quantification analysis showed that there were no significant changes in transcript level of Wg in E6+hUBE3A-expressing cells compared to the controls (Fig. 2C). These findings suggested that E6-mediated alteration of Wg is not due to transcriptional inhibition and occurs post transcriptionally either due to inhibition of translation or degradation of Wg protein.

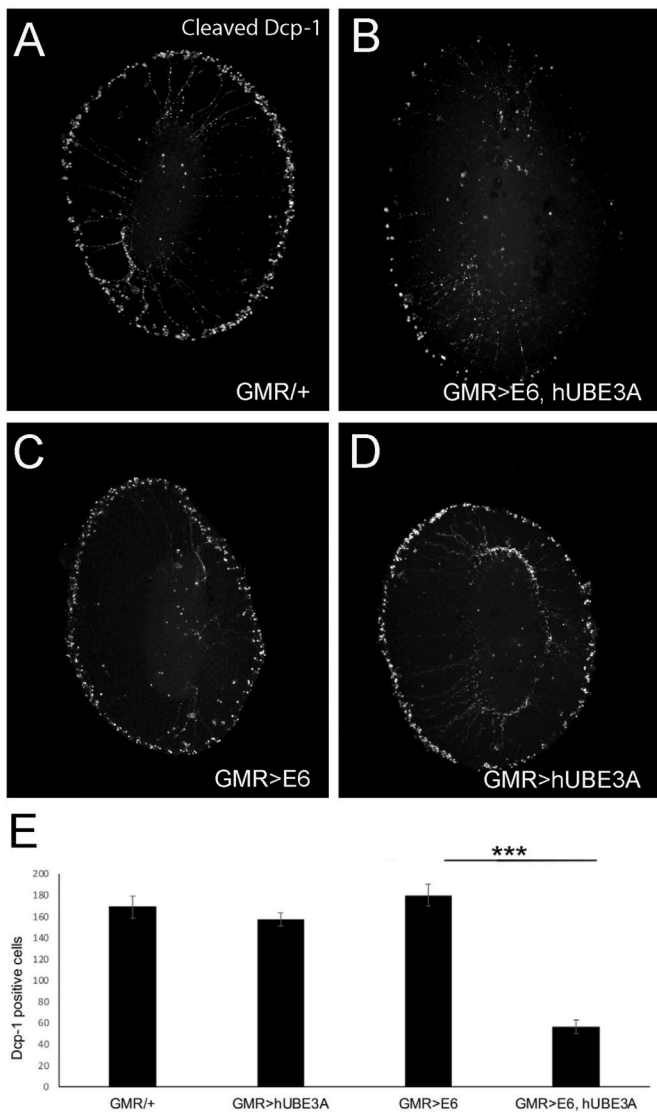


Fig. 1. Expression of HPV18 E6 oncogene and human UBE3A blocks apoptosis of peripheral ommatidia. (A–D) 42 h APF eyes expressing transgenes under the control of GMR-Gal4. (A) Control eye expressing one copy of GMR-Gal4 showing a ring of apoptotic ommatidia indicated by cleaved Dcp-1 expression. (B) Eye expressing E6+hUBE3A showing a significant reduction in apoptotic cell death. (C) Eye expressing E6 showing a ring of apoptotic ommatidia similar to A. (D) Eye expressing hUBE3A exhibiting a ring of apoptotic ommatidia similar to A. (E) Quantification of Dcp-1 expressing cells shows that E6+hUBE3A-expressing eyes have significant reduction in apoptotic cells. GMR > E6 and GMR > hUBE3A do not show any significant changes in apoptosis compared to GMR/+. p-value for these comparisons is 0.13 and 0.08, respectively. 5 eyes for each genotype were considered for quantification analysis. *** denotes $p < 0.001$.

3.3. *Drosophila* NF- κ B, Dorsal and Dif, but not Relish, functionally interact with E6 oncogene

To further understand the mechanism underlying anti-apoptotic function of E6, we performed functional genetic interaction studies between E6+hUBE3A expressing flies and flies that carry an overexpression transgene. In the offspring of these crosses, we searched for the genes whose overexpression resulted in eye size reduction of E6+hUBE3A flies. We found that when NF- κ B transcription factors Dorsal or Dif were coexpressed with E6+hUBE3A in *Drosophila* eye, the eyes were smaller compared to E6+hUBE3A-expressing eyes. Furthermore, ommatidial integrity was lost and eyes exhibited glossy

phenotype. This phenotype was more severe when compared to eye phenotype of flies expressing E6+ hUBE3A which were slightly larger than wild type (WT) eyes or eye phenotype of flies expressing Dorsal or Dif which exhibited a rough eye morphology (Fig. 3C and D compared to B, E and F). Whilst Dorsal and Dif both showed a functional genetic interaction with E6+ hUBE3A, expression of Relish (Rel) had no effect on the E6+ hUBE3A-induced eye defects. This was evident by expression of Rel which resulted in similar morphological defects as when it was coexpressed with E6+ hUBE3A (Fig. 3H and G compared to B). These results suggested that out of three NF- κ B transcription factors in *Drosophila* Dorsal and Dif, but not Rel, functionally interact with E6+hUBE3A and may interfere with anti-cell death activities of E6.

3.4. Overexpression of Dorsal and Dif but not Relish induces Wg expression leading to apoptosis, and that E6 inhibits their proapoptotic function

The Dorsal and Dif-mediated eye size reduction of E6+ hUBE3A expressing flies suggested that Dorsal and Dif may alter cell death evasion activities of E6. To address this, we first investigated whether Dorsal and Dif play any regulatory role in apoptotic cell death of the eye epithelia. NF- κ B signaling is shown to be proapoptotic and anti-apoptotic in context-dependent manner [30–33]. To learn whether Dorsal and Dif are proapoptotic or antiapoptotic we performed immunofluorescent staining on Dorsal and Dif overexpressing eyes using cleaved Dcp-1 antibody and measured the number of Dcp-1 expressing cells. Interestingly, we found that ubiquitous overexpression of either Dorsal or Dif in the eye resulted in enhancement of apoptotic cell death of peripheral ommatidia. This excess apoptotic cell elimination was predominantly evident in one half of the eye whilst the remaining regions were unaffected (Fig. 4B and C compared to A). In contrast to Dorsal and Dif we did not observe any alteration in apoptotic cell death when Relish was overexpressed (Fig. 4D compared to A). These results suggested that the *Drosophila* NF- κ B transcription factors Dorsal and Dif but not Relish have pro-apoptotic role in *Drosophila* eye development. Next, we asked whether they interfere with anti-apoptotic function of E6, promoting cell death and eye size reduction of E6+hUBE3 expressing flies shown in Fig. 3C and D compared to B. To answer this question, we measured the amount of apoptotic cell death by quantifying the number of Dcp-1 expressing cells in pupal eyes expressing either E6+hUBE3A + Dif or E6+hUBE3A + Dorsal and compared them to amount of apoptotic cell death of eye tissues expressing Dorsal, Dif or E6+hUBE3A. Interestingly, we found that Dorsal and Dif both failed to block the anti-apoptotic function of E6. This was evident by significant reduction of Dif and Dorsal-induced apoptotic cell death (Fig. 4F and G compared to B and C). These results indicated that Dorsal and Dif-mediated eye size reduction of E6+ hUBE3A expressing flies (Fig. 3C and D compared to B) is not due to interference with anti-apoptotic function of E6 and suggested a role for Dorsal and Dif in other cell death pathways that E6 does not have the ability to inhibit.

