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# The Ecdysone-inducible zinc-finger transcription factor Crol regulates Wg transcription and cell cycle progression in *Drosophila*

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The steroid hormone Ecdysone is crucial for developmental cell death, proliferation and morphogenesis in *Drosophila*. Herein, we delineate a molecular pathway linking Ecdysone signalling to cell cycle regulation in the *Drosophila* developing wing. We present evidence that the Ecdysone-inducible zinc-finger transcription factor Crol provides a crucial link between the Ecdysone steroid hormone pathway and the Wingless (Wg) signalling pathway in *Drosophila*. We identified Crol as a strong enhancer of a wing phenotype generated by overexpression of the Wg-inducible cell cycle inhibitor Hfp. We demonstrate that Crol is required for cell cycle progression: *crol* mutant clones have reduced cell cycles and are removed by apoptosis, while upregulation of Crol overrides the Wg-mediated developmental cell cycle arrest in the zone of non-proliferating cells in the wing disc. Furthermore, we show that Crol acts to repress *wg* transcription. We also show that overexpression of *crol* results in downregulation of Hfp, consistent with the identification of the *crol* mutant as a dominant enhancer of the Hfp overexpression phenotype. Taken together, our studies have revealed a novel mechanism for cell cycle regulation, whereby Crol links steroid hormone signals to Wg signalling and the regulation of crucial cell cycle targets.

**KEY WORDS: Cell cycle, *Drosophila*, Wingless signalling**

## INTRODUCTION

In mammalian cells and animal models, pathways mediated by steroid hormone receptors, such as oestrogen (Nilsson et al., 2004), and the Wnt signalling pathways (Polakis, 2000) have been long implicated in driving cell proliferation, which can lead to cancer initiation and progression. In addition, steroid hormone signals can impact on Wnt signalling to promote cancer progression (Brisken et al., 2000; Miller et al., 1998); however, the genes important for connecting these pathways are unknown.

*Drosophila melanogaster* presents an ideal model for examining the interplay between signalling pathways in cell proliferation control. In particular, in the developing wing epithilelia of third instar larvae the Wg pathway is important for the developmental cell cycle arrest at the dorsal-ventral boundary in a region known as the zone of non-proliferating cells (ZNC), which is essential for differentiation of cells along the wing margin and formation of the wing blade (Johnston and Edgar, 1998). In the wing pouch, Wg expression is highest in the cell cycle arrested cells of the ZNC, where it is required to downregulate the *Drosophila* homologue of Myc transcription factor, *dm*, which drives growth and G1- to S-phase progression in a functionally homologous manner to the *Myc* oncogene (Johnston et al., 1999). The Wg pathway also drives G2 arrest via downregulation of the *Cdc25* homologue *stg* (Edgar and Datar, 1996), which normally triggers mitotic entry by activating the Cdk1/Cyclin B kinase (Edgar and Datar, 1996). Wg-driven cell cycle exit in the ZNC, therefore, involves G1 arrest induced by downregulation of *dm* (previously *dmyc*) and G2 arrest via inhibition of *stg*.

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Ecdysone (20-hydroxy ecdysone) is a key steroid hormone in *Drosophila*, which has been predominantly studied for its role in the remodelling of larval tissues, a requirement for patterning of adult structures (Thummel, 1996). During metamorphosis, a cascade of gene transcription is triggered by ecdysone that activates dimerization of the ecdysone receptor (EcR), a member of the nuclear receptor superfamily and its receptor binding partner Ultraspiricle (USP) (Thummel, 1990; Thummel, 1995; Thummel, 1996). To date, studies on the role of the ecdysone pathway in regulating cell cycle progression have been limited. This is surprising as the ecdysone pulse is essential for adult tissue morphogenesis, a process that requires coordination of development with cell cycle, apoptosis and differentiation. Although previous studies have revealed the molecular mechanism connecting ecdysone signalling to the processes of apoptosis and differentiation (reviewed by Baehrecke, 2000; Jiang et al., 1997; Yin and Thummel, 2005), there are fewer studies showing connections between the ecdysone pulse and the developmentally regulated cell cycles essential for generating adult tissues. First, during larval development, ecdysone is required for morphogenetic furrow progression in the eye imaginal disc (Brennan et al., 1998) via the early ecdysone response Broad-complex zinc-finger transcription factor Broad (Br) (Brennan et al., 2001). In addition, ectopic BR-C expression leads to ectopic endoreplication cycles during oogenesis, suggesting that the ecdysone pathway can promote DNA replication (Tzolovsky et al., 1999). However, the molecular mechanism by which the ecdysone response affects the cell proliferation machinery is unknown.

One target of ecdysone signalling is *Crooked legs* (*crol*), which encodes a zinc-finger transcription factor (D'Avino and Thummel, 1998). *Crol* is upregulated by ecdysone during late larval/pre-pupal development in imaginal discs, salivary glands and the CNS (D'Avino and Thummel, 1998; D'Avino and Thummel, 2000). Pupal lethal *crol* mutants ($crol^{4418}$) have defects in ecdysone-induced gene expression (D'Avino and Thummel, 1998) and display

abnormal wing development associated with reduced cell adhesion (D'Avino and Thummel, 2000). However, a role for Crol in regulating proliferation has not been reported.

Here, we provide the first evidence for a link between the ecdysone pathway and Wg signalling in cell cycle regulation. We demonstrate that the ecdysone inducible gene *crol*, downregulates *wg* transcription to overcome the Wg-mediated cell cycle arrest in the ZNC, thereby leading to the upregulation of genes crucial for cell cycle progression.

## MATERIALS AND METHODS

### Fly strains and generation of UAS-crol transgenic lines

*UAS-crol* constructs contain the full-length *crol* cDNA, which encodes a 962 amino acid protein and were made as described (Quinn et al., 2001). Fly stocks were obtained from Bloomington, except *en-GAL4, UAS-GFP* and *Act<CD2<GAL4, UAS-GFP* (Laura Johnston), $axin^{E77}$ (Jessica Treisman), $stg^{AR2}$, *stg-lacZ* line 6.4 (Bruce Edgar), *PL35* (Allen Vincent), $dm^{P0}$ (Peter Gallant), $arm^{YD35}$ (Eric Wieschaus), *hsflp;arm-lacZ,M(2)z,FRT40A* (Helen McNeill) and *PCNA-GFP* and $E2F^{e01}$ (Bob Durunio).

### Generation of clones in larval wing discs

For MARCM analysis, *hsflp*, *UAS-GFP*; *FRT40A, gal80*; *tubulin-GAL4* females were crossed to *FRT40A* lines for control, *crol[k05205]* or *crol[k05205]*; *UAS-p35* males. Heatshock was at 37°C for 1 hour, 72 hours after egg deposition (aed) and larvae were aged to 120 hours aed prior to analysis. Alternatively, heatshock was at 48 hours aed and larvae were analysed at 96 hours. To generate clones in the Minute background, *FRT40A*, *crol[k05205]* males were crossed to *hsflp*; *arm-lacZ,M(2)z,FRT40A*. For flip-out clones *Act<CD2<GAL4;UAS-GFP* males were crossed to either *hsflp* control, *UAS-crol;UAS-p35* or *hsflp:UAS-EcRDN* females and analysis was carried out for 120-hour larvae, 72 hours post heatshock.

