Antagonistic Regulation of Apoptosis and Differentiation by the Cut Transcription Factor Represents a Tumor-Suppressing Mechanism in Drosophila

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Abstract

Apoptosis is essential to prevent oncogenic transformation by triggering self-destruction of harmful cells, including those unable to differentiate. However, the mechanisms linking impaired cell differentiation and apoptosis during development and disease are not well understood. Here we report that the Drosophila transcription factor Cut coordinately controls differentiation and repression of apoptosis via direct regulation of the pro-apoptotic gene reaper. We also demonstrate that this regulatory circuit acts in diverse cell lineages to remove uncommitted precursor cells in status nascendi and thereby interferes with their potential to develop into cancer cells. Consistent with the role of Cut homologues in controlling cell death in vertebrates, we find repression of apoptosis regulators by Cux1 in human cancer cells. Finally, we present evidence that suggests that other lineage-restricted specification factors employ a similar mechanism to put the brakes on the oncogenic process.

Introduction

It has been a long-standing paradigm that impaired cell fate commitment is a key initiator of cancer development [1,2], since cancer cells display reduced differentiation properties compared to normal cells, while tumor formation can be suppressed by inducing the terminal cell fate in cancer cells [3]. The molecular basis of the interplay between cell differentiation and cancer has only recently been established. Bossuyt and colleagues (2009) demonstrated that loss of the proneural transcription factor Atonal not only leads to a loss of differentiated eye tissue but also promotes tumor formation and progression in this tissue context [4]. Thus, their work provided evidence that the maintenance of a differentiated state, which is critically controlled by a cell-type specification factor, is one crucial aspect to prevent the oncogenic process, whereas loss of this master regulator, together with other mutations creating a sensitized background, leads to the initiation of tumorigenesis. In order to evade tumor development, organisms have evolved potent mechanisms to protect themselves from the effects of mutations in their soma [5]. Programmed cell death, or apoptosis, plays a crucial role in removing abnormal cells, which could develop into tumors. This is supported by the observation that most types of cancers are associated with genetic alterations that deactivate this rescue pathway, most commonly via up-regulation of anti-apoptotic genes [6].

Since loss of terminal differentiation and the inability to activate apoptosis are crucial steps in cancer development, the existence of regulatory mechanisms preventing the accumulation of cells harboring mutations in both pathways seems essential for the survival of multi-cellular organisms. Consistently, mutations in differentiation genes very often result in the activation of the programmed cell death machinery [7,8]. However, the mechanisms linking loss of differentiation and induction of apoptosis, which is crucial for the prevention of tumor formation, are still missing. Here we have used the Drosophila posterior spiracle (PS) as a model to analyze the interplay of differentiation and apoptosis at the mechanistic level. By studying the morphogenesis of this organ, we have identified a hard-wired program through which the cell-type specifying transcription factor Cut (Ct) controls in a subset of PS cells, the filzkörper cells, initiation of differentiation and simultaneous repression of apoptosis via the direct transcriptional regulation of the pro-apoptotic gene rpr. Using two well-established Drosophila in vivo eye cancer models, we demonstrate that this regulatory circuit instructed by the transcription factor Ct is a very potent mechanism to prevent and/or reduce tumor growth, as it allows the lineage-specific removal of abnormal cells at the time of their genesis. Moreover, our data show that a related regulatory wiring is used in vertebrates and that other cell-type specification factors might employ a similar mechanism for tumor suppression, thus suggesting that the coupling of differentiation and apoptosis by individual transcription factors is a widely used and
Author Summary

Apoptosis is a highly conserved cellular function to remove excessive or unstable cells in diverse developmental processes and disease-responses. An important example is the elimination of cells unable to differentiate, which have the potential to generate tumors. Despite the significance of this process, the mechanisms coupling loss of differentiation and apoptosis have remained elusive. Using cell-type specification in Drosophila as a model, we now identify a conserved regulatory logic that underlies cell-type specific removal of uncommitted cells by apoptosis. We find that the transcription factor Cut activates differentiation, while it simultaneously represses cell death via the direct regulation of a pro-apoptotic gene. We show that this regulatory interaction occurs in many diverse cell types and is essential for normal development. Using in vivo Drosophila cancer models, we demonstrate that apoptosis activation in differentiation-compromised cells is an immediate-early cancer prevention mechanism. Importantly, we show that this type of regulatory wiring is also found in vertebrates and that other cell-type specification factors might employ a similar mechanism for tumor suppression. Thus, our findings suggest that the coupling of differentiation and apoptosis by individual transcription factors is a widely used and evolutionarily conserved cancer prevention module, which is hard-wired into the developmental program.

Results

Cut inhibits rpr expression and induction of apoptosis in the PS

The PS connects the Drosophila respiratory system to the environment and consists of an internal tube, the spiracular chamber with a refractile filter, the filzkörper, which is specified by the transcription factor Ct, and an external protrusion in which the spiracular chamber is located, the stigmaphore, which is under the control of the transcription factor Spalt (Sal) (Figure 1A; Figure S1A-S1D) [9]. In 1st instar ct mutant larvae filzkörper cells are not detectable (Figure 2A, 2D; Figure S5A, S5B), which a priori suggests that Ct is primarily required for the specification of the filzkörper cell fate. However, due to the fact that Ct has also been shown to regulate programmed cell death [7], we assumed that filzkörper cells in ct mutant embryos could be completely missing due to the induction of apoptosis. To test this hypothesis, we analyzed the expression of all Drosophila pro-apoptotic genes, which revealed the specific repression of reaper (rpr) (Figure 1B, 1C; Figure S1E, S1F; Figure S2A, S2B) but not of head involution defective (hid), grim and sickle (sik) (Figure S11-S1N) transcription by Ct in embryonic filzkörper precursor cells. Strikingly, we only observed rpr de-repression in Ct-positive filzkörper, but never in Ct-neighboring, Sal-positive stigmaphore precursor cells (Figure 1B, 1C; Figure S1E, S1F), evidencing the cell-autonomous regulation of rpr by Ct. Rpr binds to Inhibitor of Apoptosis Protein (IAP), thereby releasing inhibition of caspases and promoting apoptosis [10]. Consistently we could demonstrate enhanced cell death in ct deficient filzkörper precursor cells of stage 11 embryos using the genetically-encoded caspase reporter Apoliner [11] as well as TUNEL and Acidine Orange (AO) stainings (Figure 1D, 1E, 1F, 1G; Figure S2G, S2H). Thus, rpr de-repression is followed by apoptosis induction in ct mutant embryos. To study the interplay between cell-type specification and cell death at the mechanistic level, we identified conserved Ct-dependent regulatory regions in the rpr intergenic regions using computational methods. Due to the principal requirement of the Hox transcription factor Abdominal-B (Abd-B) for PS development [9], we searched for clusters of binding sites for Abd-B and Ct and found a highly conserved 571 bp DNA element close to the rpr coding region, which we termed rpr-HRE-571 (Figure S4). Sequence-specific interaction of recombinant Ct protein with part of the enhancer module, the S2 sub-fragment, was detected by electrophoretic mobility shift assays (Figure 1K; Figure S1G). Immunostainings revealed rpr-HRE-571-GFP activity solely in stigmaphore (Figure 1I, 1L; Figure S3A, S3E, S3I, S3M) but not in Ct-positive filzkörper precursor cells (Figure 1L; Figure S3A, S3E, S3I, S3M), that do not express rpr (Figure 1B, 1H; Figure S1E).