Inhibition of proapoptotic function of Dorsal and Dif by E6 prompted us to investigate the underlying mechanism. Since E6 blocked apoptosis through alteration in Wg expression (Fig. 2) we wondered whether the proapoptotic function of Dorsal and Dif was due to their ability to induce Wg expression and that E6 inhibits their proapoptotic function by altering Wg expression. To address this question, we examined the eye tissues for level and localization of Wg. Immunofluorescent analysis using an antibody against Wg, revealed that whilst in control eyes expressing one genetic copy of GMR-Gal4 a narrow ring of Wg-expressing ommatidia was present, overexpression of Dorsal and Dif resulted in ectopic expression of Wg. This ectopic expression encompassed wider regions at the eye periphery as well as central regions of the eye. Interestingly, given the ubiquitous overexpression of Dorsal and Dif throughout the eye tissue, ectopic Wg expression was mostly evident in one half of the eye (Fig. 5B and C compared to A). These results suggested that Dorsal and Dif can induce expression of Wg in specific

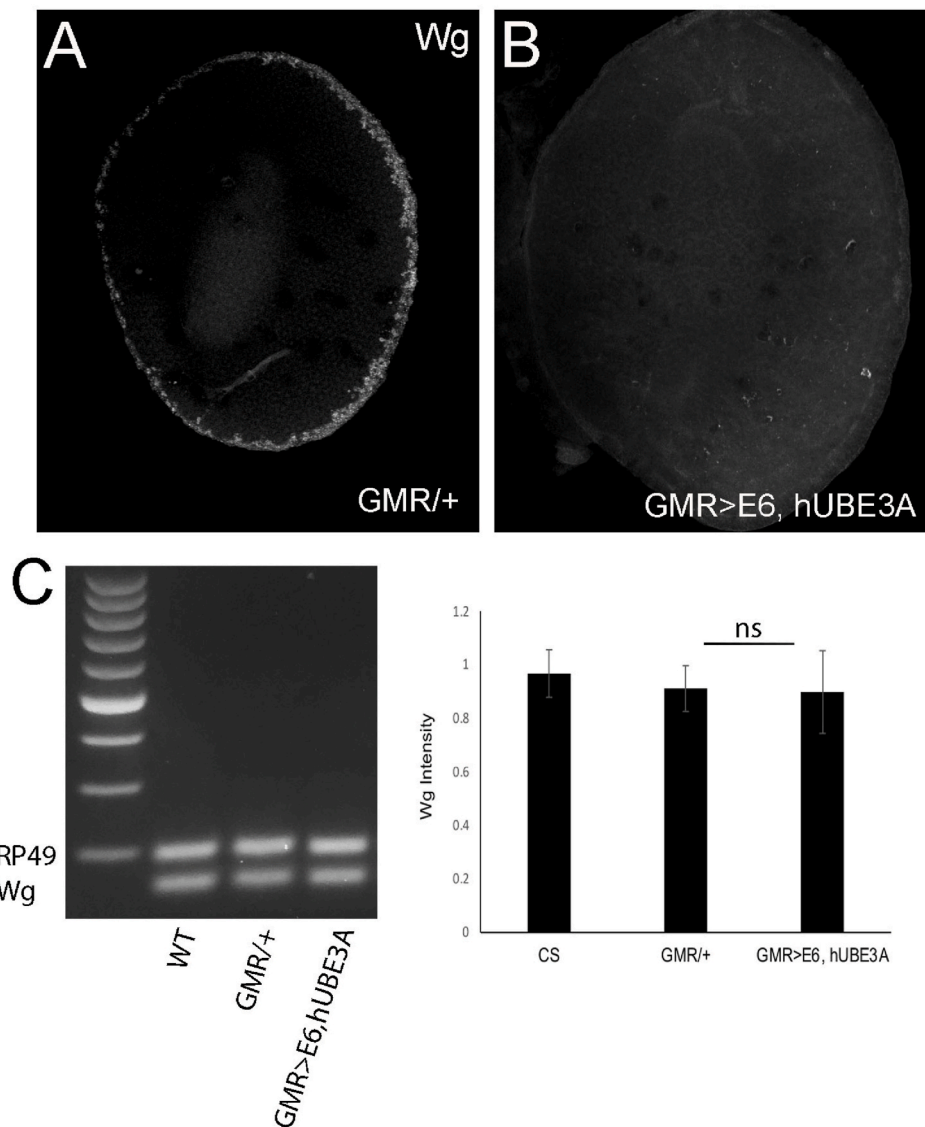


Fig. 2. HPV E6 oncogene alters Wg expression in the perimeter of the eye. (A and B) 42 h APF eyes expressing transgenes under the control of GMR-Gal4. Eyes were stained for Wg protein. (A) Eye expressing one copy of GMR-Gal4 shows a complete ring of Wg expression. (B) E6+hUBE3A-expressing eye shows absence of Wg expression at the eye periphery. (C) RT-PCR results showing no significant alteration in the transcript level of Wg in E6+hUBE3A-expressing eyes compared to wild type or eyes expressing one copy of GMR-Gal4. For quantification analysis the transcript intensity of Wg was normalized to RP49 which was used as internal control. The graph denotes quantification for three replicates and p-value >0.05.

domains of the eye which in turn leads to apoptotic cell death as shown in Fig. 4. We further show that the ability of NF- κ B transcription factors to induce Wg expression only involves Dorsal and Dif and not Relish. Relish overexpression did not lead to any ectopic Wg expression (Fig. 5D). Next, we asked whether E6-mediated reduction of cell death in eye tissues expressing either Dorsal or Dif was due to disruption in Wg expression. Immunofluorescent analysis showed that in eyes expressing E6+hUBE3A + Dorsal or E6+hUBE3A + Dif, Wg expression was nearly absent similar to condition in which E6+hUBE3A was expressed (Fig. 5F and G). These results were 100 % penetrant (n = 10) and indicated that E6 negatively regulates the pro-apoptotic function of Dorsal and Dif in part through alteration in Wg expression.

Given that Dorsal and Dif both induced Wg expression only in one half of the eye and in similar pattern, despite being ubiquitously overexpressed, suggested that they may work cooperatively in response to a signal, regulators, or effector proteins whose expression is confined to that region. To investigate whether overexpressed Dorsal and Dif each may work cooperatively with endogenous Dif and Dorsal, respectively,

to induce Wg expression, we assessed the expression of endogenous Dorsal and Dif in wild type eye. By performing in situ hybridization on 42 h APF wild type eyes we found that both Dorsal and Dif transcripts were present at the eye periphery in the areas corresponding to Dorsal and Dif-induced Wg expression (Fig. 5H and I). Furthermore, when Dorsal and Dif were co-overexpressed ubiquitously, ectopic Wg expression was still found in one half of the eye in pattern similar to Dorsal and Dif overexpression individually (Fig. 6E–G). This result further supported the notion that they may work cooperatively in response to signals, regulators, or effector proteins whose expression is confined to that region.

3.5. E6 negatively regulates the pro-apoptotic function of Dorsal and Dif by reducing the expression of Dorsal, Dif, and Wg

Our results show, for the first time, that NF- κ B transcription factors Dorsal and Dif are expressed in eye epithelia at the periphery and are proapoptotic by inducing Wg expression. Furthermore, E6 oncoprotein