### Antibody staining, BrdU and TUNEL labelling and microscopy

Crol antibody was generated to full-length Crol-GST fusion protein, in the standard manner (Quinn et al., 2001). Immunohistochemistry was carried out as previously described (Quinn et al., 2001). Antibodies used were: Wg (4D4) (Developmental Studies Hybridoma Bank), Hfp (Trudi Schupbach), E(spl)m7 (Sarah Bray), Ci (2A1) (R. A. Holmgren). Other antibodies used were anti-BrdU (Becton Dickinson) and anti-phosphohistone H3 (Upstate Biotech.). All fluorescently labelled samples were analysed by confocal microscopy (Zeiss LSM Meta).

### Quantitative real-time PCR

mRNA was isolated from 20 sets of third instar larval heads either overexpressing *UAS-crol* with *Actin-GAL4* or from control tissue (*Actin-GAL4/+*), and cDNA generated using the first-strand synthesis kit (Invitrogen). *wg* primer sets for Q-RTPCR were 5′-gagatctccacaagcgaacc-3′ and 5′-ccaatcacacggaagttgg-3′. Q-RTPCR was carried out with SYBR-GREEN (Applied Biosystems) and the default conditions for ABI prism. Quantification was achieved by normalization within each sample with cycle time for the GAPDH primers (5′-ccgatgcgaccaaatccat-3′ and -5′ agccatcacagtcgattc-3′).

## RESULTS

### Identification of Crooked legs as a positive cell cycle regulator

We previously characterised Hfp [homologue of mammalian transcription factor FIR (Liu et al., 2006)] as a downstream target of Wg and demonstrated its cell cycle inhibitory behaviour (Fig. 1A) (Quinn et al., 2004). In order to identify Hfp interactors, which would be potential regulators of cell cycle, we carried out a genetic screen. Briefly, we screened deficiency and mutant collections for dominant modifiers of a reduced wing phenotype that results from overexpression of *UAS-Hfp* in the posterior compartment (PC) of the larval wing disc, with the *en-GAL4* driver (Fig. 1C). As a strong

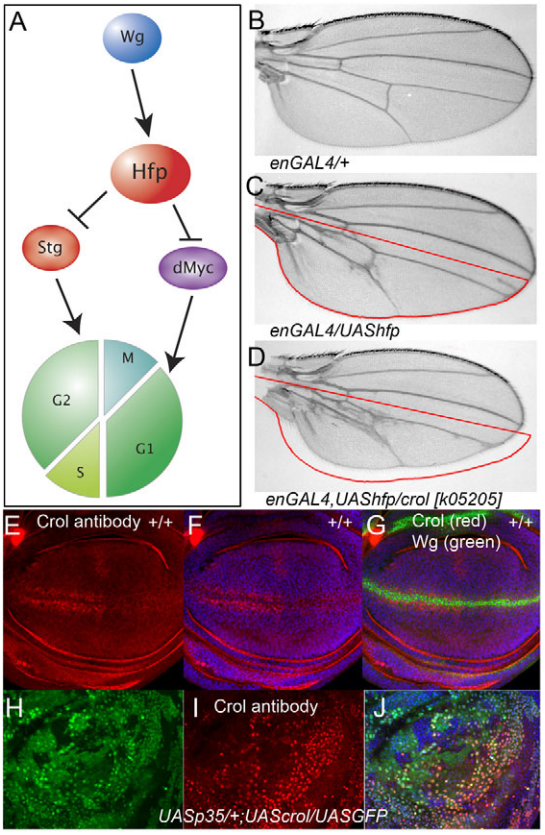


**Fig. 1. Crol interacts with the Wg-inducible cell cycle inhibitor Hfp.** (**A**) Hfp is upregulated by Wg and inhibits G1- to S-phase transition by downregulating *dm* and mitosis via Stg. (**B-D**) Halving the dose of *crol* enhances the *hfp* overexpression wing phenotype. Adult wings for *en-GAL4/+* control (B), *en-GAL4, UAS-hfp/+* (C) and *en-GAL4, UAS-hfp/crol[k05205];* (D). A tracing of the posterior compartment from *en-GAL4, UAS-hfp/+* has been superimposed on the *en-GAL4, UAS-hfp/crol[k05205]* wing. (**E-G**) Crol antibody (red) on wild-type third instar wing disc, merged with DNA (blue, F) and with Wg (G, green). (**H-J**) *UAS-crol;UAS-p35* flip-out clones marked with GFP (H), with Crol antibody (red, I) and merged with DNA (blue, J).

enhancer of the *hfp* overexpression phenotype, we identified the gene *Crooked legs* (*Crol*) (Fig. 1D), which encodes a zinc-finger transcription factor (D'Avino and Thummel, 1998). Crol has been shown to be normally required for wing development; however, expression from the *crol4418-lacZ* enhancer trap was not detected in the wing disc (D'Avino and Thummel, 1998). In order to determine whether Crol is normally expressed in the wing, we stained wild-type wing discs with an anti-Crol antibody. Crol protein was detected in the nucleus of most wing disc cells from both the pouch and the hinge (Fig. 1E,F). Co-staining with Wg (Fig. 1G) revealed a decrease in Crol expression within the Wg-expressing cells comprising the ZNC, when compared with cells flanking the ZNC. We demonstrated the specificity of the Crol antibody by showing increased Crol protein in flip-out clones over-expressing the *UAS-crol* transgene (Fig. 1H,I). The detection of Crol in the pouch and hinge is consistent with Crol being required to regulate transcription during wing development. We therefore sought to test whether enhancement of the *hfp* overexpression phenotype was due to Crol normally being required for cell cycle in the wing.