To validate the in vivo interaction of Ct with the identified enhancer module, we interfered with Ct-enhancer interaction in two ways: we mutated Ct binding sites within the rpr-HRE-571-S2 fragment (Figure 1J), a truncated module with identical activity as the rpr-HRE-571 enhancer (Figure 1M; Figure S3B, S3F, S3J, S3N), and eliminated all three sites in a small deletion version of the rpr-HRE-571 enhancer, termed rpr-HRE-571-S1 (Figure 1J). In both cases, GFP expression was ectopically activated in Ct-positive filzkörper precursor cells (Figure 1N, 1O; Figure S3C, S3D, S3G, S3H, S3K, S3L, S3O, S3P). These experiments demonstrated that Ct directly represses rpr transcription and thus apoptosis in the filzkörper precursor cells of the PS in a cell-autonomous manner by interacting with a small enhancer module located in the rpr intergenic region.

Repression of apoptosis by Ct is required for differentiation of filzkörper cells

Since our result showed that filzkörper cells are very efficiently eliminated by apoptosis in the absence of Ct function, we next asked whether Ct primarily acts as a repressor of programmed cell death or whether this factor is also required for the differentiation of filzkörper cells. To this end, we analyzed ct deficient cells, which were kept alive by expressing the caspase inhibitor p35 [12] in ct mutant embryos using the PS-specific driver ems-GAL4 [13]. In order to follow the cells normally under the control of Ct, these cells were GFP-labeled using the same driver, which is active only in a subset of Ct-expressing cells (Figure 2G, 2K). Our experiments revealed that Ct- and GFP-positive filzkörper cells found in the wild-type situation (Figure 2A, 2F, 2F', 2B, 2G, 2G', 2K; Figure S5A, S5D, S5G) are eliminated in ct mutant embryos (Figure 2D, 2J, 2J', 2L, 2I; Figure S5B, S5E, S5H), whereas they remained viable when apoptosis is blocked (ct'; ems:p35) (Figure 2E, 2J, 2J). However, these ct deficient, undead cells had developmental defects, as they did not properly invaginate and did not achieve their terminal cell fate as indicated by reduced expression of the apical cell polarity marker Crumbs (Crb) and the cell adhesion molecule DE-Cadherin (Figure 2F'), 2J; Figure S5D, S5F, S5G, S5J). Consistently, these cells never adopted a filzkörper cell fate (Figure 2E; Figure S5C). These defects were a consequence of blocking cell death in ct deficient, undifferentiated cells and were not due to a general response to the apoptosis inhibitor p35, as the filzkörper of ems:p35 control embryos (Figure 2B, 2G, 2G') was indistinguishable from those of wild-type embryos (Figure 2A, 2F, 2F'). Local activation of apoptosis was sufficient to induce cell death in filzkörper cells, as expression of a rpr transgene resulted in their elimination (Figure 2G, 2H, 2H'). Taken together, our results revealed that Ct carries out two functions during PS morphogenesis: it allows the survival of uncommitted precursor cells by the transcriptional repression of the pro-apoptotic gene rpr and subsequently it drives these cells into a filzkörper-specific cell fate.
Simultaneous regulation of apoptosis and cell fate commitment is a general function of Cut

Ct is expressed in many different cell and tissue types [14], thus we tested the Ct switch function in diverse developmental contexts. Ct activity was eliminated in the Drosophila eye using RNAi (Figure 3C, 3G), resulting in an overall reduction of the eye size (Figure 4A, 4B), and a loss of interommatidial bristles (Figure 3A, 3E), which normally express Ct (Figure 3C). Consistently, expression of the bristle shaft progenitor marker DE-Cadherin [15] was lost in GMR:ctRNAi pupal retinas (Figure 3B, 3F).

Figure 1. Cut directly represses rpr and apoptosis in the PS primordium. (A) Posterior spiracle (PS) of a 1st instar wild-type Drosophila larva. The filzkörper is highlighted by red asterisks. (B, C) rpr mRNA (green) expression in stage 11 wild-type (B) and ct mutant (C) embryos. Spalt (Sal) protein (blue) labels stigmatophore precursor cells, Cut (Ct) protein (red, nuclear) marks spiracular chamber and filzkörper precursor cells and the apical membrane marker Crb (red) outlines the cells. Small, green arrows in (C) mark rpr positive spiracular chamber and filzkörper precursor cells in the eighth abdominal segment (A8) of ct mutant embryos. (D, E) Over-expression of the apoptosis sensor UAS-Apoliner using the arm-GAL4 driver in stage 11 wild-type (D) and ct mutant (E) embryos. Small, green arrows in (E) mark apoptotic cells in PS precursor cells (A8) of ct mutant embryos. (F, G) TUNEL stainings in wild-type (F) and ct mutant (G) embryos. Closed arrowhead in (G) marks TUNEL-positive cells in ct mutants, which are absent in wild-type embryos (F). (H, I) Co-localization of GFP protein and rpr mRNA (H) or Cut protein (I) in stage 15 rpr-HRE-571 embryos. White circles mark the PS primordium. (J) Top: conservation blot of rpr-HRE-571 genomic region obtained from the UCSC genome browser (http://genome.ucsc.edu/). Species used for generating blot are also shown in Figure S2A. Bottom: diagram of the rpr-HRE-571 deletion constructs tested. (K) EMSA using S2 sub-fragment with Ct binding sites either in wild-type (wt probe) or mutated (mut. probe) version and no protein (‒), purified MBP protein (M), and purified Cut-MBP fusion protein consisting of the Cut repeat 3 and the Cut homeodomain (C). The black arrowheads indicate the specific DNA-protein complexes. Loading of equal amounts of labeled wild-type and mutated oligonucleotides is illustrated by formation of comparable amounts of unspecific DNA-protein complexes (black arrow). (L–O) Reporter gene expression in the PS of stage 15 embryos driven by the fragments described above. In the S2-Ctbs-GFP, line Ct binding sites within the rpr-HRE-571-S2 fragment are mutated. Spalt (Sal) and Cut (Ct) proteins label stigmatophore (blue) or spiracular chamber and filzkörper cells (red). Closed, yellow arrowheads in (N) and (O) mark reporter gene expression in filzkörper cells, whereas open, yellow arrowheads in (L) and (M) mark missing GFP expression.

