Developmental Biology

Extramacrochaetae regulates Notch signaling in the *Drosophila* eye through non-apoptotic caspase activity

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Abstract

Many cell fate decisions are determined transcriptionally. Accordingly, some fate specification is prevented by Inhibitor of DNA binding (Id) proteins that interfere with certain master regulatory transcription factors. We report that the *Drosophila* Id protein Extra macrochaetae (Emc) also affects developmental decisions by regulating caspase activity. Emc, which prevents proneural bHLH transcription factors from specifying neural cell fate, also prevents homodimerization of another bHLH protein, Daughterless (Da), and thereby maintains expression of the *Death-Associated Inhibitor of Apoptosis* (*diap1*) gene. Multiple effects of *emc* mutations, on cell growth and on eye development, were all caused by reduced Diap1 levels and corresponding activation of caspases. These effects included growth of unspecified imaginal disc cells, acceleration of the morphogenetic furrow, failure of R7 photoreceptor cell specification, and delayed differentiation of non-neuronal cone cells. Within *emc* mutant eye clones, morphogenetic furrow speed was increased by elevated Notch signaling, while decreased Notch signaling inhibited R7 specification and cone cell differentiation. This was all due to caspase-dependent increase in levels of Delta protein, a transmembrane ligand that both trans- activates and cis-inhibits Notch. Thus, *emc* mutations reveal the importance of restraining caspase activity, even in non-apoptotic cells, to prevent abnormal development.

eLife assessment

This **important** work presents data showing that all non-proneural phenotypes of the Inhibitor of DNA binding (Id) protein emc are mediated through inappropriate non-apoptotic caspase activity. Using the developing Drosophila retina as a model the authors **convincingly** show that emc acts by transcriptionally regulating the Death-Associated Inhibitor of Apoptosis 1 (diap1) gene, which impacts on Notch signaling by caspase-dependent increase of Delta protein. These findings are interesting for the caspase/apoptosis field as they add more non-apoptotic functions of caspases to the list, as well as for the Id field, which examines how Id proteins inhibit cell differentiation.

Introduction

Certain families of master regulatory transcription factor control major developmental decisions in metazoa. This includes the bHLH proteins encoded by proneural genes, which were discovered in *Drosophila* and control neural fate specification and differentiation from sponges to humans(Weinberger et al., 2017; Baker & Brown, 2018; Dennis, Han, & Schuurmans, 2019; Johnson, 2020). One of the ways proneural gene activities are regulated, thereby modulating the location and timing of neurogenesis, is through Inhibitor of DNA binding proteins (Id proteins), which are HLH proteins lacking basic DNA-binding sequences. Using their HLH domains, Id proteins form inactive heterodimers with proneural bHLH proteins, preventing DNA binding and transcriptional regulation of target genes by the resulting heterodimer (Benezra, Davis, Lockshon, Turner, & Weintraub, 1990; Ellis, Spann, & Posakony, 1990; Garrell & Modolell, 1990; Cabrera, Alonso, & Huikeshoven, 1994; Ellis, 1994; Norton, 2000). Accordingly, mutations in Id protein genes, of which there are 4 in mammals and one in *Drosophila*, permit enhanced proneural bHLH function, deregulating neurogenesis at many developmental stages and in many tissues. Mammalian Id gene expression is particularly associated with proliferative and stem cell states, and is frequently affected in cancer(Ling, Kang, & Sun, 2014; Wang & Baker, 2015a; Roschger & Cabrele, 2017; Oproescu, Han, & Schuurmans, 2021).

An outstanding question has been whether heterodimerization with proneural bHLH proteins represents the only mechanism of Id protein function. This question arises because not all phenotypes of Id gene mutations obviously represent gain of proneural gene function.