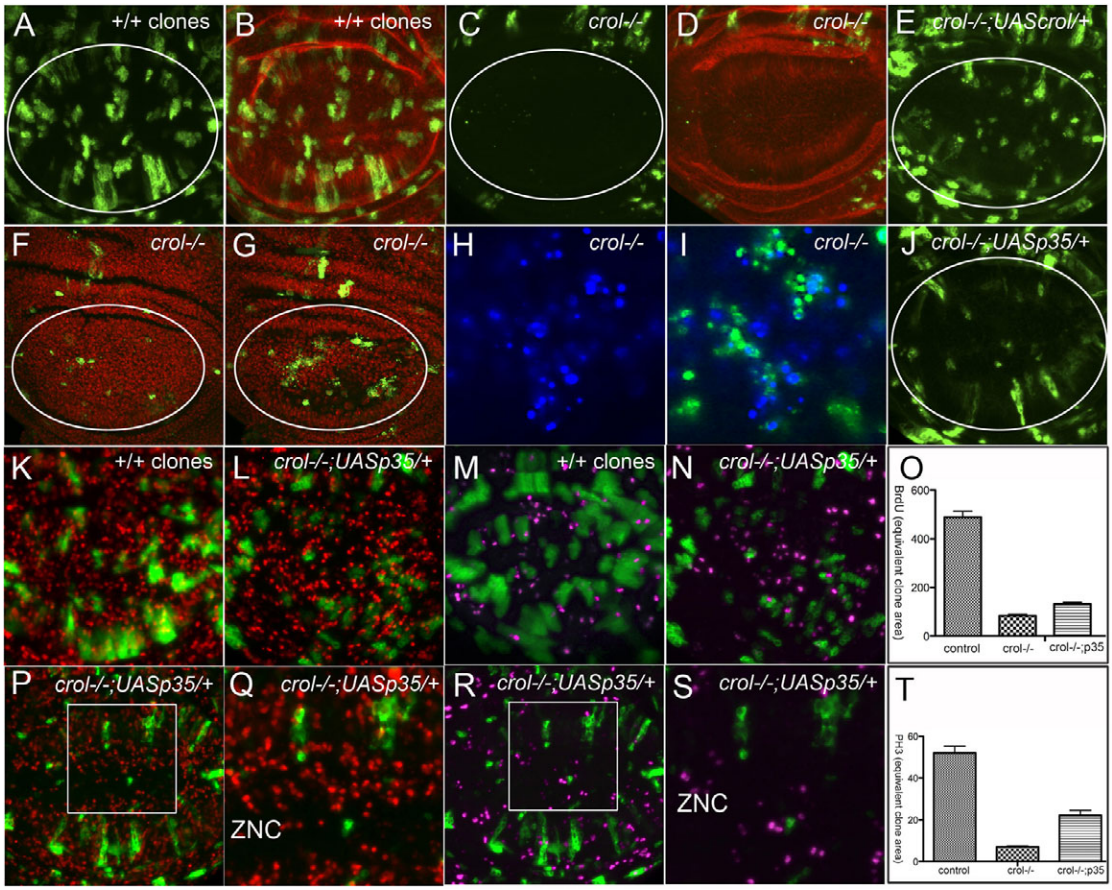


**Fig. 2. *crol* is required for cell cycling.** (A-N,P-S) Clones generated using MARCM (positively marked with GFP). (**A**,**B**) 120-hour wild-type clones; (**C**,**D**) 120-hour $crol^{-/-}$ clones. (**E**) 120-hour *crol* mutant clones expressing *UAS-crol*. (F-J) *crol* clones die by apoptosis. (**F**) Apical section of 96-hour *crol* clones. (**G**) Basal section of the disc in H, counterstained with propidium iodide (red). (**H**) Higher magnification to show pyknotic nuclei with DAPI (blue) and (**I**) merged with GFP. (**J**) 120-hour $crol^{-/-}$ clones overexpressing *UAS-p35*. In B and D, discs have been co-stained with phalloidin-TRITC to distinguish the hinge from the pouch; the pouch is marked by a white line in A,C,E-G,J. (K-T) Cell cycle analysis of *crol* mutant clones in the *UAS-p35* background. (**K-N**) 96-hour discs 48 hours after heat-shock. BrdU labelling (red) in control clones (K) and $crol^{-/-}$ clones (L) or PH3 staining (purple) in control (M) and *crol* mutant clones (N). (**P**,**Q**) $crol^{-/-}$ clones in 120-hour discs with BrdU (P) and a higher magnification (Q) to show cells flanking the ZNC, which corresponds to the white square in P. (**R**,**S**) $crol^{-/-}$ clones in 120-hour discs with PH3 and a higher magnification (S) to show mitotic cells adjacent to the ZNC, which corresponds to the white square in R. (**O**,**T**) Quantification of BrdU (O) and PH3 (T) in 96-hour discs. Counts are from equivalent clone areas (three sets of 70,000 pixels) from control (BrdU 488.6±24.3, PH3 52.0±3.2), $crol^{-/-}$ (BrdU 84.24±5.2, PH3 7.02±0.6) and $crol^{-/-}$; *UAS-p35* (BrdU 131.61±7.8, PH3 22.14±2.4). A statistically significant decrease was observed between $crol^{-/-}$ and control clones for BrdU ($P$<0.0001) and PH3 ($P$<0.0001), and for $crol^{-/-}$; *UAS-p35* and control clones BrdU ($P$<0.0001) and PH3 ($P$<0.0002).

## Crol is required for cell cycle progression

To determine whether Crol was required for cell cycle in the wing disc, we generated mutant clones for the embryonic lethal *crol* mutant ($crol^{k05205}$) in third instar larvae, using MARCM (Lee et al., 2000). Wing discs from 120 hour larvae contained very few $crol^{-/-}$ clones in the pouch (Fig. 2C,D), which differentiates into the wing blade, compared with wild type (Fig. 2A,B). By contrast, *crol* mutant clones were observed in imaginal tissue destined to give rise to hinge (Fig. 2C,D). As both control and *crol* mutant clones are induced at the same developmental time point [heat shock induced Flp-mediated recombination at 72 hours after egg deposition (aed)], this result suggests that $crol^{-/-}$ cells within the wing pouch, but not in the hinge region, either fail to proliferate and/or are removed by apoptosis. Although previous analysis had verified that $crol^{k05205}$ contained a single *P* element in the *crol* promoter (Spradling et al., 1999), we wanted to show that the phenotype was due to loss of Crol function. Indeed, expression of the *UAS-crol* transgene in $crol^{k05205}$ mutant clones resulted in rescue of clonal size and survival (Fig. 2E). Thus, the reduced size of the clonal tissue within the pouch is due to loss of *crol*.

The absence of *crol* mutant clones in 120-hour discs 48 hours after clone induction, suggested they might be removed from the wing pouch epithelium via apoptosis. Analysis of 96 hour wing discs (48 hours after clone induction) revealed small *crol* clones in the apical sections of the wing epithelium (Fig. 2F) and in the basal section of the wing pouch GFP marked *crol* mutant cells with a pyknotic morphology were observed (Fig. 2G-I). Thus, *crol* mutant clones are more readily eliminated from the wing disc epithelium during later stages of wing disc development. In order to inhibit apoptosis in *crol* mutant clones, we co-expressed a *UAS-p35* transgene to prevent caspase-dependent cell death (Hay et al., 1994). In the p35 background, more *crol* mutant cells were observed (Fig. 2J), suggesting that *crol* mutant tissue is normally removed by apoptosis. It is important to note, however, that inhibition of caspase-

dependent death does not completely restore the size and number of *crol* mutant clones (compare Fig. 2J with the control in Fig. 2A), suggesting that apoptosis is not the sole explanation for the reduction in size and number of *crol* mutant clones.

The small size of *crol*$^{-/-}$ clones was also associated with reduced proliferation, as cell cycle analysis carried out for *crol*$^{-/-}$*;UASp35* clones in 96-hour discs revealed reduced numbers of S phases (BrdU, Fig. 2L compared with control Fig. 2K) and mitoses (PH3, Fig. 2N compared with control Fig. 2M). Reduced cell cycle progression was also observed in clones from 120-hour wing discs (BrdU in Fig. 2P,Q and PH3 in Fig. 2R,S). Counts of cycling cells from equivalent clone areas in 96-hour discs revealed a significant reduction in S phase (Fig. 2O) and mitosis (Fig. 2T) for both the *crol* mutant alone (BrdU, $P<0.0001$ and PH3, $P<0.0001$) and *crol* mutant plus *UAS-p35* (BrdU, $P<0.0001$ and PH3, $P<0.0002$), when compared with control clones. Thus, *crol* mutant clones have fewer cell cycles and are normally eliminated from the wing pouch epithelium by apoptosis, as expression of p35 within the mutant clone allows survival despite their reduced proliferation. In summary, these data show that *crol* mutant cells have a proliferative disadvantage, which is consistent with Crol normally playing a role in positively regulating cell cycle progression.