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Expression of the apoptotic executor activated Caspase-3 was significantly increased in eye discs of ey::cRNAi 3rd instar larva (Figure 3D, 3H), and a significant induction of rpr RNA levels was observed using quantitative Real Time-PCR (qRT-PCR) (Figure 3K). Co-expression of either the apoptosis inhibitor p35, which rescues the eye size (Figure 4C), or of a rprRNA construct along with the cRNAi transgene resulted in a survival of ct deficient cells, as evidenced by the expression of the bristle shaft progenitor marker DE-Cadherin (Figure 3J). However, reminiscent to the phenotypes in the PS (Figure 2E, 2J), these cells were unable to adopt their terminal fate due to the absence of the cell specification factor Ct, and consequently fully differentiated interommatidial bristles were absent (Figure 3J). Similar results were obtained in other cell types specified by Ct (Figure S6), suggesting that the Ct-dependent switch between cell-type specification and programmed cell death is of general relevance.

Simultaneous and antagonistic regulation of differentiation and apoptosis represents a cancer prevention mechanism

By analyzing the Ct-spr interaction in two well-established in vivo Drosophila cancer models, we asked whether the combined transcriptional regulation of differentiation and apoptosis repression by Ct could represent a cancer prevention mechanism. In the oncogenic “eyeful” model [16], eye tumors occurred in 72.5% of control flies, with 4.9% of them showing macroscopically visible secondary tumor growths derived from the developing retina (Figure 4H, 4N) due to the eye-specific over-expression of the Notch ligand Delta (Dl) and the two epigenetic regulators longitudinal lacking (lola) and pipsqueak (psq) [16]. In contrast, pre-oncogenic ey:Dl flies over-expressing Dl exclusively in eye tissue [16] never displayed any eye tumors or invasive tumors but only mildly overgrown eyes (Figure 4D, 4N). Eye-specific inhibition of Ct activity alone only caused a small increase in primary and secondary tumor incidences in both sensitized backgrounds (Figure 4E, 4H, 4N), however, these numbers were dramatically increased when Ct function and the ability to activate apoptosis were simultaneously inhibited (Figure 4F, 4G, 4K, 4L, 4M, 4N). Consistently, increased numbers of apoptotic cells were found in tumorous tissue with reduced Ct levels (ey::Dl2xctRNAi) (Figure 4P, 4Q), demonstrating that the coupled regulation of differentiation and apoptosis by a single transcription factor is an important mechanism to suppress cancer.

However, despite increased apoptosis activation in ey::Dl2xctRNAi eye imaginal discs (Figure 4P), which should result in a reduction of tumor growth, tumor formation in these animals was increased (Figure 4N, 4Q). Using the proliferation marker Phosphorylated histone H3 (PH3), we could demonstrate that the tumor growth induced by differentiation loss is due to excessive cell proliferation (Figure 4P), which is in line with previous results [4]. What is the molecular basis for this phenotype? RT-PCR analysis of candidate genes involved in cell cycle and growth control using ey::Dl and ey::DlctRNAi eye imaginal discs revealed a strong induction of phosphoinositide 3-kinase (PI3K) upon Ct depletion (Figure 4O). It

Figure 2. Cut-dependent repression of apoptosis is required for cell survival and differentiation. (A–E) Cuticle preparations of the different genotypes with focus on the PS of 1st instar Drosophila larvae. Closed, orange arrowheads in (A) and (B) mark the filzkörper, whereas open, orange arrowheads in (C, D and E) indicate the absence of this structure in the respective genotypes. (F–L) Labeling of the different parts of the PS primordium of stage 15 embryos in the different genetic backgrounds using the filzkörper marker Ct (red, nuclear), the stigmatophore marker Sal (blue) and the apical membrane marker Crb (red). In (C, I, J, K and L) GFP expression (green) driven by the ems-GAL4 driver is shown in the different genetic backgrounds. Red asterisks in (F) mark the invaginated cells of the PS of 1st instar larvae. Closed, orange arrowheads in (A) and (B) mark the filzkörper, whereas open, yellow arrowhead in (C) and (L) GFP-positive cells in along with the which rescues the eye size (Figure 4C), or of a (Figure 3K). Co-expression of either the apoptosis inhibitor p35, ey::ctRNAi and a significant induction of ey::ctRNAi flies over-expressing Dl exclusively in eye tissue [16] never displayed any eye tumors or invasive tumors but only mildly overgrown eyes (Figure 4D, 4N). Eye-specific inhibition of Ct activity alone only caused a small increase in primary and secondary tumor incidences in both sensitized backgrounds (Figure 4E, 4H, 4N), however, these numbers were dramatically increased when Ct function and the ability to activate apoptosis were simultaneously inhibited (Figure 4F, 4G, 4K, 4L, 4M, 4N). Consistently, increased numbers of apoptotic cells were found in tumorous tissue with reduced Ct levels (ey::Dl2xctRNAi) (Figure 4P, 4Q), demonstrating that the coupled regulation of differentiation and apoptosis by a single transcription factor is an important mechanism to suppress cancer.

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has been shown before that PI3K overexpression in the ey::Dl pre-oncogenic background leads to tumor formation [17] and that PI3K is a limiting factor for RasV12 DlgRNAi induced tumor growth [18]. Thus we tested its contribution to tumor formation in Ct-induced oncogenic eyes by reducing its level in ey::Dl;2xctRNAi animals. Interestingly, we not only found a rescue of the tumorous eye growth, but also a dramatic increase in the occurrence of smaller sections of one ommatidium of 50 h pupal retinas of control Dcr2; ey::lacZ animals. (Figure 4B). Taken together, these results show that the Ct-dependent tumor growth is in part mediated by the up-regulation of the PI3K signaling pathway and that this pro-tumorigenic effect counteracts the anti-tumorigenic apoptosis effect of Ct.