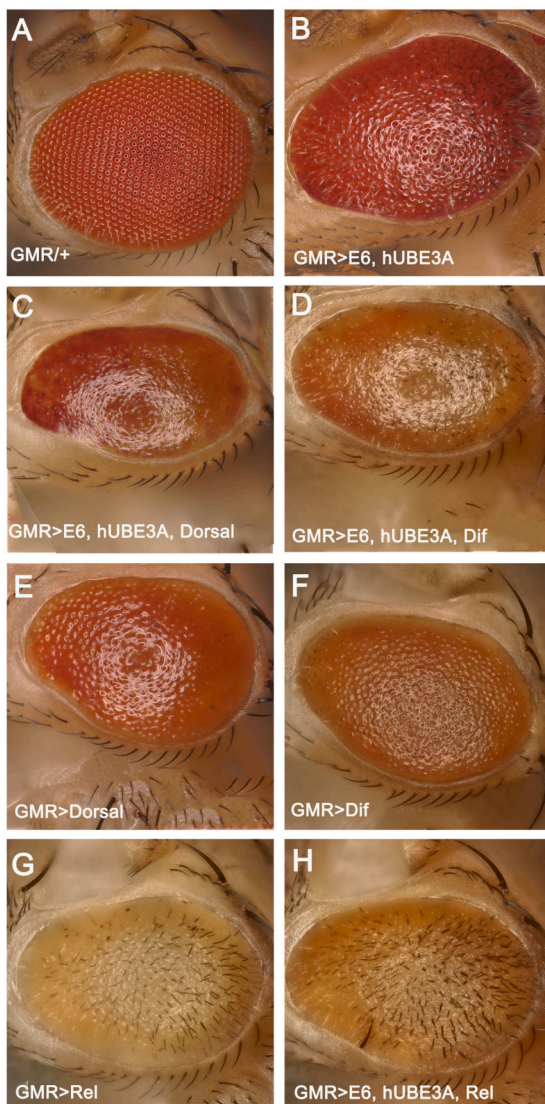


Fig. 3. NF- κ B transcription factors Dorsal and Dif but not Relish functionally interact with E6 oncogene. All transgenes are expressed under control of GMR-Gal4. (A) Adult eye expressing one genetic copy of GMR-Gal4 showing an intact eye morphology. (B) E6+hUBE3A expressing eye exhibiting a rough and disorganized morphology. (C) Coexpression of Dorsal and E6+hUBE3A results in eye size reduction and loss of ommatidial integrity manifested as glossy eye. (D) Coexpression of Dif with E6+hUBE3A results in eye size reduction and loss of ommatidial integrity. (E, F) Overexpression of Dorsal (E) and Dif (F) in wild type eye leads to rough eye morphology. (G) Overexpression of Rel in wild type eye results in loss of pigmentation and disorganized ommatidial morphology. (H) Coexpression of Rel with E6+hUBE3A exhibits morphological defects that resembles the combined effect of Rel and E6+hUBE3A expression. All eyes are from female flies and in the same magnification.

inhibits the proapoptotic function of Dorsal and Dif by altering Wg expression. The question remained to be answered was whether E6-mediated Wg downregulation was due to alteration in the expression of Dorsal and Dif. To address this question, we conducted RT-PCR analysis on eye tissues that either were wild type, expressed one copy of GMR-Gal4, or coexpressed E6+hUBE3A. This analysis revealed that E6+hUBE3A function significantly reduced the transcript levels of Dorsal and Dif when compared to controls in which either the GMR-Gal4 was expressed or were wild type (Fig. 6A and B). This result suggested that Wg downregulation in E6+hUBE3A expressing cells could be due to E6-induced reduction of Dorsal and Dif expression. However, as it is shown in Fig. 2, reduction of Dorsal and Dif transcript level did not lead

to decreased transcript level of Wg. This result suggested that although anti-apoptotic function of E6 may partly involve downregulation of proapoptotic genes Dorsal and Dif, this downregulation does not affect Wg transcript level. Hence, elimination of Wg protein in E6+hUBE3A expressing eyes does not appear to be due to E6-induced downregulation of Dorsal and Dif. This result was further supported when downregulation of endogenous Dorsal and Dif was compensated by their overexpression. Immunofluorescence analysis using a Wg antibody revealed that when Dorsal and Dif were co-overexpressed in the absence of E6+hUBE3A they induced Wg expression in pattern similar to Dorsal and Dif overexpression individually (Fig. 6C–G). However, when they were co-overexpressed in the presence of E6+hUBE3A Wg protein was nearly absent like the condition in which only E6+hUBE3A was expressed (Fig. 6H compared to G). These results altogether suggested that either Dorsal and Dif do not directly regulate transcription of Wg or E6-induced reduction of Dorsal and Dif levels is not sufficient to affect the transcription of Wg. Therefore, E6-induced downregulation of Wg to block apoptosis is likely posttranscriptional or posttranslational.

We also examined the adult eye phenotype of flies that co-overexpressed Dorsal and Dif and compared that to eye phenotype of flies that either overexpressed Dorsal or Dif. We found that Dorsal and Dif co-overexpression resulted in eye degeneration which was evident by loss of pigmentation and ommatidial integrity (Fig. S1E). This phenotype was distinct from the adult eye phenotypes of flies overexpressing either Dorsal or Dif in which the ommatidial integrity was lost but no loss of pigmentation was seen (Figs. S1C–D). Furthermore, we show that the adult eye phenotype of flies expressing E6+hUBE3A + Dorsal + Dif was mostly like the eye phenotype of flies co-overexpressing Dorsal and Dif. The eyes had no pigmentation, lost ommatidial integrity, and exhibited necrotic sites (Fig. S1F). These results confirmed our previous results (Figs. 3 and 4) and suggested that Dorsal and Dif may play role in other cell death mechanisms in addition to apoptosis and that E6 is likely to lack the ability to block this function of Dorsal and Dif.

3.6. Dorsal promotes autophagy while Dif induces the formation of acidic vacuoles and necrosis, a cell death mechanism that E6 fails to inhibit

To investigate whether Dorsal and Dif play a role in other cell death mechanisms that are independent of apoptosis, we first performed lysotracker staining. Lysotracker staining, which marks cellular acidic compartments including lysosomes and autolysosomes, is a widely used tool for detecting programmed cell death including apoptosis, autophagy and necrosis [34,35]. Increased lysosome activity is correlated with increased cell death [36]. By performing lysotracker staining we found that in the control eyes that express one genetic copy of GMR-Gal4 a ring of lysotracker-positive cells was evident at eye perimeter (Fig. S2A'). Interestingly, not all lysotracker-positive cells were colocalized with apoptotic cells marked by the presence of cleaved Dcp-1 (Figs. S2a–a"). This result suggested that the elimination of peripheral ommatidia is not solely due to apoptosis and other cell death mechanisms that involve lysosomal activity are also involved. Next, we examined E6+hUBE3A-expressing eyes to understand whether E6 had any effect on these lysotracker-positive cells. Lysotracker staining revealed that in E6+hUBE3A expressing eyes no lysotracker-positive cells were present at the eye periphery (Figs. S2B–b"). This result indicated that E6 oncogene blocks apoptotic and lysosomal-mediated cell death of perimeter ommatidia.

To investigate whether Dorsal and Dif play a role in induction of lysosomal-mediated cell death at eye periphery we overexpressed Dorsal and Dif individually and jointly using GMR-Gal4. Lysotracker staining showed that Dorsal overexpression led to a dramatic increase in lysotracker-positive cells at eye edge some of which colocalized with apoptotic cells and some did not (Fig. S 2C–c"). Conversely, Dif overexpression reduced the lysotracker-positive cells at eye periphery (Figs. S2d–d") and instead induced the formation of large number of