## Ectopic overexpression of Crol drives cell cycle progression

To determine whether Crol might drive proliferation, we overexpressed *UAS-crol* in the posterior compartment (PC) of the wing disc with *en-GAL4*. Increased S phases were evident in the PC of the wing pouch ZNC in cells over-expressing *UAS-crol* (Fig. 3C,D) compared with the control (Fig. 3A,B). In addition, PH3 staining of the same disc revealed mitotic cells within the PC ZNC (Fig. 3G,H) compared with the control (Fig. 3E,F). As cells within

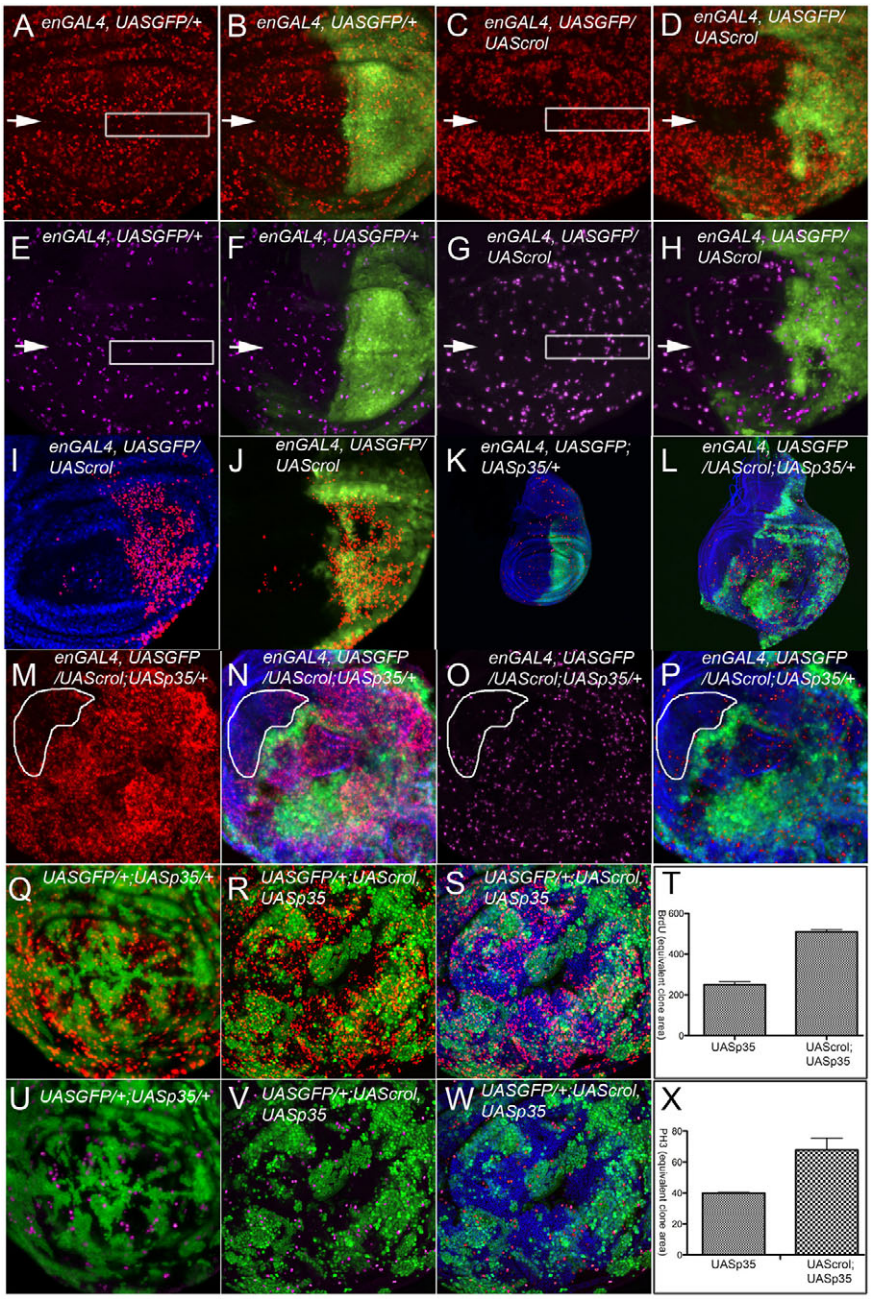


**Fig. 3. Overexpression of Crol promotes cell cycle.** (**A**,**B**) BrdU (red) of control *en-GAL4, UAS-GFP/+*. (**C**,**D**) BrdU of *en-GAL4, UAS-GFP/UAS-Crol*. (**E**,**F**) PH3 (purple) of control. (**G**,**H**) PH3 of the same disc for which BrdU is shown in C and D. In A-H, the arrow indicates the ZNC. (**I**,**J**) TUNEL labelling (red) of the basal layer of an *en-GAL4, UAS-GFP/UAS-Crol* disc and counterstained with DAPI (blue) in I. (**K**) *en-GAL4, UAS-GFP/+;UAS-p35/+* control and (**L**) *en-GAL4, UAS-GFP/UAS-Crol;UAS-p35/+*. Discs in K and L are both taken at 10× magnification and stained with PH3 (red) and DAPI (blue). (**M-P**) 120-hour *en-GAL4, UAS-GFP/UAS-Crol;UAS-p35/+* taken at 20× magnification compared with 40× for discs in A-H. (M) BrdU (red), (O) PH3 (purple). The merge with GFP and DNA (blue) is shown with BrdU in N and PH3 in P. In M-P, a white line marks the AC of the pouch. (**Q-X**) Cell cycle analysis of 120-hour discs with flip-out clones 72 hours after induction. (Q) *UAS-p35* with BrdU (red), (R) *UAS-crol;UAS-p35* with BrdU (red) and merged with DAPI in S. (T) Quantification of BrdU in 120-hour discs. Counts are from equivalent GFP-positive areas (three sets of 70,000 pixels) for *UAS-p35* (249.7±14.2) and *UAS-crol;UAS-p35* (509.8±10.9). (U) *UAS-p35* with PH3 (purple), (V) *UAS-crol;UAS-p35* with PH3 (purple) and merged with DAPI in W. (X) Quantification of PH3 (as above) from *UAS-p35* (39.9±0.5) and *UAS-crol;UAS-p35* (67.9±7.7). A statistically significant increase was observed between *UAS-crol;UAS-p35* and *UAS-p35* clones for BrdU ($P<0.0001$) and PH3 ($P<0.0033$).

the PC ZNC have normally exited the cell cycle and are arrested in G1 phase prior to differentiation, this suggests that overexpression of *crol* interferes with developmentally regulated cell cycle exit in the wing pouch.

Despite the finding that *crol* overexpression results in ectopic cell cycles in the ZNC, the PC of *en-GAL4/+;UAS-crol/+* adult wings (Fig. 4A) is smaller than wild type. This may be explained by the observation that increased cell doubling times can lead to compensatory cell death (Reis and Edgar, 2004). Indeed, TUNEL labelling revealed massive levels of apoptosis within *crol*-expressing cells of the PC (Fig. 3I,J). Although we have shown that overexpression of *crol* causes wing imaginal cells to die by apoptosis, it is unlikely that the primary role of Crol is to drive apoptosis, as cells lacking *crol* have decreased cell cycles and increased cell death rather than enhanced survival (Fig. 2). Therefore the increased apoptosis in cells overexpressing *crol* is most probably a consequence of ectopic proliferation, rather than a direct affect of *crol* in driving cell death.