Cell adhesion properties are critical for migratory behavior of tumor cells

We found cell clusters expressing the eye differentiation marker ELAV at abnormal, ectopic positions in undifferentiated tissue of 3rd instar eye-antennal discs (Figure S7), and it had been shown before that changes in the adhesive properties of cells are critical in inducing migratory behavior [19,20]. Consistently, transcriptome profiling experiments revealed a reduction in the expression of cell adhesion genes in eye-imaginal discs of Ct depleted animals in comparison to control animals (ey::Dl) (Figure 5A). To test the significance of this finding, we interfered with the function of α-PS4 integrin, one of the genes identified as Ct responsive (Figure 5A), by reducing its expression and the expression of its heterodimeric interaction partner β-PS integrin (mys) [21] in the ey::Dl pre-oncogenic background. We observed an increase in primary and secondary tumor formation in both situations, while reducing the activity of a related but Ct-independent integrin, the α-PS2 integrin (gf), did not have any effect (Figure 5B). Since decreasing the activity of another Ct responsive cell adhesion gene, namely Tissue inhibitor of metalloproteinases (Timp), also induced an increase in secondary tumors (Figure 5B), we asked if restoration of cell adhesion would be able to rescue this phenotype in the Ct loss-of-function setting. To this end, we expressed one of the major cell adhesion genes regulated by Ct, DE-Cad (Figure 5C), in eye cells of ey::2xctRNAi; p35 animals, which display high rates of invasive tumors (Figure 4N, Figure 5D), and observed a reduction of secondary tumor growth rate by more than 50% (Figure 5D). These results demonstrate that regulation of cell adhesiveness is one of the essential Ct-dependent mechanisms to suppress tumor spread. In vertebrates, invasive tumor growth requires the detachment of abnormal cells from tumor tissue and their circulation in the bloodstream [22]. To test if secondary tumor formation mediated by loss of Ct function is dependent on a similar mechanism, we analyzed the hemolymph, the insect
Figure 4. The Ct switch function represents a cancer prevention mechanism. (A–M) Adult compound eyes of the respective genotypes are shown. (L) eyeful::ctRNAi; p35 flies show high frequency of long range metastasis (marked by yellow arrowhead), a close-up of which is shown in (M). Eyes of such eyeful::ctRNAi; p35 flies show undifferentiated and overproliferated eye tissue (marked by light blue arrowhead). (N) Quantification of primary and secondary tumor formation in different genetic backgrounds. (O) Relative transcript levels of selected genes involved in cell cycle control, DNA damage response, growth control and epigenetic regulation in eye-antennal discs of 3rd instar larvae of pre-oncogenic control animals (ey::Di) and animals with reduced Ct activity (ey::Di;2xctRNAi). (P) Expression of the apoptosis marker Caspase-3 (Casp-3) and the proliferation marker Phosphorylated histone H3 (PH3) in representative 3rd instar eye-antennal discs of ey::Di and ey::Di;2xctRNAi animals. An increase in Casp-3 and PH3 positive cells is seen in the area below the dashed, yellow line highlighting the morphogenetic furrow. (Q) Top panel: representative pictures of eyes from ey::Di;PI3KRNAi and ey::Di;2xctRNAi,PI3KRNAi animals. Bottom panel: quantification of tumorous eye growth, secondary tumor growth and “small eye” phenotype in ey::Di;2xctRNAi and ey::Di;2xctRNAi,PI3KRNAi animals. doi:10.1371/journal.pgen.1002582.g004
Apoptosis Regulation in Development and Disease

| genes | expression ratio | cell adhesion |
|-------|-----------------|---------------|
| αPS4  | 0.753 ↓         |                |
| Timp  | 0.511 ↓         |                |
| Con   | 0.590 ↓         |                |
| Cad88C| 1.457 ↑         |                |
| CG32458| 0.529 ↓       |                |
| CG11637 | 0.489 ↓       |                |
| Sgs1  | 0.217 ↓         | ng1:2cRNAi     |
| Sgs3  | 0.474 ↓         | ng2:2cRNAi     |
| Sgs4  | 0.372 ↓         | ng3:2cRNAi     |
| Sgs5  | 0.291 ↓         |                |
| Sgs7  | 0.896 ↓         |                |
| Sgs8  | 0.548 ↓         |                |
| CG40006| 0.625 ↓        |                |
| CG5847| 0.511 ↓         |                |
| ng1   | 0.201 ↓         |                |
| ng2   | 0.136 ↓         |                |
| ng3   | 0.887 ↓         |                |
| Eig71Ee| 0.425 ↓        |                |

B

tumorous eye growth
secondary tumour growth

C

control
ey::2cRNAi

D

% of animals

E

hemolymph
eye-imaginal disc
bleeding
analysis of hemolymph

F

GFP+ cells/10,000 events
ey::GFP
ey::GFP:cr345p35
ey::GFP:cr345p35

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expressing GFP in eye-imaginal discs (either ey::lacZ or ey::GFP) pictures of tumor growth in eye-antennal discs of 3rd instar larvae of control animals (Dcr2; ey::lacZ) and in animals with reduced Ct activity (Dcr2; ey:2xctRNAi). (D) Quantification of secondary tumor growth rates in different genetic backgrounds. Co-expression of E-Cad strongly reduces invasive tumor growth rates in eyeful-GFP;p35 vs. p35 flies. (E) Schematic drawing of a 3rd instar larva expressing GFP in eye-imaginal discs (either ey::GFP or eyeful-GFP;ctRNAi;p35). Locations of GFP-labeled eye-imaginal discs and the insect circulatory fluid, the hemolymph, are indicated by arrows. For analysis of the hemolymph, the insect circulatory fluid is extracted by bleeding out the larvae after cutting at the posterior end (indicated by dashed, blue line). (F) Left: Quantification of GFP-positive cells in the hemolymph of wild-type, ey:GFP and eyeful-GFP;ctRNAi;p35 3rd instar larvae. Right: Relative GFP transcript levels in the hemolymph of ey::GFP and eyeful-GFP;ctRNAi;p35 3rd instar larvae. doi:10.1371/journal.pgen.1002582.g005

“blood”, in our fly lines. Strikingly, we detected a significant increase in GFP-labeled eye-imaginal disc cells in the hemolymph of animals forming invasive tumors (eyful+GFP;ctRNAi;p35) in comparison to control animals (ey::GFP) (Figure 5E, 5F), suggesting that tumor cells in flies indeed circulate through the bloodstream and invade ectopic locations. In sum, these results demonstrate that transcriptional coupling of differentiation and apoptosis is a cell-intrinsic mechanism to ensure normal development and to prevent tumor initiation, progression and invasion, which is at least in part achieved by fine-tuning the adhesive properties of cells required for tissue integrity.

Antagonistic coupling of cell fate commitment and apoptosis is a general and evolutionary conserved cancer prevention mechanism

We next explored whether the effective regulation of programmed cell death by Ct has been conserved during evolution. The vertebrate homologue of Cut, Cux1, has a well-documented function in cell differentiation during normal development as well as in tumor initiation and progression in specific cancer types [23]. In addition, several studies show that Cux1 represses apoptosis during normal vertebrate development [24,25,26], and just recently it has been demonstrated that Cux1 knock-down leads to activated apoptosis and to reduced growth of xenograft tumors in vivo [25,27]. To further investigate the mechanistic basis of Cux1 function in mediating apoptosis repression in vertebrates, we suppressed Cux1 in Panc1 pancreatic cancer cells (Figure 6A) and determined the transcriptional response of human apoptosis genes. Strikingly, mRNA levels of the pro-apoptotic gene puma were consistently elevated, whereas the anti-apoptotic gene Bcl-2 was down-regulated upon Cux1 depletion (Figure 6A). Since BH3-only proteins, like Puma, and Bcl-2 are important for the release of the vertebrate functional equivalent of Rpr, Smac/DIABLO, from mitochondria [28] and since Cux1 binding sites are present close to the puma coding region (Table S1), these results suggest that the regulatory wiring of differentiation and apoptosis, at the level of Cut, is functionally conserved in vertebrates.