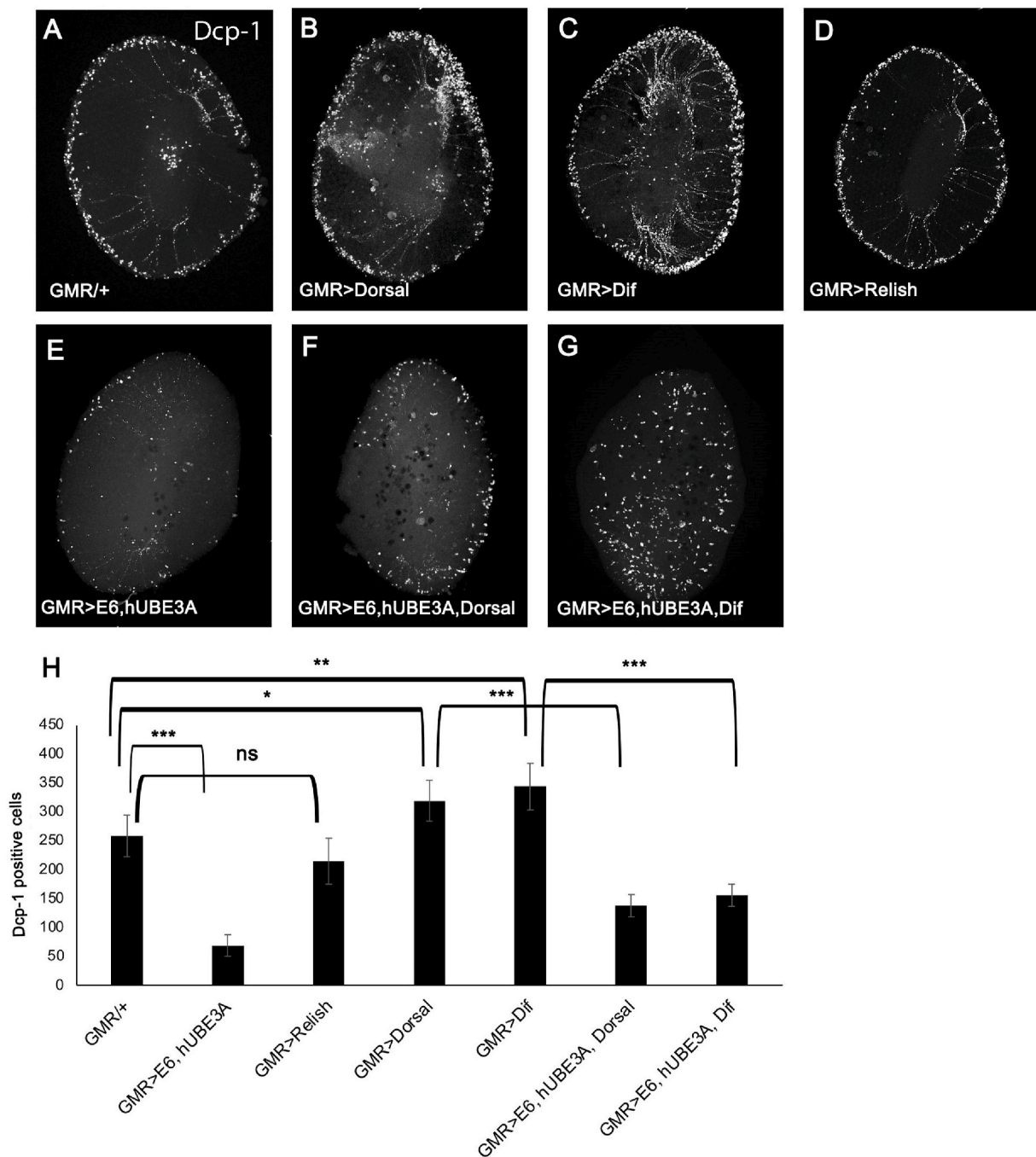


Fig. 4. Overexpression of Dorsal and Dif but not Relish induces ectopic apoptosis, an action that is suppressed by E6+hUBE3A function. 42 hrs APF eyes expressing transgenes under the control of GMR-Gal4. Eyes were stained for cleaved Dcp-1 protein to detect apoptotic cell death. **(A)** Control eye expressing one copy of GMR-Gal4 showing a ring of apoptotic ommatidia. **(B)** Eye overexpressing Dorsal showing ectopic apoptotic cell death predominantly in one half of the eye. **(C)** Eye overexpressing Dif showing ectopic apoptotic cell death predominantly in one half of the eye. **(D)** Eye overexpressing Relish showing no ectopic apoptosis compared to control eye in A. **(E)** Eye expressing E6+hUBE3A shows a significant reduction in apoptotic cell death. **(F)** Eye coexpressing E6+hUBE3A and Dorsal showing a significant reduction in apoptotic cell death compared to B. **(G)** Eye coexpressing E6+hUBE3A and Dif showing a disorganized pattern of apoptotic cells compared to C in addition to a significant cell death reduction that is evident in the quantification analysis shown in H. **(H)** Quantification of Dcp-1 expressing cells shows that overexpression of Dorsal and Dif but not Relish leads to a significant enhancement of apoptotic cell death and that this increased cell death is significantly reduced by the E6+hUBE3A function. 5 eyes for each genotype were considered for quantification analysis. * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$.

acidic vacuoles (Figs. S2D–D’). In order to see whether Dif-induced formation of vacuoles and Dorsal-mediated increased lysosomal activity occur independently or concomitantly, we performed co-overexpression analysis of Dorsal and Dif. We found that although co-overexpression of Dorsal and Dif did not affect their proapoptotic function (Figs. S2E, S2C, S2D), it blocked the Dorsal-induced increased lysosomal activity (Figs. S2e–e’’) and enhanced Dif-induced

vacuolization (Fig. S2E’, S2D’). These results indicated that Dorsal-induced increased lysosomal activity and Dif-induced vacuolization are likely not independent events and may involve the function of both Dorsal and Dif. Furthermore, it suggested that the NF- κ B transcription factors Dorsal and Dif can induce other forms of cell death in addition to being proapoptotic. These findings were intriguing and prompted us to investigate whether Dorsal and Dif-mediated eye size reduction of

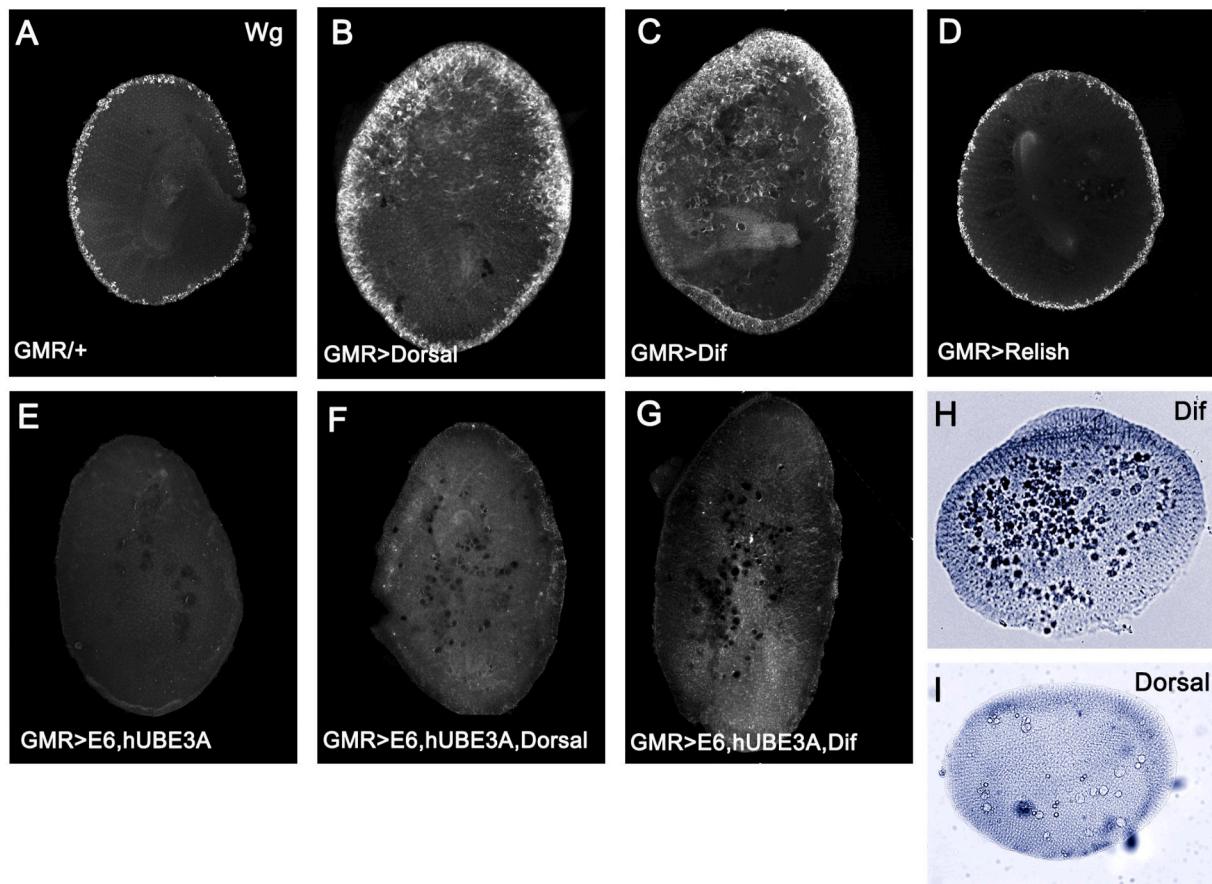


Fig. 5. Dorsal and Dif induce Wg expression, an action that is inhibited by E6+hUBE3A function. 42 hrs APF eyes expressing transgenes under the control of GMR-Gal4. Eyes were stained for Wg protein. (A) Eye expressing one copy of GMR-Gal4 shows a complete ring of Wg expression. (B) Eye overexpressing Dorsal showing ectopic Wg expression at the periphery as well as central region of the eye. This ectopic Wg expression is present in one half of the eye. (C) Eye overexpressing Dif showing ectopic Wg expression at the periphery as well as central region of the eye. This ectopic Wg expression is present in one half of the eye. (D) Eye overexpressing Relish showing no ectopic Wg expression. (E) E6+hUBE3A-expressing eye showing absence of Wg expression at the eye periphery. (F) Eye coexpressing E6+hUBE3A and Dorsal showing absence of Dorsal-induced ectopic Wg expression. (G) Eye coexpressing E6+hUBE3A and Dif showing absence of Dif-induced ectopic Wg expression. (H) Wild type eye exhibiting Dif transcript localization at eye periphery. (I) Wild type eye showing the presence of Dorsal transcript in eye periphery.