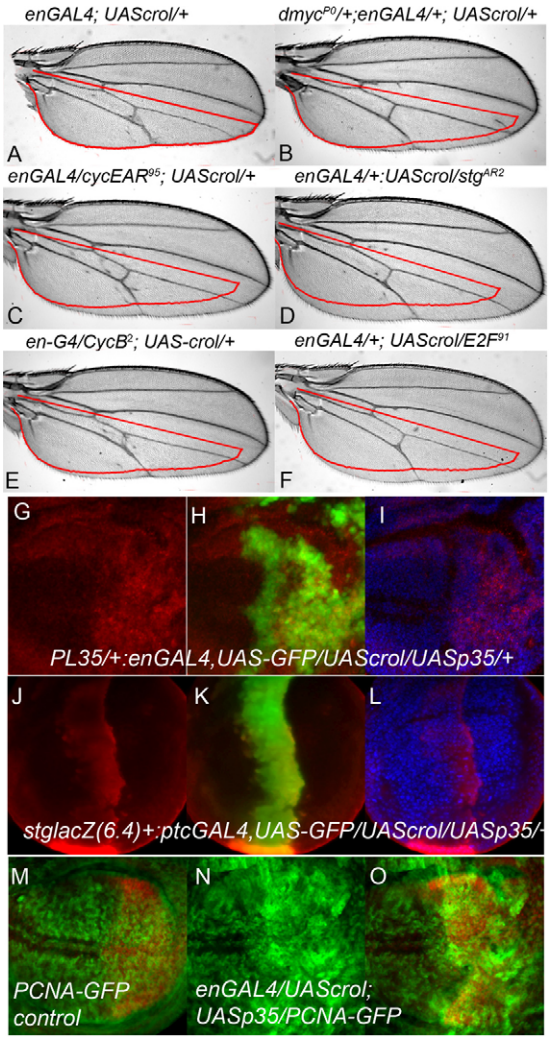


**Fig. 4. Crol regulates G1-S and G2-M cell cycle genes.** (**A**) Adult wings overexpressing *UAS-crol* with *en-GAL4* and with the following cell cycle mutants: (**B**) *dm^PO*, (**C**) *cycE^AR95*, (**D**) *stg^AR2*, (**E**) *cycB^2*, (**F**) *E2F1*. A tracing of the PC from *en-GAL4/+, UAS-crol/+* has been superimposed to show relative modification of compartment size. (**G-I**) β-gal (red) of 120-hour wing discs for *PL35 /+; en-GAL4,UAS-GFP/UAS-crol;UAS-p35/+*. (**J-L**) β-gal (red) on 96-hour wing discs for *stg-lacZ 6.4/+; Ptc-GAL4,UAS-GFP/UAS-crol; UAS-p35/+*. (**M**) Control PCNA-GFP in the 120-hour wing disc with the PC marked by En (red). (**N**) *en-GAL4/UAS-crol; UAS-p35/PCNA-GFP*, merged with En antibody in red (**O**).

## Overproliferation results when apoptosis is blocked in cells overexpressing crol

The co-expression of *UAS-p35* to inhibit caspase-dependent apoptosis in *crol*-expressing cells revealed the contribution of apoptosis to the reduced PC. Indeed, we found a substantial increase in the size of the PC from 120-hour larvae overexpressing *UAS-crol* in the presence of *UAS-p35* (Fig. 3L compared with control Fig. 3K), which was associated with a massive increase in S phase (Fig. 3M,N) and mitosis (Fig. 3O,P). Analysis of 96-hour wing discs co-expressing *UAS-crol* and *UAS-p35* also revealed an increase in mitotic cells (see Fig. S1A-C in the supplementary material) and S phases in the PC (see Fig. S1D-F in the supplementary material). Importantly, *crol* overexpression results in ectopic mitoses (see Fig. S1G-I in the supplementary material) and S phase cells (see Fig. S1J-L in the supplementary material) within the PC ZNC. Thus, *crol* over-expression can override the developmental signalling that promotes cell cycle exit in the ZNC. In order to further characterise the overgrowth, we carried out cell cycle analysis of flip-out clones. As found for wing discs co-expressing *crol* and p35 in the PC, discs containing clones co-expressing *UAS-crol* and *UAS-p35* were also overgrown and clonal tissue had increased BrdU (Fig. 3R,S, compare with control in Fig. 3Q) and PH3 (Fig. 3V,W, compared with the control in Fig. 3U). Quantification revealed a significant increase in both BrdU (Fig. 3T; $P<0.0001$) and PH3 (Fig. 3X; $P<0.0033$) from equivalent clone areas for *UAS-crol*;*UAS-p35* clones compared with *UAS-p35* alone.

Collectively, these data (Figs 2 and 3) suggest that Crol is necessary for normal cell cycle progression, and overexpression of *crol* can drive the cell cycle in the larval wing disc, in a manner that overcomes developmental cell cycle inhibition. Furthermore, the accelerated rate of cell cycle progression in tissue overexpressing *crol* results in a high degree of compensatory apoptosis that, if blocked, leads to massive tissue overgrowth.

Interestingly, in the p35 background, some cells in the PC lose expression of GFP, which is first evident as small patches of non-GFP cells at 96 hours (see Fig. S1A-F in the supplementary material) and more striking at 120 hours (see Fig. S1G-L in the supplementary material). Increased proliferation is observed in both the GFP-positive cells still overexpressing *crol* and in the patch of cells that have lost GFP. Although it is unclear why patches of cells lose GFP, staining with anti-En revealed continued expression of En, suggesting that these cells maintain their posterior identity (see Fig. S1M-O in the supplementary material). It is possible these cells have acquired an ‘undead’ state owing to the expression of *p35*, as caspase-inhibited cells initiate apoptosis but do not die. ‘Undead’ cells can cause additional proliferation of surrounding cells owing to transient signals sent from the ‘undead’ cell (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004). Thus, it is possible that cells co-expressing *crol* and *p35* might exhibit ‘undead’ properties and affect secreted signalling molecules to cause non-autonomous affects on the surrounding cells. Indeed, increased proliferation is observed in cells adjacent to clones co-expressing *UAS-crol* and *UAS-p35* (Fig. 3R,S,V,W). Importantly, expression of p35 alone in the PC does not affect proliferation (see Fig. S2 in the supplementary material). Thus, *crol* overexpression drives proliferation, but additional cells are

normally removed by compensatory apoptosis (Reis and Edgar, 2004). If apoptosis is blocked with p35, these cells may continue to proliferate or acquire an 'undead state'. Therefore, expression of p35 in cells overexpressing *crol* results in tissue overgrowth because (1) extra cells resulting from overproliferation are not removed by compensatory apoptosis and (2) the potentially 'undead' cells can promote further growth by sending growth promoting signals to neighbouring cells.

### The *crol* overexpression phenotype is sensitive to the dose of cell cycle regulators

Thus far we have shown that loss of *crol* results in reduced cell cycles and that overexpression of *crol* leads to ectopic proliferation. To investigate whether the effects of increased Crol were sensitive to cell cycle regulators, we used the adult wing phenotype resulting from *crol* overexpression. The *crol* overexpression adult wing phenotype represents the effects of *crol* on proliferation and on cell death seen in larval wing disc development (Fig. 3), and results in a reduced PC (Fig. 4A). To examine the interaction between cell growth/cycle genes and *crol*, we tested whether various cell cycle mutants could modify the *crol* overexpression phenotype. Halving the dose of *dm* (Johnston et al., 1999) suppressed the *crol* phenotype (Fig. 4B), suggesting *dm* is required downstream of Crol and is rate-limiting for Crol-induced G1- to S-phase progression. This was not specific to *dm*, suppression of the *crol* phenotype was also achieved by halving the dose of the essential S-phase Cyclin *cycE* (Knoblich et al., 1994) (Fig. 4C). Suppression of the *crol* phenotype also resulted from halving the dose of either *stg/Cdc25* (Edgar and Datar, 1996) or the G2-M Cyclin *CycB* (Lehner and O'Farrell, 1990) (Fig. 4D,E, respectively). Thus, the *crol* overexpression phenotype is also sensitive to the dose of G2-M regulators, in accordance with our finding that overexpression of *crol* increases mitoses. Consistent with the interaction of both G1-S and G2-M regulators with the *crol* overexpression phenotype, we found suppression by halving the dose of the *E2F1* transcription factor (Fig. 4F), which upregulates both *cycE* and *stg* to drive cells through both S phase and mitosis (Reis and Edgar, 2004).