Does this regulatory layout represent a general mechanism employed by other differentiation factors? This would require a whole suite of cell-type specifying transcription factors to repress cell death genes by interacting with distinct enhancer modules located in their regulatory regions. In addition, these modules should follow a similar functional logic to the tpr-HRE-717 enhancer, in that cell-type specific gene activation is counteracted by strong repressing inputs from linked cis-elements (Figure 1L-1O). In line with this, we found that a different conserved enhancer module on the Drosophila tpr regulatory region (tpr-HRE-707) drove expression in CNS midline cells of stage 14 embryos (Figure 6F), which never express tpr at this and subsequent developmental stages (Figure 6L) [29]. However, extending the enhancer to include additional cis-elements (tpr-HRE-707+156) (Figure 6D) resulted in loss of enhancer activity (Figure 6H). Using the JASPAR database [30], we found consensus binding sequences for POU-domain containing transcription factors on the extended enhancer module, and one of these factors, Ventral veins lacking (Vvl), is known to function in midline glial cells and to repress apoptosis [31,32]. Our analysis revealed a partial overlap of Vvl and reporter gene expression in tpr-HRE-707 embryos (Figure 6J), and consistently ectopic tpr transcripts were detected in several midline cells of vvl mutants (Figure 6L, 6M). Due to the existence of GFP-positive cells not expressing Vvl (Figure 6J), we assume that not only Vvl but also other POU transcription factors interact with the tpr-HRE-707+156 enhancer to repress tpr transcription in midline cells. Revisiting the tpr-HRE-717 enhancer module revealed that extension of the enhancer also led to a complete loss of reporter activity (compare Figure 1L, Figure 6E, 6G). Thus, complete repression of tpr transcription in the PS requires two inhibitory inputs: one active in filzkörper cells, which we had identified to be mediated by Ct, and one so-far unknown repressor functional in stigmatophore cells. Importantly, the functional analogy of Vvl and Ct also extended to the tumor suppression activity, since, like in the case of Ct (Figure 4N), primary and secondary tumor frequencies were increased when the ability to activate apoptosis and Vvl function was impaired at the same time (Figure 6K). Furthermore, we identified two unrelated cell-type specific transcription factors in animals (Bop, Rve) which showed similar behavior with regards to tumor suppression (Figure S8). Together with the fact that the regulatory sequences flanking the Drosophila tpr coding region show significantly less sequence divergence than expected and a high occurrence of conserved transcription factor binding motifs (Figure 6B, 6C), these findings lead us to propose that coupling of differentiation and cell death repression via a single transcription factor represents a general cancer prevention mechanism (Figure 7), which could be employed by a large number of developmental regulators in diverse organisms.

Discussion

Programmed cell death is an integral aspect of animal development [33]. Genetic studies in C. elegans, Drosophila and mouse have shown that apoptosis is used to sculpt tissues and to remove excessive and unwanted cells, thus defining the morphology required for diverse physiological functions [34]. In this context, apoptosis is usually regulated by cell signaling pathways [33,35,36]. In addition to its role in tissue morphogenesis, apoptosis is also required to eliminate potentially deleterious cells, which in most cases involves complex multi-step control mechanisms [33,37]. One such situation generating harmful cells is the inability to differentiate or adopt the
appropriate cell fate, which very often results in uncontrolled cell proliferation and cancer development, and thus requires the immediate killing of these cells. However, even though it is established that apoptosis is a protective mechanism against tumorigenesis in cases of aberrant cell differentiation [1,34,38], the interplay of the two processes at the mechanistic level has remained unclear. In our study, we show that the simultaneous and antagonistic regulation of differentiation and apoptosis is a hard-wired developmental program and carried out by individual transcription factors, such as Cut. Our results demonstrate that impairment of differentiation in the cell lineage specified by Cut instantaneously triggers locally restricted apoptosis by releasing transcriptional repression of the pro-apoptotic gene rpr in these cells. Due to its immediate effect, the coupling of differentiation and apoptosis on the transcriptional level represents one of the fastest and most direct mechanisms to eliminate abnormal cells in status nascendi and thereby immediately interferes with their potential to develop into harmful cells.

Figure 6. Functional and evolutionary conservation of coupling differentiation and apoptosis on the transcription factor level. (A) Relative mRNA expression of eight apoptosis genes after lentivirus transduced stable Cux1 (p200) knock-down in human Panc1 cancer cells. RT-PCRs are shown for two independent Cux1 knock-down lines, KDa in blue and KDb in red. Results are shown as the expression ratios between shCux1/shCtreated cells and are representative for three independent experiments. Western blot shows knock-down efficiencies in both independent stable Cux1 (p200) knock-down lines (KDa, KDb) and Cux1 expression in an shRNA control knock-down. Stronger effects of KDa (reduced) versus KDb (almost complete) p200 Cux1 knock-down on target gene expression is very likely due to the processed p110 Cux1 isoform, which can have opposite transcriptional effects to the p200 full-length form [62]. (B, C) Distribution of average conservation (B) and average number of conserved DNA binding motifs per 1000 bp (C) in all non-coding regions of the D. melanogaster genome. The red bars highlight the rpr intergenic regions, showing that 93% of all D. melanogaster non-coding regions are less conserved (B) and 89% of all non-coding regions have fewer conserved DNA binding sites per 1000 bp (C) in all non-coding regions of the D. melanogaster genome. The following regulatory regions tested are marked in different colors: rpr-HRE-571 (light-red), rpr-HRE-571+210 (dark-red), rpr-HRE-707 (light-blue) and rpr-HRE-707+156 (dark-blue). (E–H) Reporter gene expression driven by the fragments described above. The rpr-HRE-571 enhancer drives reporter gene expression in the PS (E), which is abolished in the rpr-HRE-571+210 reporter line (G). Similarly, reporter gene expression in CNS midline cells in the rpr-HRE-707 line (F) is completely suppressed in the rpr-HRE-707+156 transgenic line (H). Closed, yellow arrowheads in (E) and (F) mark presence of reporter gene expression, whereas open, yellow arrowheads in (G) and (H) mark absence of GFP expression. (I, J) Co-localization of GFP with Sal in the rpr-HRE-571 (I) and with Vvl in the rpr-HRE-707 (J) reporter lines. (K) Quantification of primary and secondary tumor formation in different genetic backgrounds. Only when Vvl function is reduced and apoptosis is simultaneously inhibited, tumors and metastasis develop. (L, M) rpr transcripts are not found in CNS midline cells of stage 14 wild-type embryos (L) but in vvl mutants (M).
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Interestingly, apoptosis induction as a consequence of aberrant cell-type specification is not only mediated by the cell death promoting gene \( rpr \) but also by \( hid \) \[8\]. However, despite the same trigger, which is the inability to properly differentiate, the transcriptional basis for inducing the expression of one of these two apoptosis genes seems to be quite different: in \textit{Drosophila} early developmental mutants only the expression of the pro-apoptotic gene \( hid \) is up-regulated \[8\], whereas our study shows that exclusively the transcription of \( rpr \) is induced when a factor specifying a distinct cell type is lost. Although it is currently unknown how \( hid \) expression is regulated at the transcriptional level, this raises the possibility that the apoptosis gene \( hid \) acts a safeguard when broad positional information at the onset of embryogenesis is absent, whereas \( rpr \) might take over this function later in development when individual and specific cell types are defined by transcription factors restricting cell fate choices.