E6+hUBE3A expressing flies (Fig. 3C and D compared to B) may have resulted from the inability of E6 to block apoptosis-independent pro-death function of Dorsal and Dif. Hence, we expressed E6+hUBE3A in eyes co-overexpressing Dorsal and Dif. Lysotracker staining revealed that while E6 blocked the Dorsal + Dif induced apoptotic cell death at eye periphery, it failed to block the vacuolization (Figs. S2F–F’). This finding suggested that while E6 can block the proapoptotic function of Dorsal and Dif it lacks the ability to interfere with their role in other cell death pathways that are independent of apoptosis.

Since only a portion of the lysosomes found at the eye periphery colocalized with apoptotic cell death marker, cleaved Dcp-1, we investigated whether other lysosomal-mediated cell death such as autophagy was involved. Although autophagy is primarily considered a cell survival mechanism by removing the damaged or unnecessary organelles and aberrant protein aggregates, but it is also known to induce cell death [37]. To detect autophagy, we expressed an autophagosome marker, Atg8a-GFP using GMR-Gal4. We found that Atg8a-GFP was present in many ommatidia of the eye periphery and that a portion of them colocalized with lysosomal marker, lysotracker (Fig. 7A–A’). This result suggested that elimination of incomplete ommatidia from the eye periphery involves autophagy in addition to apoptosis. We next asked whether Dorsal plays a role in induction of autophagy at the eye periphery as its overexpression had resulted in a dramatic increase in lysotracker-positive cells, many of which did not colocalize with

apoptosis marker, Dcp-1. To address this question, we expressed Atg8a-GFP in eyes overexpressing Dorsal. Our results showed that increased expression of Dorsal enhanced autophagy which was evident by the presence of increased number of autophagic ommatidia. Many of these autophagic cells colocalized with lysosome marker (Fig. 7B–B’). This finding indicated that Dorsal not only is proapoptotic but can also induce autophagy of incomplete ommatidia at the eye periphery. Given that Dif overexpression dramatically reduced the Dorsal-associated enhanced lysosomal activity with no effect on apoptosis, we asked whether Dif blocks autophagic function of Dorsal. Hence, we expressed Atg8a-GFP in eyes co-overexpressing Dorsal and Dif. Our results indicated that Dif overexpression led to a dramatic reduction in the autophagic cells at the eye periphery (Fig. 7C–C’). These findings suggested that autophagy plays a role in elimination of incomplete ommatidia from the eye periphery and that Dorsal is a positive regulator of autophagy. Conversely, Dif is likely to play a negative regulatory role in this process as its overexpression blocked Dorsal-induced autophagy.

Previous studies have shown that vacuolization is associated with different forms of cell death such as necrosis and autophagy [38,39]. Our analysis revealed that Dif and Dif + Dorsal induced vacuoles are not autophagic as they lacked Atg8a-GFP (data not shown). Therefore, we examined them for necrosis. To detect necrosis, we used propidium iodide (PI), a dye that enters the cell when cell membrane integrity is disrupted. Since loss of membrane integrity is hallmark of necrosis, PI is