### Crol upregulates *dm*, *stg* and *E2F1*-dependent gene transcription

In order to test whether Crol was driving proliferation via upregulating transcription of essential cell cycle genes, we used *lacZ* enhancer traps for *dm* (*PL35*) (Bourbon et al., 2002) and *stg* (*stg-lacZ 6.4*) (Lehman et al., 1999). Although we found that β-gal staining for *PL35-dm-lacZ/+* and *stg-lacZ/+* was barely above background (data not shown), when we overexpressed *UAS-crol* with *en-GAL4* in the *UAS-p35* background, increased *dm-lacZ* staining was observed in the PC of the wing disc (Fig. 4G-I). In addition, using *ptc-GAL4* to co-express *UAS-crol* and *UAS-p35* in the *stg-lacZ* reporter background showed increased β-gal expression in the Ptc domain (Fig. 4J-L). These findings are consistent with overexpression of *crol* resulting in upregulation of *dm* transcription to drive S-phase progression and of *stg* transcription to drive M-phase progression.

To determine whether overexpression of *crol* affects the *E2F1* transcription factor, we used a GFP reporter for E2F1 transcriptional activity [the proliferating cell nuclear antigen GFP reporter, *PCNA-GFP* (Thacker et al., 2003)]. We observed a massive increase in PCNA-GFP staining across the entire PC of the wing discs expressing *UAS-crol* with *en-GAL4* (Fig. 4N,O) compared with the control (Fig. 4M), showing Crol acts to upregulate E2F transcriptional activity.

### Crol blocks the Wg signalling pathway required for inhibition of cell proliferation

The finding that *crol* overexpression drives ZNC cells through the cell cycle combined with the knowledge that Wg signalling is important for cell cycle arrest in the ZNC (Johnston and Edgar, 1998) suggested Crol might act to block the Wg pathway. To determine whether Crol might mediate cell cycle via affects on the Wg pathway, we tested for dominant modification of the *crol* overexpression wing phenotype by Wg pathway mutants (Fig. 5). We found that halving the dose of genes required for Wg activation, such as the β-catenin homologue *armadillo* (*arm*) (Tolwinski and Wieschaus, 2001), resulted in enhancement of the *crol* overexpression phenotype (Fig. 5B). Conversely, halving the dose of inhibitory components of the Wg pathway, such as *Axin* (Willert et al., 1999) or *Sgg/GSK3β* (Siegfried et al., 1992), resulted in suppression of the wing phenotype (Fig. 5C,D, respectively). The *crol* overexpression phenotype is therefore sensitive to the dose of Wg pathway components, in a manner consistent with Crol acting to antagonise Wg signalling in order to overcome cell cycle inhibitory effects of Wg in the wing pouch.

### Reducing Wg signalling restores *crol*$^{-/-}$ clonal size

To determine whether the failure to proliferate in *crol* mutant clones was dependent on increased Wg signalling within the clone, we tested whether the growth deficiency could be suppressed by halving the dose of *arm*. Analysis of 120-hour third instar wing discs revealed that growth of *crol* mutant clones was restored (Fig. 5E-G) compared with the *crol* mutant alone (Fig. 2C,D). Thus, the proliferative disadvantage of the *crol* mutant clones is sensitive to the dose of the Wg pathway transcription factor Arm, suggesting that failure of the *crol* mutant clones to proliferate is dependent on signalling through the Wg pathway.

### Crol downregulates wg transcription and is essential for suppression of Wg

To determine whether *crol* acts to repress Wg, we first tested whether overexpression of *UAS-crol* in the PC with *en-GAL4* affected *wg-lacZ* enhancer-trap activity (Fig. 5H-M). The level of β-gal staining was reduced in the PC cells overexpressing *crol* when compared with either the AC of the same disc (Fig. 5K-M) or the PC from the control (Fig. 5H-J). We counterstained with DAPI to show that the reduced staining was not due to the loss of cells via apoptosis, as we observe abundant cells in the apical region of the disc (Fig. 5M). This suggests that *wg* promoter activity is downregulated in response to *crol* overexpression in the wing pouch.

Although these findings are consistent with Crol behaving as a repressor of *wg* gene transcription, we wanted to determine whether Crol was necessary for downregulation of *wg*. As we had previously found that *crol* mutant cells proliferated more slowly than surrounding wild-type tissue, we attempted to ameliorate this proliferative disadvantage by generating *crol* clones in a minute [*M(Z)2*] background. Although homozygous *crol* clones were eliminated from the pouch in a wild-type background, clones induced at the equivalent stage in the slow-growing *Minute* background were detected in the pouch (Fig. 5Q-S). Nevertheless, control clones induced at the same time were much larger (Fig. 5N-P), suggesting that *crol*$^{-/-}$ cells grow much more slowly than *Minute* heterozygous cells. In particular, we noticed that the only *crol*$^{-/-}$ clones comprising more than one or two cells were adjacent to the endogenous Wg domain, from either the pouch or the hinge (Fig. 5Q-S). However, regardless of the position of the *crol* mutant clone, the level of Wg protein was elevated (Fig. 5Q-S) when compared