Given the well-known role of the vertebrate homologue of Cut, Cux1, in tumor initiation and progression in specific cancer types \[23\], we addressed whether the switch function of the cell specification factor Cut is also relevant in a pathological context. We found that simultaneous inhibition of Cut function and apoptosis within a sensitized background increases tumor formation and metastasis to secondary sites in the animal. In contrast, down-regulation of Cut and inhibition of apoptosis in a normal developmental context, such as in the \textit{Drosophila} PS or the developing eye, only results in the survival of the Cut deprived cells, but not in tumor development. These results demonstrate that cells, which are unable to undergo the cell lineage-specific differentiation program, have to be eliminated, since they have the potential to develop into cancerous cells when other genetic or micro-environmental changes accumulate \[19,27,39\]. But why do differentiation-deprived cells form tumors in a cancer-prone tissue environment despite the ability to activate the apoptotic rescue pathway? This is due to the fact that the transcription factor Cut, as part of its selector gene function, coordinately regulates multiple cellular processes, including differentiation, apoptosis, cell adhesion, but also proliferation, which are all required for proper cell fate specification and the maintenance of a differentiated state (thereby preventing tumor formation). If, however, Cut activity is abolished, all its downstream functions are affected, leading not only to the activation of apoptosis, but also to reduced differentiation and adhesion properties and the activation of cell proliferation, which is, in the case of Cut, mediated (at least in part) by the PI3K signaling pathway. Thus, loss of Cut function stimulates tumor growth in a sensitized background, since the pro-tumorigenic effects of deregulated proliferation and cell adhesiveness out-compete the anti-tumorigenic apoptosis effects at work. However, when the anti-tumorigenic effect is eliminated in the differentiation-compromised cancer tissue, tumorigenesis is strongly enhanced, which resembles a prevalent situation in aggressive human cancers characterized by the loss of differentiation, the resistance to apoptosis activation and the mis-regulation of adhesion properties \[1,40,41\].

Several lines of evidence suggest that the dual role of Cut in differentiation and apoptosis for cancer prevention is conserved in evolution. First of all, the two vertebrate homologues of Cut, Cux1 and Cux2, code for homeobox-containing transcription factors, which are crucially involved in cell-type specific terminal differentiation \[14,23,42\]. Both, Cux1 and Cux2, have similar binding specificities to \textit{Drosophila} Cut \[43\], they also operate as transcript...
tional repressors and activators of genes in multi-lineage differentiation pathways [26] and, like *Drosophila* Cut, they act as downstream effectors of the Notch signaling pathway [44,45]. In addition to their well-established role in development and differentiation, there are also several examples linking the vertebrate Cut homologue Cux1 to apoptosis and cancer. First, inhibition or partial disruption of Cux1 function in mice leads to increased apoptosis rates in vivo [24,26]. Second, Cux1 regulates normal hematopoiesis, in part by modulating the levels of survival and apoptosis factors [26]. Third, Cux1 plays a prominent role in cancer progression [23]. And fourth, induced down-regulation of Cux1 in subcutaneous xenograft tumors leads to activation of apoptosis and to reduced tumor growth [25]. Our results now show that the Cut-Rpr regulatory wiring of apoptosis and differentiation is conserved in vertebrates. In mammalian cells, the Rpr functional homologue, Smac/DIABLO, which is normally compartmentalized within mitochondria, has to be released to execute its pro-apoptotic function by binding to and inactivating Inhibitors of Apoptosis (IAPs) [46]. This process requires the permeabilization of the outer mitochondrial membrane (MOMP), which is achieved by the interaction of pro-apoptotic proteins like Puma with anti-apoptotic proteins like Bcl-2, which normally inhibit MOMP [47]. We now show that down-regulation of Cux1 in pancreatic cancer cell lines leads specifically to the transcriptional induction of the pro-apoptotic gene *puma* and the down-regulation of the anti-apoptotic gene *Bcl-2*. Thus, two crucial regulators for Smac/DIABLO release are controlled by Cux1 on the transcriptional level, showing that the basic design principle of the Cut-Rpr regulatory wiring is conserved but has been adapted to the system requirements in evolution. In future, it will be intriguing to study this mechanism in diverse cellular backgrounds, including stem cells, which neither die nor differentiate.

**Materials and Methods**

**Bioinformatics**

To identify Abd-B binding sites we used the method of Wasserman and Sandelin (2004) [48] with the Abd-B Position Frequency Matrix [49] ([http://jaspar.genereg.net/](http://jaspar.genereg.net/) and 90% cut-off. Abd-B binding site clusters were identified if at least three Abd-B sites were present in a 400 bp window. Conserved enhancers were identified using PhastCon score [50]. Within conserved regions, Ct binding sites were identified using published sequence data [49]. Vd and Cux1 binding sites were identified using the Vd4 and Cux1 Position Frequency Matrices available at the JASPAR database [49] ([http://jaspar.genereg.net/](http://jaspar.genereg.net/)).

**Genetics**

*Drosophila melanogaster* strain *Oregon R* was used as wild type. Amorphic allele *dpn/Em* [51], *ems-Gal4* and *ems-Gal4*, UAS-GFP/ TM6B [13] were obtained from J. Castelli-Gair Hombria, UAS-Dor2; ey-Gal4 from B. Dickson, ey-Gal4/TM6B [32] from H. Pi, UAS-Apoliner5 [11] from J. P. Vincent, UAS-eh2b/CyO [53], UAS-eh2b (Grueber and Jan, unpublished) from Y.N. Jan and ey-Gal4, UAS-DI/CyO and eyefish flies (ey-Gal4, G898A, UAS-DI/CyO) from M. Domiguez [16]. UAS-Doctor2; C96-Gal4 (BL-25757), UAS-Cd8::GFP (BL-5130) from Bloomington stock center. GMR-Gal4, UAS-p35, UAS-Abd-B, arm-Gal4, UAS-trp, UAS-iacZ were described elsewhere [54,55,56,57]. Other UAS-RNAi lines were obtained either from BDSC, VDRC or TRiP: UAS-Apoliner5 (v8024), UAS-Dor2; ey-Gal4 (v12045), UAS-eh2b (v02126), eyefish (v0316), H (v24466), zPS2 (f) (BL2754), zPS4 (v109783), BPS (mys) (HMS00403), P3K (v107390) and Temp (v109427). Five independent transgenic lines were analyzed for each reporter construct.