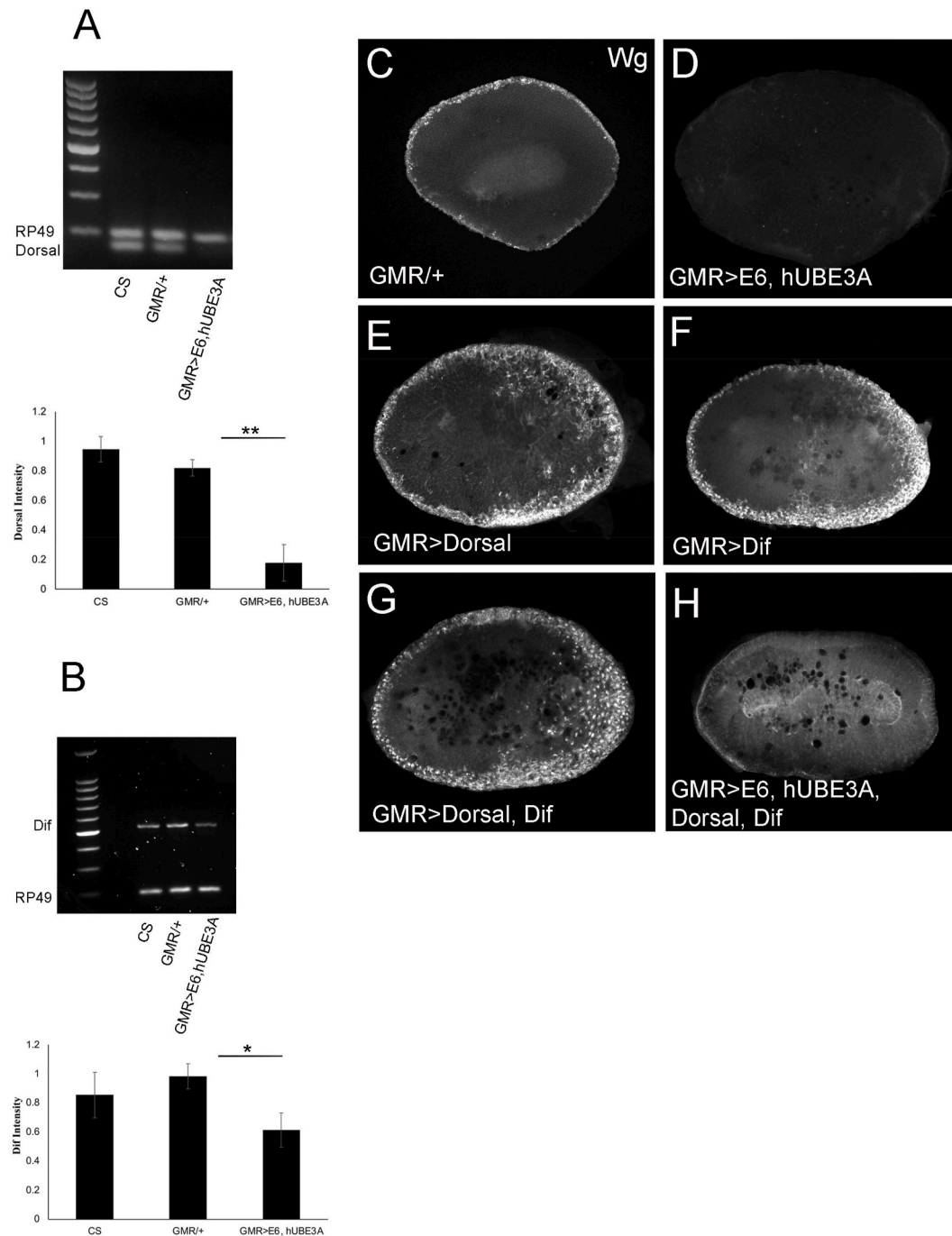


Fig. 6. E6 reduces the transcript levels of Dorsal and Dif and that increasing the expression of Dorsal and Dif cannot prevent E6-mediated Wg elimination. (A) RT-PCR results showing significant reduction in the transcript level of Dorsal in E6+hUBE3A-expressing eye compared to wild type or eye expressing one copy of GMR-Gal4. (B) RT-PCR results showing significant reduction in the transcript level of Dif in E6+hUBE3A-expressing eye compared to wild type or eye expressing one copy of GMR-Gal4. For quantification analyses 3 replicates were included and the transcript intensity of Dorsal and Dif was normalized to RP49 which was used as internal control. * denotes $p < 0.05$, ** denotes $p < 0.01$. (C–H) 42 h APF eyes expressing transgenes under the control of GMR-Gal4. Eyes were stained for Wg protein. (C) Eye expressing one copy of GMR-Gal4 shows a complete ring of Wg expression. (D) E6+hUBE3A-expressing eye showing absence of Wg expression at the eye periphery. (E) Eye overexpressing Dorsal showing ectopic Wg expression at the perimeter as well as central region of the eye. This ectopic Wg expression is present in one half of the eye. (F) Eye overexpressing Dif showing ectopic Wg expression at the perimeter as well as central region of the eye. This ectopic Wg expression is present in one half of the eye. (G) Eye co-overexpressing Dorsal and Dif showing ectopic Wg expression at the eye perimeter as well as central region. (H) Eye expressing E6+hUBE3A + Dorsal + Dif showing absence of Dorsal, Dif-induced Wg expression.

used to detect necrotic cells [40]. Staining of eyes with PI revealed that while neither of control eyes of GMR-Gal4 nor Dorsal overexpression contained any necrotic cells, Dif overexpression led to induction of necrosis which was evident by the presence of PI-positive cells (Fig. 8D compared to C and A). This result suggested that Dif but not Dorsal plays

a positive role in induction of necrosis. Next, we asked whether Dif cooperates with Dorsal to induce necrosis. Hence, we co-overexpressed Dorsal and Dif and examined the eyes for presence of necrotic cells. PI staining showed that co-overexpression of Dif and Dorsal enhanced the production of necrotic cells (Fig. 8E compared to D). This result

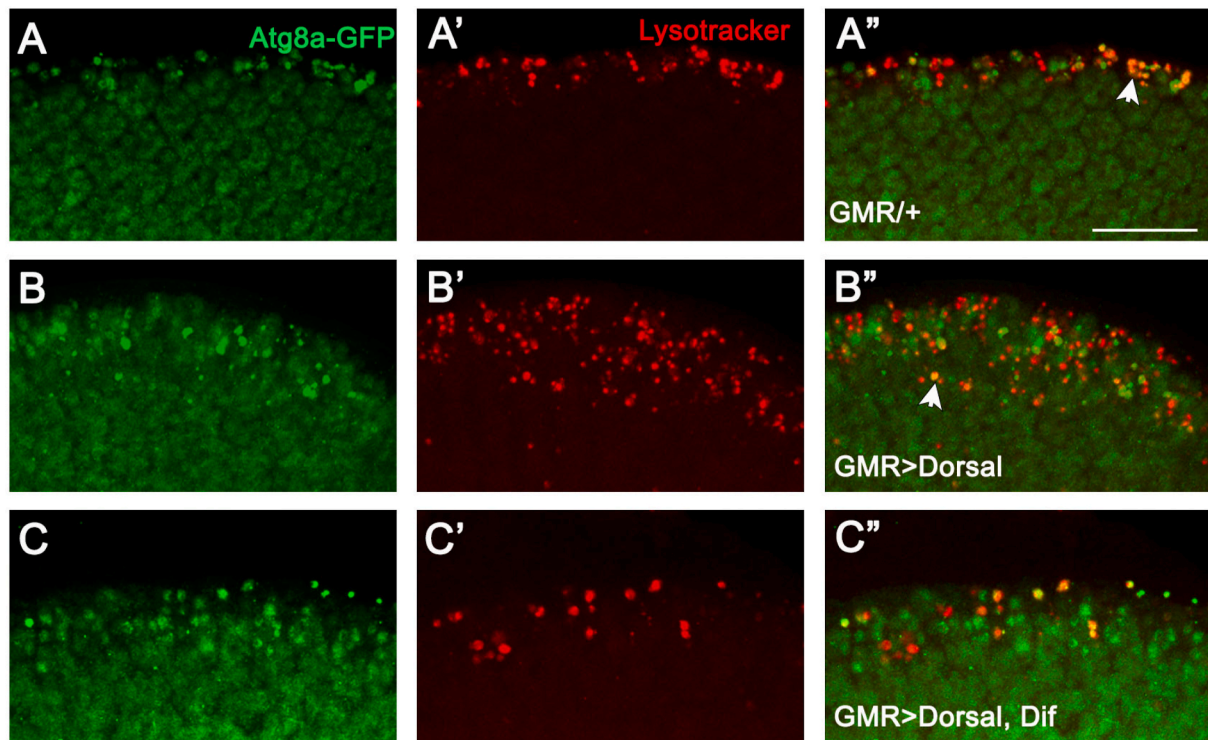


Fig. 7. Dorsal induces autophagy of the peripheral ommatidia, an action that is inhibited by Dif. 42 hrs APF eyes expressing transgenes under the control of GMR-Gal4. Eyes were stained for GFP and lysotracker. (A-A'') Eye expressing Atg8a-GFP showing the presence of autophagosomes at the perimeter of the eye (A). Significant number of autophagosomes colocalize with lysosomes (arrow in A''). (B-B'') Eye overexpressing Dorsal showing an increase in autophagosomes (B) and lysosomes (B') at the eye margin when compared to A and A'. Similar to A'' many of Dorsal-induced autophagosomes colocalize with lysosomes (arrow in B''). (C-C'') Eye co-overexpressing Dorsal and Dif showing a reduction in autophagosomes (C) and lysotracker-positive units (C') at eye periphery. Scale bar indicates 100 μ m.

suggested that Dif is likely to recruit Dorsal for induction of necrosis.

Our results shown in [Supplementary Fig. 2](#) indicated that combined expression of Dorsal and Dif can induce lysosomal-mediated cell death of E6 expressing eye tissue. We investigated whether Dorsal and Dif co-overexpression can similarly induce necrotic death of E6 expressing eye tissue. To address this question, we performed PI staining on eye tissues expressing E6+hUBE3A + Dorsal + Dif and compared that to PI staining of eye tissues expressing Dorsal + Dif. We found that eyes expressing E6+hUBE3A + Dorsal + Dif contained large proportion of necrotic cells to the same extent as the eyes co-overexpressing Dorsal and Dif. This was in contrast to E6+hUBE3A expressing eyes which did not show any PI-positive cells ([Fig. 8F](#) compared to E and B). These results were further supported by the eye degeneration phenotype of adult flies coexpressing Dorsal and Dif and the inability of E6 to inhibit this phenotype ([Fig. S1F](#)). These findings collectively indicated that although E6 oncogene can interfere with the proapoptotic function of Dorsal and Dif it lacks the ability to interfere with their role in induction of apoptosis-independent cell death events.