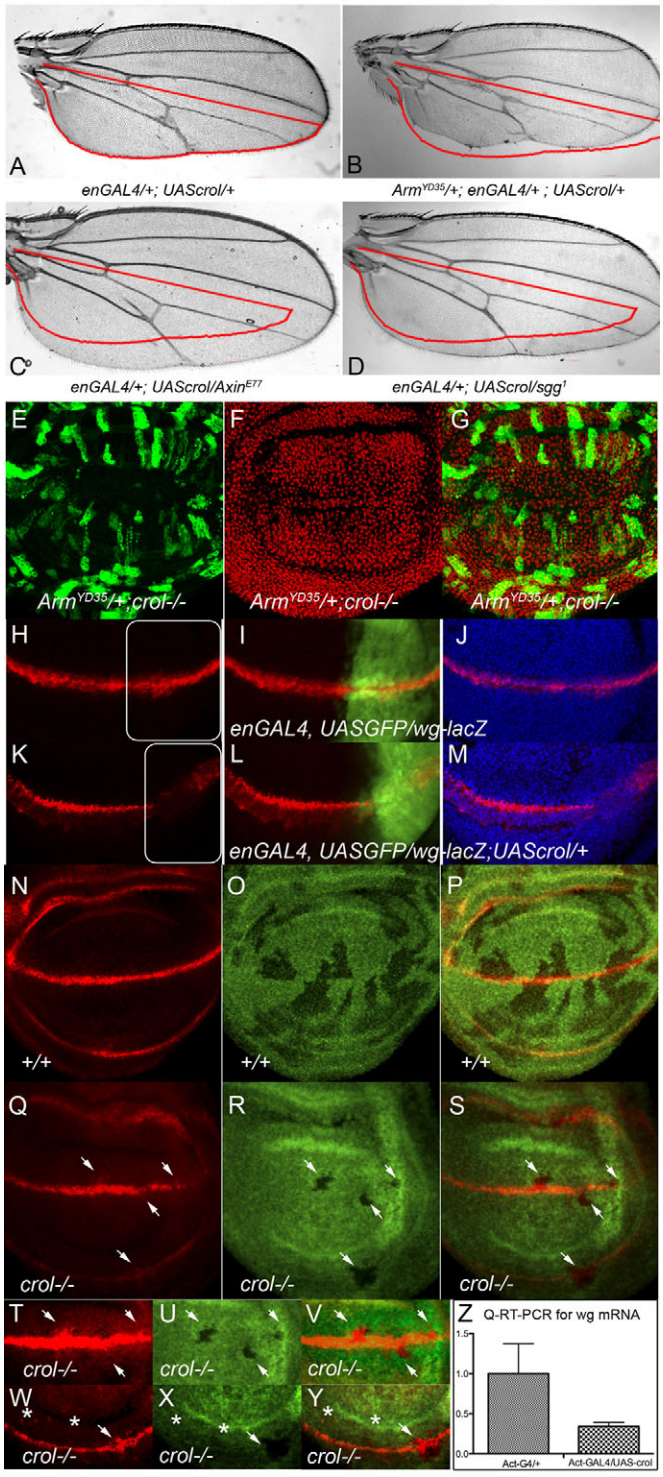


**Fig. 5. Crol inhibits Wg transcription.** (**A**) *crol* overexpression wing phenotype alone and with (**B**) $arm^{YD35}$/+; (**C**) $Axin^{E77}$/+ and (**D**) $Sgg^{1}$/+. (**E-G**) *crol* mutant clones in the $arm^{YD35}$/+ background, positively marked with GFP and counterstained for DNA (red), compare with Fig. 2C,D. β-gal staining (red) for control *en-GAL4, UAS-GFP/wg-lacZ* (**H-J**) and *en-GAL4, UAS-GFP/wg-lacZ; UAS-Crol/+* (**K-M**). (J,M) Merge with DAPI to show cells present in the apical region of the disc. Wg antibody (red) on control clones (**N-P**) and *crol* mutant clones generated in the *Minute* background (**Q-S**) are marked by the absence (arrows) of βgal (green). (**T-Y**) Arrows indicate *crol* clones adjacent to the ZNC (T-V) and the hinge (W-Y), and ectopic Wg in small clones are highlighted with asterisks (W-Y). (**Z**) Q-RT-PCR for *wg* mRNA in third instar larval imaginal tissues: *Actin-GAL4/+* (1.0±0.21); *Actin-GAL4/UAS-crol*, (0.34±0.03). PCR was carried out in triplicate and normalized using GAPDH. The level of Wg mRNA is significantly reduced in *Actin-GAL4/UAS-crol* compared with *Actin-GAL4/+* ($P$=0.0376).

with control clones (Fig. 5N-P); Wg protein associated with the Wg domains in the ZNC (Fig. 5T-V) or in the hinge (Fig. 5W-Y) was expanded and ectopic Wg expression was seen in the small one or two cell clones isolated from endogenous Wg domains (Fig. 5W-Y). In summary, these data suggest that Crol is normally required for repression of Wg expression.

To quantitate the effect of Crol on *wg* transcription, we carried out quantitative real-time PCR (Q-RT-PCR) to measure the level of *wg* mRNA in third instar larval imaginal discs (wing, eye-antennal, leg) and brain tissues. We compared the level of *wg* mRNA from tissue overexpressing the *UAS-crol* transgene with the weak global driver *Actin-GAL4* with control tissue (*Actin-GAL4/+*). Analysis was carried out in triplicate and *wg* mRNA levels were normalized using GAPDH. There was a significant decrease in *wg* mRNA levels in third instar imaginal tissues overexpressing *crol* (Fig. 5Z, $P$<0.0376) and we therefore conclude that Crol is capable of downregulating *wg* transcription in larval imaginal tissues.

## Crol acts to block Hfp expression in the wing disc

Our previous studies have demonstrated that Hfp is upregulated in response to Wg signalling and is required for cell cycle arrest in the ZNC (Quinn et al., 2004). Hfp negatively regulates G1- to S-phase progression by downregulating *dm* and inhibits mitosis via Stg. We therefore tested whether increased proliferation driven by Crol was associated with changes to Hfp protein levels (Fig. 6). Hfp protein is normally detected in the nuclei of all wing imaginal disc cells (Fig. 6A-C); however, a clear reduction in Hfp was observed in PC cells expressing *UAS-crol* (Fig. 6D-F). Thus, Crol leads to downregulation of Hfp. Given this result, the mechanism by which the *crol* mutant dominantly enhances the *hfp* overexpression phenotype (Fig. 1) may be via its effect on increasing Wg levels, which would in turn result in increased Hfp levels and further cell cycle inhibition.

## Wg regulation is not achieved via indirect affects on the Hh or Notch pathways

The Hedgehog (Hh) and Notch (N) pathways are key upstream regulators of Wg in the wing disc. To determine whether Crol might regulate *wg* indirectly, via affects on the Hh or N pathways, we tested whether these pathways were altered in *crol* mutant clones or tissue overexpressing *crol*.

The zinc-finger transcription factor Cubitus interruptus (Ci) is the mediator of Hh-dependent transcriptional activation of *wg*. Ci is both necessary and sufficient to drive expression of Hh-responsive genes, including the upregulation of *wg* transcription (Aza-Blanc and Kornberg, 1999). Upregulation of *wg* could therefore occur via Hh signalling. However, ectopic Ci protein was not detected in *crol* mutant clones, suggesting that Crol does not affect *wg* transcription indirectly via the Hh pathway (see Fig. S3A-C in the supplementary material).

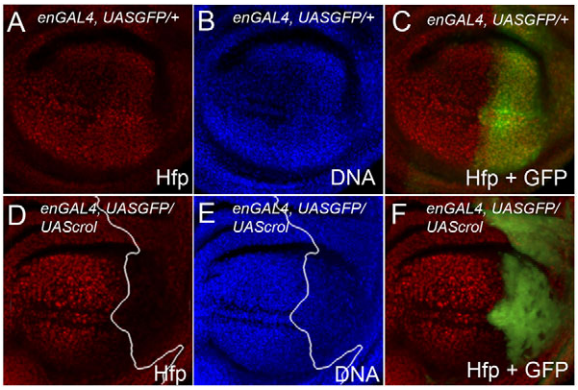


**Fig. 6. *crol* overexpression results in downregulation of the cell cycle inhibitor Hfp.** (**A**,**C**) Hfp antibody (red) on *en-GAL4, UAS-GFP/+*; (**D**,**F**) *en-GAL4, UAS-GFP/+; UAS-Crol/+*; (**B**,**E**) DNA (blue) to show the presence of cells.

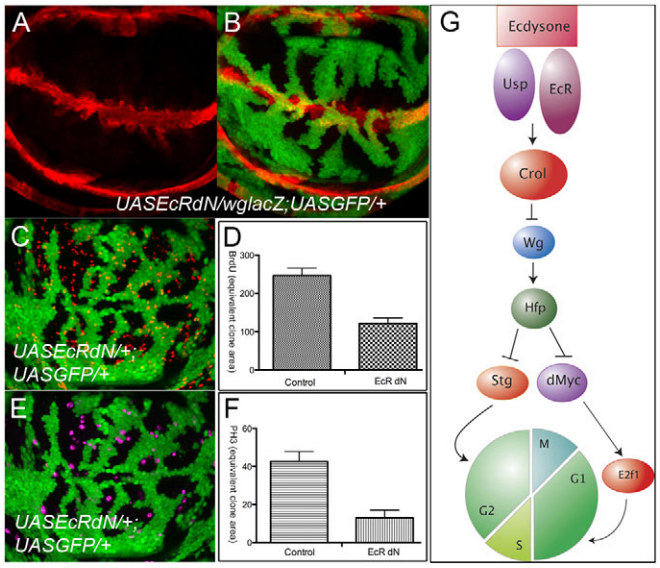


**Fig. 7. Signalling via EcR is required for repression of Wg and cell cycle progression.** (**A**,**B**) β-gal (red) for *UAS-EcRdN* flip-out clones in the *wg-lacZ* background. (**C**) BrdU (red) for *UAS-EcRdN* clones and (**D**) quantification of BrdU in 120 hour larval wings (see above); *UAS-EcRdN* clones (121.7±14.4) have significantly less BrdU than the control (246±19.7; *P*=0.0001). (**E**) PH3 (purple) for *UAS-EcRdN* clones and (**F**) quantification of PH3; *UAS-EcRdN* clones (13.0±4.0) have significantly less PH3 than the control (42.65±5.3; *P*=0.0001). (**G**) Model for Crol connecting steroid hormone signalling to cell cycle progression. Crol is upregulated in response to ecdysone signalling and increased Crol results in decreased *wg* mRNA expression. Reduced Wg signalling results in less Hfp, which leads to increased *dm* expression to drive S phase and mitosis via increased Stg.