Interpretation is complicated in mammals by the number of proneural bHLH genes and of Id protein genes, both functionally redundant so that single mutant phenotypes reflect only subsets of protein function(Ling et al., 2014; Wang & Baker, 2015a). In *Drosophila*, it is clear that mutating the sole Id protein gene *extra macrochaetae* (*emc*) affects some tissues where no proneural gene is known to function. The first example noted was the requirement for *emc* for normal growth of imaginal discs(Garcia-Alonso & Garcia-Bellido, 1988). Imaginal discs are larval progenitors of adult tissues and remain proliferative and undifferentiated until late in the final larval instar; no proneural gene is active in these proliferating cells. A second example is that *emc* is required for ovarian follicle cell development (Adam & Montell, 2004), which does not depend on proneural bHLH genes. Further examples are noted in *Drosophila* eye development. The *emc* gene is required to restrain the rate at which the morphogenetic furrow, a wave of fate specification that traverses across the eye imaginal disc as retinal patterning and differentiation begin(Brown, Sattler, Paddock, & Carroll, 1995). *emc* is also required for the specification of the R7 photoreceptor cells and the non-neuronal cone cells, neither of which depends on any known proneural bHLH gene (Bhattacharya & Baker, 2009). Most recently, *emc* was also found to be

required for left-right asymmetry of the *Drosophila* gut(Ishibashi et al., 2019). Thus, *Drosophila* provides opportunities to investigate how at least one Id protein functions independently of proneural bHLH proteins, and potentially reveal new mechanisms of developmental regulation.

Insights into how Emc acts independently of proneural genes have progressively emerged from several studies. The first concerned the proper proliferation of imaginal disc cells(Garcia- Alonso & Garcia-Bellido, 1988). Independence of imaginal disc growth from proneural bHLH genes is confirmed by the lack of requirement for *da*, the only E protein in *Drosophila*(Bhattacharya & Baker, 2011; Andrade-Zapata & Baonza, 2014). E proteins are ubiquitously-expressed bHLH proteins that are heterodimer partners for proneural bHLH proteins. In Drosophila, proneural bHLH proteins cannot function without Da, so normal growth of *da* mutant cells implies independence from all Da heterodimer partners. Remarkably, it is actually ectopic Da activity that causes poor growth in *emc* mutant cells, normal growth being restored in *emc da* doubly mutant cells (Bhattacharya & Baker, 2011). Without *emc*, Da levels are elevated and by themselves activate transcription of *expanded* (*ex*), a component of the Salvador-Hippo-Warts (SHW) tumor suppressor pathway. This increased SHW pathway activity is what inhibits growth of imaginal disc cells(Wang & Baker, 2015b). Thus, one role of *emc* is to restrain Da(Reddy, Bhattacharya, & Baker; Bhattacharya & Baker, 2011; Wang & Baker, 2015a; Li & Baker, 2018).

In the course of these studies, we noticed that *ex* mutations affected the number of sensory organ precursor (SOP) cells in the thorax(Wang & Baker, 2015b). We showed that this was due to activity of Yorkie (Yki), the transcription factor target of SHW signals, in the *ex* mutant cells. This in turn increased transcription of a direct Yki target gene, *Death-Associated Inhibitor of Apoptosis 1* (*diap1*)(Wang & Baker, 2019). Diap1 is a ubiquitin ligase that prevents accumulation of a processed form of Dronc, an apical caspase in *Drosophila*(Muro, Hay, & Clem, 2002; Wilson et al., 2002; Yan, Wu, Chai, Li, & Shi, 2004; Li, Wang, & Shi, 2011). Caspases are proteases that are well known for driving apoptosis by cleaving cellular substrates. They are expressed as inactive zymogens that are then activated by signals (initiator caspases) or by cleavage by caspases (effector caspases) in a feed-forward process that is expected to kill cells rapidly once a threshold of caspase activity has been crossed(Fuchs & Steller, 2011). Many studies show that caspases can also mediate non-apoptotic processes, although what differentiates apoptotic from non-apoptotic outcomes is uncertain, as the same caspase cascade of initiator and effector caspases seems to be involved (Yamaguchi & Miura, 2015; Aram, Yacobi-Sharon, & Arama, 2017; Nakajima & Kuranaga, 2017; Baena-Lopez, Arthurton, Xu, & Galasso, 2018; Colon-Plaza & Su, 2022). Non-apoptotic caspase activities are of particular interest because of their contributions to multiple diseases(Su, 2020). In *ex* mutants, the increased *diap1* transcription does not affect cell death much, but causes an increase in *wg* signaling activity in the thorax(Wang & Baker, 2019). Wg signaling promotes SOP cell determination in the thorax, and it has been shown previously that Wg signaling is antagonized by caspases, through a mechanism that is non-apoptotic but whose direct target is not yet certain(Baker, 1988; Kanuka et al., 2005; Wang & Baker, 2019).