4. Discussion

High-risk HPV E6 oncoprotein has been shown to play an important role in HPV-induced cancer partly through inhibition of apoptosis [2]. E6 alters intrinsic and extrinsic apoptotic pathways to promote the survival of infected cells [41]. In our study we reveal that E6 oncoprotein exerts anti-apoptotic function in *Drosophila* eye epithelia similar to its action in human cells. This suggests a conserved mechanism of action for E6 to evade cell death. Our results show that the *Drosophila* homologs of human NF- κ B RELA, Dorsal and Dif are both proapoptotic in the eye epithelia. They induce the expression of Wg signaling leading to apoptotic cell death of perimeter ommatidia ([Fig. 9](#)). Dorsal and Dif-induced Wg expression is confined to regions of the eye that

correspond to Dorsal and Dif expression shown by in situ hybridization. The presence of Dorsal and Dif in the same eye regions suggests that they may work in conjunction to induce Wg expression. To support this notion, Dorsal and Dif have been shown to heterodimerize *in vitro* and harbor transcriptional activation structural domains (TAD) which can activate transcription of genes [42]. Furthermore, the promoter of some Wnt family members have binding sites for the NF- κ B transcription factors [43,44]. Taken together, it is likely that Dorsal and Dif may form a heterodimer, which binds to the promoter of Wg to activate its expression. Whether Wg promoter harbors binding site for Dorsal and Dif remains to be elucidated. However, our results show that down-regulation of Dorsal and Dif transcripts has no effect on the transcription of Wg. This finding suggests that either the remaining low dose of these transcription factors is sufficient to activate Wg expression or they regulate Wg expression through an indirect mechanism rather than binding to Wg promoter. NF- κ B transcription factors are known to bind the promoters of Wnt target genes, potentially influencing their expression. This can have downstream effects on the Wnt pathway, as NF- κ B may either upregulate or downregulate the expression of genes that are also regulated by Wnt signaling [45–48]. Wg expression in peripheral ommatidia has been shown to be essential for their elimination by inducing the expression of proapoptotic genes hid, reaper and grim [27]. These ommatidia destined for elimination are often incomplete ommatidia which show unfitness relative to their adjacent complete ommatidia. It is not known what signaling activates Wg-mediated elimination of peripheral ommatidia. Toll pathway involving Dorsal and Dif has been shown to be necessary for elimination of unfit cells known as “loser” cells to maintain tissue homeostasis and plays a protective role in response to infection [49]. Inhibition of Toll signaling in loser cells by RNAi-induced downregulation of its components including Toll-related receptors (TRRs), its ligand Spätzle (Spz), or NF- κ B proteins Dorsal or Dif blocks apoptotic death of loser cells [50,51]. In this context Dorsal and

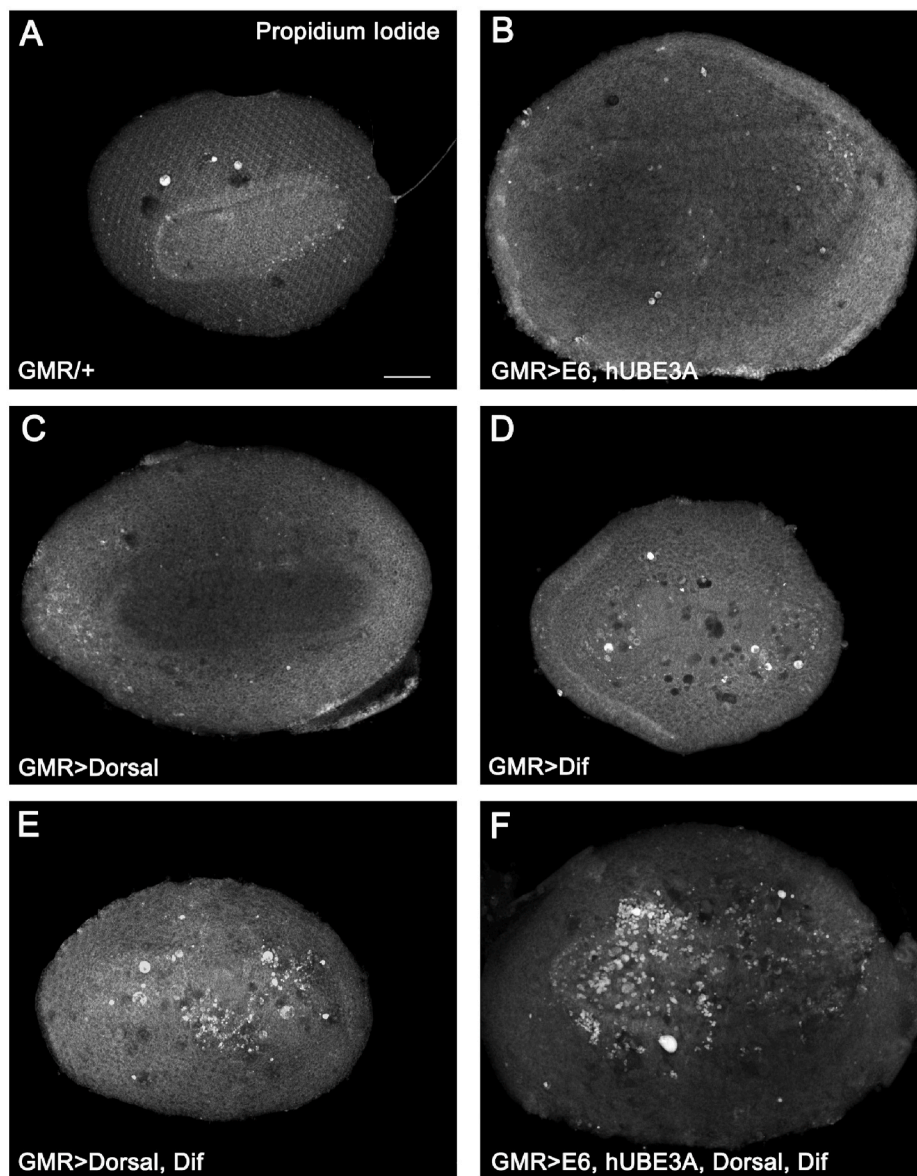


Fig. 8. Dif induces necrosis, a function that E6 oncoprotein fails to inhibit. 42 hrs APF eyes expressing transgenes under the control of GMR-Gal4. Eyes were stained for propidium iodide. (A) Eye expressing one copy of GMR-Gal4 shows almost no necrotic cells. (B) E6+hUBE3A-expressing eye showing no necrosis. (C) Eye overexpressing Dorsal contains no necrotic cells. (D) Eye overexpressing Dif displaying presence of PI-positive cells. (E) Eye co-overexpressing Dorsal and Dif showing a great amount of necrosis. (F) Eye co-overexpressing Dorsal and Dif in conjunction with E6+hUBE3A shows a significant number of necrotic cells when compared to E and B. Scale bar indicates 100 μ m.

Dif have been shown to induce the expression of proapoptotic gene reaper. Based on our findings showing an induction of Wg expression and apoptosis by Dorsal and Dif, it is likely that Toll pathway in peripheral ommatidia activates Wg signaling which in turn leads to activation of proapoptotic genes hid, reaper and grim to eliminate these unfit cells (Fig. 9). Our results also indicate that in this context of incomplete ommatidia elimination IMD pathway mediated by NF- κ B Relish is not proapoptotic and does not play a role in activation of Wg signaling. Unlike Dorsal and Dif, overexpression of Relish did not result in induction of apoptosis or Wg expression. Our previous studies have demonstrated a role for IKK β , a serine/threonine kinase that phosphorylates and activates Relish, in E6+UBE3A-induced pathogenesis [52,53]. Lack of functional genetic interaction between Relish and E6+UBE3A suggest that IKK β contributes to E6 oncogenesis with a mechanism independent of its function in IMD pathway.

Our study further shows that the proapoptotic function of Dorsal and Dif is inhibited by E6 oncoprotein. E6 inhibits apoptosis by reducing the

level of Wg protein with no effect on its transcript level (Fig. 9). Furthermore, E6 attenuates the transcript levels of Dorsal and Dif. The exact mechanism of how E6 alters the expression of Dorsal, Dif and Wg is not known. Our results suggest that E6-mediated downregulation of Wg protein is not dependent on Dorsal and Dif expression levels. This is supported by our result showing that when E6-mediated Dorsal and Dif downregulation was compensated by their overexpression, Wg protein was still absent. Therefore, one possible explanation for downregulation of Dorsal and Dif could be reduction in Wg signaling. To support this possibility, previous studies have shown a reciprocal regulation between Wnt and NF- κ B signaling pathways and that these two pathways modulate each other in context-dependent manner [54]. Recently, a positive feedback loop between Wnt and NF- κ B signaling pathways have been reported in which activated Wnt signaling activates NF- κ B which in turn induces Wg expression [55]. The second possible scenario for downregulation of Dorsal and Dif could be a mechanism that does not depend on Wg signaling. The HPV oncoproteins E6 and E7 have been