Notch activity also plays a role in cell cycle arrest during wing development (Johnston and Edgar, 1998). Notch is activated in cells along the dorsoventral (DV) boundary (ZNC) of the wing disc, where it is required for Wg expression (de Celis et al., 1996). However, decreased levels of the Notch target, En(spl)m7 were not observed in *crol* overexpressing cells, suggesting Notch signalling is not downregulated by Crol (see Fig. S3D-F in the supplementary material). Taken together, these results suggest that Crol does not affect *wg* transcription indirectly via effects on the Notch or Hh pathways.

### The Ecdysone pathway is required for Wg repression and proliferation

To test whether EcR signalling leads to altered *wg* transcription and rates of proliferation in the wing disc, we analysed flip-out clones overexpressing *UAS-EcRdN* [*UAS-EcRA.F645A* (Cherbas et al., 2003)] (Fig. 7). In the *wg-lacZ* background, we observed increased levels of β-gal staining within clones adjacent to the Wg domain in both the pouch and the hinge (Fig. 7A,B). These findings are consistent with the EcR normally being required to inhibit *wg* transcription in the wing imaginal disc. As predicted, the increased level of Wg was associated with a significant reduction in cell cycle progression in *UAS-EcRdN* clones, measured by BrdU (Fig. 7C, quantified in 7D, $P<0.0001$) and PH3 (Fig. 7E, quantified in Fig. 7F, $P<0.0001$). These results are consistent with EcR normally being required for repression of Wg and therefore cell cycle progression in the wing pouch. However, unlike $crol^{-/-}$ clones, *UAS-EcRdN* clones are not out-competed and eliminated by apoptosis, which might be a consequence of reduced levels of the cell death targets normally activated by the EcR pathway (reviewed by Baehrecke, 2000; Jiang et al., 1997; Yin and Thummel, 2005). Therefore, in contrast to loss of Crol function, blocking the ecdysone pathway upstream of Crol results in survival. Thus, in this background, the increase in *wg* expression is associated with decreased proliferation, but not with reduced survival.

## DISCUSSION

We have demonstrated that the Ecdysone-inducible gene *crol* regulates Wg and cell cycle. Crol is necessary for cell cycle progression and increased Crol results in ectopic proliferation in the wing disc. We find that overexpression of *crol* causes increased apoptosis in wing discs, which is most probably a consequence of increased proliferation being accompanied by compensatory apoptosis. Indeed, it is unlikely that the primary role of Crol is to drive apoptosis as cells mutant for *crol* have increased cell death rather than enhanced survival. Modification of the *crol* overexpression phenotype by cell cycle genes (*dm*, *cycE*, *stg*, *cycB* and *E2F1*) and the finding that overexpression of *crol* results in increased *dm* and *stg* transcription, and *E2f1* activity provides further support that Crol positively regulates proliferation. Crol most probably affects cell cycle in the wing via downregulation of *wg* transcription, which is unlikely to be due to indirect affects on either the Notch or Hh pathways. Our future studies are therefore aimed at determining whether Crol achieves repression of Wg by directly binding the *wg* promoter to downregulate *wg* transcription.

The Wg signal within the wing imaginal disc differentially affects proliferation. In the pouch, Wg drives cell cycle exit and differentiation, while upregulation of Wg signalling in the hinge drives proliferation (Johnston and Sanders, 2003). In accordance with Crol being required to repress Wg throughout the wing disc, Crol protein is present in both the pouch and hinge, and loss of Crol results in increased Wg in both domains. In agreement with the proliferation driven by Crol being mediated by the Wg signal, we observe differential affects on proliferation for *crol* mutant clones in the pouch compared with the hinge. We observe frequent $crol^{-/-}$ clones in the hinge, most probably as a consequence of Wg promoting proliferation in these cells (Johnston and Sanders, 2003). Consistent with Wg repressing proliferation in the pouch, we see very few $crol^{-/-}$ clones in this region.

However, if the only function of Crol was to inhibit *wg* transcription, it would be expected that loss-of-function *crol* clones would behave similarly to clones with increased Wg in regard to proliferation and survival. However, although Wg promotes survival (Johnston and Sanders, 2003), $crol^{-/-}$ clones are removed by apoptosis, which is most probably due to cell competition (de la Cova et al., 2004). As Crol has also been shown to be required for integrin expression, removal of $crol^{-/-}$ cells from the wing epithelium may also be a consequence of a potential reduction in cell adhesion (D'Avino and Thummel, 2000). Indeed, loss of integrin-mediated cell adhesion to the extracellular matrix (ECM) has been shown to cause apoptosis, a process known as anoikis (Jan et al., 2004). Thus, in $crol^{-/-}$ clones, increased Wg leads to reduced proliferation, but reductions to integrin levels and cell adhesion would be expected to reduce survival.

Our current working model for how Crol connects steroid hormone signalling to cell cycle progression is shown in Fig. 7. First, Crol is upregulated in response to ecdysone (D'Avino and Thummel, 1998) and then the increased level of Crol results in decreased *wg* transcription. Reduced Wg signalling results in downregulation of Hfp, which results in increased *dm* expression and S-phase progression (Quinn et al., 2004). Increased Dm levels also lead to upregulation of its cell cycle targets *cycE*, *cycD* and *cdk4*, resulting in inactivation of *Rbf* and increased activity of the S phase transcription factor E2f1 (Duman-Scheel et al., 2004). Hfp downregulation also leads to increased mitosis owing to increased Stg protein levels (Quinn et al., 2004). Thus, we have uncovered a novel mechanism for cell cycle regulation, whereby Crol acts to link steroid hormone signals to the Wg pathway and the regulation of crucial cell cycle targets.

Our finding that the early response ecdysone pathway target Crol is crucial for developmental cell cycle progression adds significantly to previous studies, which have made connections between the ecdysone pulse and developmentally regulated apoptosis and differentiation. Our finding that the ecdysone-inducible gene *crol* is required for cell cycle progression, provides evidence that the Ecdysone pulse is essential for all aspects of adult tissue morphogenesis, being required for coordination of developmental progression with cell cycle, apoptosis and differentiation. Of particular developmental importance, *crol* induction by the ecdysone pulse at the larval-pupal transition (Thummel, 1996), may be crucial for driving the final rounds of proliferation of the epithelial cells of the pupal wing blade prior to their final exit from the cell cycle between 20 and 24 hours after pupariation, before they undergo terminal differentiation (Buttitta et al., 2007; Milan et al., 1996).

Connections between mammalian steroid hormone pathways and regulation of cell cycle genes are associated with a variety of hormone-dependent diseases, including cancer (Nilsson et al., 2004). In murine embryonic stem (ES) cells, oestrogen has been shown to promote proliferation associated with increased mRNA expression of proto-oncogenes, including *Myc* (Han et al., 2006). Our work suggests that steroid hormone regulation of proliferation via the Myc family is conserved between flies and humans. However, whether mammalian steroid hormone signalling pathways regulate the human homologue of Crol (called ZNF84) to affect *wg/Wnt* transcription, *hfp/Fir* levels and cell cycle progression remains to be determined.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/16/2707/DC1

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