Mammalian cell culture and lentivirus-mediated Cux1 knock-down

Panc-1 human pancreatic cancer cells (Department of Surgery, Medical Faculty, University of Heidelberg) and 293 T cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, non-essential amino acids, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 μg/ml amphotericin B. shRNA directed against human Cux1 was generated using the following complementary oligonucleotides (forward and reverse): Cux1_KDa:

5′-CCCGAAGAAGAAGACCTCCAGAGGTACATCCTCGAGGTGTTGCATTGCAGTTCTCCTCTTTTTGTG3′ and

5′-ATTCACAAAAAAAGAGAAGAGATCCCAGAGATCATTTCAGTTTGAGATATTTTTTGTG3′ and

5′-ATTCACAAAAAAAGAAGATCCTCCTGTTGAAACCTCAGGTTGGTTTCAACGAGAACAGTTTTTTGTG3′ and

5′-CCCGAAGAATTCCTTCGTGTTTGAAACCTCAGGTTGTTTGAAACCTCAGGTTGGTTTCAACGAGAACAGTTTTTTGTG3′ and

shRNA control (C),

5′-CCCGAAATTCGCCAGCTGTTCACTACCTCAGGATGATTGGAAACCAGAAGATTTTGTTG3′ and

5′-CCCGAAATTCGCCAGCTGTTCACTACCTCAGGATGATTGGAAACCAGAAGATTTTGTTG3′ and

pLKO lentiviral vectors containing shRNA were transfected into 293 T cells together with pSAX2 (packaging vector) and pMD2.G (VSV-G envelope protein expression vector) using the calcium-phosphate transfection kit (Sigma). Panc-1 cells were infected using lentivirus-containing 293 T cell supernatant and Cux1 protein levels were assessed by Western blotting using anti-Cux1 (Sigma-Aldrich) and anti-β-Actin (GeneFex) antibodies.

**Plasmid constructs**

All enhancer fragments were PCR amplified from genomic DNA and cloned in *pHPelican-GFP* [58] or *pHPelican-GFP_DEST* [59]. For binding site mutations, a two-step overlap PCR was performed. For mutating Ct binding sites within the *rpr*-HRE-571-S2 enhancer fragment, mutation introduced into the Cut-Rpr regulatory wiring is conserved but has been adapted to the system requirements in evolution. In future, it will be intriguing to study this mechanism in diverse cellular backgrounds, including stem cells, which neither die nor differentiate.

**Real-time PCR**

Real Time PCR was performed following standard protocols using SYBR green. Expression was normalized to GAPDH for mammalian cells and to endogenous *actin3C* mRNA for imaging disc analysis. Relative expression levels are based on three biological replicates.

**Histology and scanning electron microscopy**

*Drosophila* embryos were fixed as described [56]. Eye discs or wing discs were dissected in PBS and fixed in 4% paraformaldehyde/PBS for 10 min for immunostaining. In situ hybridization and immunohistochemistry were performed as described [56]. Fluorescent mRNA/protein double labeling and fluorescent duplex in situ hybridizations were done as described previously [60]. Primary Antibodies used were: mouse anti-Ct 2B10 (1:200, DSHB), mouse anti-Crb cq4 (1:200, DSHB), rat anti-DE-Cad DCAD2 (1:100, DSHB), mouse anti-ELAV (1:200, DSHB), rat anti-Sal (1:800 kind gift from R. Barrio), rabbit anti-PH3 (1:200, Santa Cruz), rabbit anti-Cleaved Caspase-3 (1:50 Cell Signalling), mouse anti-GFP (1:1000, Roche), rabbit anti-GFP (1:2000, Sigma), anti-DIG POD (1:200, Roche), Streptavidin HRP.
Protein purification and electrophoretic mobility shift assays (EMSA)

Cut CR3HD (4849-5412 of ctRNA from Y.N. Jan) was cloned into pMAL2c-2x vector (NEB) and expressed as Maltoose Binding Protein (MBP) fusion proteins. EMSAs were carried out as described in Söhbe et al. (2009) [56]. The following oligonucleotides (S2 subfragment) were used for analyzing the Cut binding sequence in EMSA (only forward strand is shown):

Wild-type. 5’GCACCTTTTGCCTGCAGTTCACTCGG-TTACGTTGCTTTGTGTCATAAAAATGCT3’

Mutated. 5’GCACCTTTTGCCTGCAGTTCACTCGG-GGAGTGTCATTGTTGTCATAAAAATGCT3’

Cut consensus sequences are underlined, exchanged nucleotides in the mutated versus the wild-type sequence are shown in bold.

Quantification of GFP labeled eye cells in hemolymph of 3rd instar larvae

ey::CD8-GFP or eyeful+CD8-GFP;ctRNAi::p35 3rd instar larvae were dissected by rupturing the larval cuticle at the posterior end with a pair of fine forceps, the hemolymph was collected in ice-cold Schneider’s medium (Invitrogen GIBCO) containing 1X Complete protease inhibitor mixture (Roche). Hemolymph cells were analyzed via FACSAria to quantify GFP-labeled cells.

Microarray

Eye-antennal discs of ey::Dl or ey::Dl;2xctRNAi::p35 3rd instar larvae were dissected in cold PBS, total RNA was extracted using standard procedures. Microarray analysis was conducted at the Genomics Core Facility, EMBL, Heidelberg, Germany. Microarray data analysis was performed using the R package as described previously [54].

Supporting Information

Figure S1 Cut directly represses trp transcription in a cell-autonomous manner. (A–D) Expression of the posterior spiracle markers Spalt (Sal) (green), which labels the stigmaphore precursor cells, and Cut (Ct) (red), which marks the spiracular chamber and filzkörper precursor cells, in stage 11 (A), stage 13 (B), stage 14 (C) and stage 16 (D) embryos. In (A), (B) and (D) lateral views of the posterior spiracle primordia are shown, whereas in (C) a dorsal view is presented. (E, F) trp mRNA expression (green) in posterior spiracle primordia of stage 14 wild-type (E) and ct mutant (F) embryos is shown (lateral view). Spalt (Sal) protein (blue) labels stigmaphore precursor cells, Cut (Ct) protein (red, nuclear) marks spiracular chamber and filzkörper precursor cells and the apical membrane marker Crb (red) outlines the cells. Small, white arrow in (F) marks additional tracheal cells found at the posterior end in ct mutant embryos; yellow circle in (F) highlights trp expression in ct mutant embryos. (G) EMSA using S2 sub-fragment with Ct binding sites either in wild-type (wt probe) or mutated (mut. probe) version and no protein (–). purified MBP protein (M), and purified Cut-MBP fusion protein consisting of the Cut repeat 3 and the Cut homeodomain (C). The black arrowheads indicate the specific DNA-protein complexes, the black arrow highlights unspecific DNA-protein complex. Loading of equal amounts of labeled wild-type and mutated oligonucleotides is illustrated by formation of comparable amounts of unspecific DNA-protein complex (indicated by black arrow). (JPG)