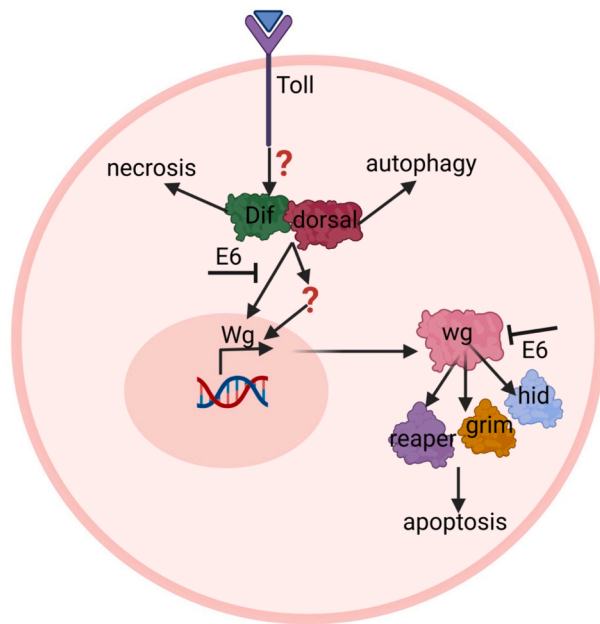


Fig. 9. Schematic representation of summary of results. We propose that *Drosophila* homologs of NF- κ B Dorsal and Dif induce expression of Wg (human Wnt-1) either directly or indirectly which in turn results in activation of pro-apoptotic genes *hid*, *reaper* and *grim* and apoptosis. E6 inhibits Wg-induced apoptosis by downregulation of Dorsal and Dif as well as Wg protein. We further propose that Dorsal promotes autophagy while Dif promotes necrosis. E6 lacks the ability to interfere with Dif-induced necrosis suggesting that inducing necrosis through NF- κ B activation may have therapeutic potential for HPV-induced cancers. The cartoon was generated using BioRender.

shown to regulate NF- κ B expression, however, there are conflicting data with some showing stimulation [56,57] and others suppression of NF- κ B activation [58,59]. Our results support a negative regulation of NF- κ B expression by E6 oncoprotein (Fig. 9).

Wnt signaling plays important role in cancer initiation, promotion, and progression. *Drosophila* Wg is a homolog of human Wnt-1 which is involved in canonical Wnt/ β -Catenin pathway [60,61]. This signaling pathway is a highly conserved pathway across species and is involved in development, proliferation, migration, differentiation, apoptosis, autophagy, cell adhesion and polarity [62–64]. Previous studies have reported activation of Wnt/ β -Catenin pathway in human HPV-positive cancer cells and that this activation is directly regulated by E6 and E7 oncoproteins [65,66]. Further studies showed that E6 requires the assistance of human UBE3A(E6AP) to be stabilized and induce augmentation of Wnt/ β -Catenin signaling in HPV-positive cells [67]. E6 directly interacts with components of Wnt/ β -Catenin pathway such as Dvl2 to disassemble β -Catenin degradation complex and promote canonical Wnt pathway activation [68]. Similarly, E6 in conjunction with UBE3A induces degradation of *NHERF1*, a PDZ protein that suppresses Wnt/ β -Catenin signaling, to activate canonical Wnt pathway [69]. Growing body of studies have revealed that Wnt signaling can have oncogenic and tumor-suppressing functions in context-dependent manner. Modulation of Wnt signaling either through β -Catenin or via mechanisms independent of β -Catenin can inhibit cell proliferation and induce apoptosis in cancer cells [70,71]. Crosstalk of Wnt pathway with other pathways including Hippo, Notch, *EGFR/RAS/RAF/MEK/ERK*, *c-myc*, *p53*, and *FOXO* is important for anti-tumor or tumor promoting outcome of Wnt signaling pathway [70,72]. Our results suggest a pro-apoptotic role for Wnt signaling likely through crosstalk with NF- κ B signaling in response to HPV infection. This notion is supported by our findings showing E6 oncoprotein induces downregulation of NF- κ B transcription factors and Wnt to evade apoptosis. Further studies are needed to dissect the mechanism of NF- κ B/Wnt crosstalk and their role

in HPV infection.

Another interesting finding in our study was the role of Dorsal and Dif in autophagy and necrotic cell death. We show that Dorsal promotes autophagy while Dif counteracts it. Dif instead is an inducer of necrotic cell death (Fig. 9). Our study reveals that in the wild type eye, autophagy is occurring alongside apoptotic cell death to eliminate the perimeter ommatidia. Moreover, we find that Dorsal overexpression increases lysosomes and autolysosomes many of which colocalize with autophagosome marker Atg8a. In contrast, Dif co-overexpression with Dorsal leads to a reduction in autophagosomes and autolysosomes. Instead Dif induces the formation of acidic vacuoles and necrotic cells. These acidic vacuoles or necrotic cells are not detected in Dorsal overexpressing eyes and are only observed when Dif is either overexpressed alone or in conjunction with Dorsal. These results suggest that Dorsal and Dif in addition to their proapoptotic role in *Drosophila* eye play an important role in autophagy and necrosis. Previous studies have suggested role for NF- κ B in autophagy. Both Toll and IMD pathways have been shown to regulate autophagy. For instance, NF- κ B transcription factor Relish, has been shown to induce expression of Atg1, which is sufficient for activation of autophagy and degradation of *Drosophila* salivary glands [73]. Similarly, the Toll pathway, which involves NF- κ B transcription factors Dorsal and Dif has been demonstrated to be important for maintaining optimal level of autophagy in *Drosophila* brain [74]. Furthermore, NF- κ B-mediated autophagy has been implicated in innate immune response to recognize and clear pathogens including viruses [75–77]. Our results show that Dorsal and Dif do not share the same function in autophagy. Dorsal promotes autophagy whereas Dif suppresses it and activates necrosis. The mechanism of when each of these two cell death mechanisms are activated remains to be elucidated. Previous studies have shown that necrosis can inhibit autophagy through several mechanisms including activation of NF- κ B signaling [78,79] and dysregulating SNARE-mediated autophagosome-lysosome fusion. Hence, one possible reasoning for our result is that the role of Dif and Dorsal in autophagy and necrosis is context-dependent. Our results show that when E6 oncoprotein was coexpressed in conjunction with Dorsal, Dorsal-induced lysosome increase was blocked and instead an accumulation of necrotic cells was observed (data not shown). This result together with previous studies which suggested regulation of necrosis by autophagy and vice versa [79,80] indicate that during autophagy Dif may be inactivated to allow Dorsal to induce autophagy. In response to infection or other specific stimulus Dif is activated and either forms heterodimer with Dorsal or functions independently to induce necrosis. Taken together, we have uncovered new roles for Dorsal and Dif in cell death by showing their involvement not only in apoptosis but also in autophagy and necrosis.

In this study we also show that E6 oncoprotein, while blocking the Dorsal/Dif-induced apoptosis, lacks the ability to inhibit the Dif-induced vacuolization and necrotic cell death. We find that in E6-expressing tissue the endogenous expression level of Dorsal and Dif is reduced. This result suggests that downregulation of Dorsal and Dif is a strategy used by E6 to not only block their proapoptotic function but also to inhibit autophagy and necrosis. Hence, increasing their expression in conjunction with E6 can overcome the inhibitory action of E6 on Dif-induced necrosis. This notion is supported by our findings showing that when E6 is expressed in conjunction with Dorsal, Dif or Dorsal/Dif it fails to block necrosis. Taken together, these results shed light on how E6 may combat cell death in the human cell and that human homologs of Dorsal and Dif, RELA, may be considered for further investigation in HPV positive human cells for their role in activation of cell death mechanisms that HPV oncogene E6 lacks the ability to intervene.

CRedit authorship contribution statement

Mojgan Padash Barmchi: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Rami N.**

Hassan: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Mehrnaz Afkhami:** Visualization, Validation, Methodology, Investigation. **John P. Masly:** Writing – review & editing, Resources. **Harrison Brown:** Visualization, Validation, Methodology, Investigation. **Quincy P. Collins:** Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Michael J. Grunsted:** Writing – review & editing, Visualization, Validation, Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tvr.2025.200316>.

Data availability

Data will be made available on request.

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