Figure S2 Ct represses trp transcription and apoptosis activation. (A–F) trp RNA expression in stage 11 wild-type (A, C, E), ctRNAi::p35 (B, D) and arm::ct (F) embryos. trp transcription is ectopically activated in the posterior spiracle primordium (B) and the gut primordium (D) in ct mutant embryos (marked by red arrowheads), and is globally repressed when Ct is ubiquitously mis-expressed (F). (G, H) Acridine Orange (AO) staining of stage 13 wild-type (G) and ct mutant (H) embryos highlights up-regulation of programmed cell death in the PS primordium of ct mutant embryos. (I–N) hid (I, J), gran (K, L), skl (M, N) RNA expression in stage 11 wild-type (I, K, M) and ctRNAi::p35 (J, L, N) mutant embryos. Red boxes indicate posterior spiracle primordium in respective embryos. (JPG)

Figure S3 Cut directly represses trp and apoptosis in the PS primordium. (A–D) trp expression in the posterior spiracle primordium of different reporter lines at developmental stage 15. Spalt (Sal) and Cut (Ct) proteins label stigmaphore (blue) or spiracular chamber and filzkörper precursor cells (red). Closed, yellow arrowheads in (C) and (D) mark reporter gene expression in filzkörper cells, whereas open, yellow arrowheads in (A) and (B) mark missing GFP expression. (E–H) Single color images of the different reporter lines showing only Sal expression. (I–L) Single color images of the different reporter lines showing only Ct expression. (M–P) Single color images of the different reporter lines showing only GFP expression. (JPG)

Figure S4 Location and conservation of the trp-HRE-571 element. (A) Conservation graph of the trp intergenic region obtained from the UCSC genome browser (http://genome.ucsc.edu/). The trp-HRE-571 element, which is marked by a light-red box (3 L: 18344438..18385008), is located 6 kb downstream of the trp coding sequence (marked by a dark-red box). The coding region of the pro-apoptotic gene grn is marked by a dark-blue box. (B) Alignment of the trp-HRE-571 region from five different Drosophila species (D. melanogaster, D. yakuba, D. pseudoobscura, D. viridis, D. grimshawi). Abd-B binding sites are marked by purple, Ct binding sites by orange boxes. (JPG)

Figure S5 Ct function is required for filzkörper differentiation. The following genotypes are shown: wild type (A, D and G), ctRNAi::p35;ems::GFP (C) and ctRNAi::p35;ems::GFP + F and I. (A–C) Cuticle preparations of the different genotypes with focus on the posterior spiracle of 1st instar Drosophila larvae. Closed, orange arrowhead in (A) marks the filzkörper, whereas open, orange arrowheads in (B) and (C) indicate the absence of this structure in the respective genotypes. (D–F) Ct and Crb staining in the respective embryos are shown to highlight the morphology of the filzkörper in the different genotypes. (G–I) DE-Cad staining in the respective genotypes. Closed, red arrowheads in (G) indicate the presence of the filzkörper, whereas open, red arrowheads in (H) and (I) highlight the absence of this structure in ctRNAi::p35;ems::GFP (H) and ctRNAi::p35;ems::GFP + F (I) embryos. (JPG)

Figure S6 Ct represses apoptosis in wing margin bristles and in external sensory organs of the notum. (A–D) Close-up of Drosophila notum in wild-type (A), Eq::ctRNAi::p35 (B), Eq::ctRNAi::p35;ems::GFP (C) and in Eq::ctRNAi::p35; trpRNAi::p35 animals (D). Open, yellow circles in (B, C and
D) mark the absence of external sensory organs, whereas closed, yellow circle in (A) highlights the presence of these structures in the different genotypes. Note that in (B) most bristles are missing, whereas in (C) and (D) some bristles form, which have cell polarity defects. Expression of Cut in mechanosensory organs of the notum has been shown before [63]. (E–H) Close-up of *Drosophila* adult wing with focus on wing margin between the wing veins L2 and L3 in wild-type (E), *C96* (F), *C96::ctRNAi*; p35 (G) and in *C96::ctRNAi*, *rprRNAi*; *hid* (H) animals. Closed, blue arrowheads in (E), (G) and (H) highlight the presence of mechanosensory bristles at the wing margin, whereas the open, blue arrowhead in (F) marks their absence in the respective genotype. Importance of Cut function for wing margin development has been shown before [64,65,66,67]. Despite the fact that Cut is expressed in a narrow region along the wing margin [64,65,66,67], we observed a loss of cells outside that region. One likely explanation for this phenotype is the known requirement of Cut to maintain expression of the secreted factor Wingless (Wg) at the wing margin [64,65,66,67], thus we assume that neighboring cells which normally receive the Wg signal undergo apoptosis in a cell non-autonomous manner. (I, J) Quantification of mechanosensory bristles on notum (I) and between wing veins L2 and L3 (J) in the different genetic backgrounds. 15–20 flies were scored for each genotype.

**Figure S7** Invasiveness of Ct depleted cells. (A–D) Co-localization of the eye differentiation marker Embryonic Lethal Abnormal Vision (ELAV) and the proliferation marker Phosphorylated histone H3 (PH3) in 3rd instar eye-antennal discs. Blue circle in (D) marks loss of ELAV expression in *eyeless*:ctRNAi 3rd instar eye-antennal discs, yellow circle in (B) marks ELAV-positive cells at ectopic location in *eyeless*:ctRNAi; p35 3rd instar eye-antennal discs. (JPG)

**Figure S8** Simultaneous regulation of differentiation and apoptosis represents a general cancer prevention mechanism. Top: Representative pictures of tumorous eye growth in flies of indicated genotypes. Bottom: Quantification of primary tumor growth in the respective genotypes. Genes were tested were selected based on their function as cell-type specifying transcriptional regulators active in the *Drosophila* eye. Genes: *wt*: central veins lacking; *gvo*: grouch; *ct*: cut; *H*: Hairless. (JPG)

**Table S1** Putative binding sites for vertebrate Cux1 within the non-coding regions of the *puma* gene. (DOC)

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**Author Contributions**

Conceived and designed the experiments: ZZ IL AH-B NB. Performed the experiments: ZZ SS NH AH-B NB DB. Analyzed the data: ZZ NH IL AH-B NB FP DB. Contributed reagents/materials/analysis tools: IL. Wrote the paper: FP IL.

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