Since *emc* mutations elevate *ex* expression(Wang & Baker, 2015b), we wondered whether *emc* also changes Diap1 levels and affects caspase activities. Increased *ex* expression in *emc* mutants would be expected to reduce transcription of the *diap1* gene and potentially increase caspase activity, the opposite of what occurs in *ex* mutants. This predicts that caspases could contribute to aspects of the *emc* mutant phenotype, especially those that do not depend on proneural bHLH genes. Remarkably, we found that caspase activity was responsible for all the aspects of the *emc* phenotype that we tested. This included reduced growth of imaginal disc cells, accelerated morphogenetic furrow progression, and loss of R7 and cone cells fates in the eye. The effects on eye development all reflected caspase-dependent expression of the Notch ligand Delta, which is known to contribute to morphogenetic furrow progression, and R7 and cone cell fate specification. Thus, caspase-dependent non-apoptotic signaling underlies multiple roles of *emc* that are independent of proneural bHLH proteins.

Materials and Methods

Drosophila Strains

The following stocks were employed in this study and were maintained at 25°C unless otherwise stated - *hsflp;;emc^{AP6} FRT80/TM6B, hsflp;;dronc^{i29}FRT80/TM6B, hsflp;;dronc^{i29}emc^{AP6}, hsflp;;Df(3L)H99/TM3, hsflp;;Df(3L)H99 emc ^{AP6}/TM3, Ubi-GFP M(3)67C FRT80, FRT42 M(2)56F Ubi-GFP, FRT82 M(3)95A Ubi-GFP, hsflp;; Ubi-GFPFRT80, hsflp; Su(H)$^{\Delta 47}$FRT40, pie^{eB3} FRT40* (Baker et al., 1992), *psn[v1]FRT80/TM6B, Fz3-RFP*;D/TM6B (kind gift from Yu Kimata, University of Cambridge). We obtained genomic Ci construct from Kalderon lab and used BestGene Inc. to target the transgene on the third chromosome and then recombined these flies to generate *hsflp;;ci+M(3)67Calz FRT80/TM6B;ci94/y+spa*.

Mosaic Analysis

Mosaic clones were obtained using FLP/FRT mediated mitotic recombination (Golic, 1991; Xu & Rubin, 1993). For non-Minute genotypes, larvae were subjected to heat shock for 30 minutes at 37°C, 60 ± 12 hours after egg laying. For Minute genotypes, heat shock was performed 84 ± 12 hours after egg laying for 50 minutes. Larvae were dissected 72 hours after heat shock. All flies were maintained at 25°C unless otherwise stated.

Immunohistochemistry and histology

Unless otherwise noted, preparation of eye and wing imaginal discs for immunostaining and confocal imaging were performed as described previously (Baker, Li, Quiquand, Ruggiero, & Wang, 2014). Antibodies from Developmental Studies Hybridoma Bank (DSHB): anti-Ptc (mouse, 1:40), anti-Elav (mouse, 1:100), anti-Elav (rat, 1:50, anti-Cut (mouse, 1:50), anti-Delta C594.9B (mouse, 1:2000), anti-βGal (mouse, 1:100), mouse anti-βGal (1:100, DSHB 40-1a) and anti-Ci (rat, 1:10). Other antibodies: anti-phospho-Smad1/5 (rabbit, 1:100, Cell Signaling), anti- DIAP1 (mouse, 1:50) (gift from Hyun Don Ryoo, NYU), anti-Dcp1 (rabbit, 1:100, Cell Signaling), anti-Sens (guinea pig, a gift from Hugo Bellen used at 1:50), anti-Da (mouse, 1:200), rabbit anti-Emc (1:40), anti-GFP (rat, 1:50 from Nacalai Tesque # GF090R), rabbit anti-GFP (1:500), rabbit anti-ß-Galactosidase (1:100, Cappel), E(spl)bHLH (1:50,mAb323) and guinea pig anti-runt (1:500). Secondary antibodies conjugated with Cy2, Cy3 and Cy5 dyes (1:200) were from Jackson ImmunoResearch Laboratories and Alexa 555 (1:500). Multi-labelling images were sequentially scanned with Leica SP8 confocal microscopes and were projected and processed with ImageJ. All images were assembled into figure format using Adobe Illustrator 2020.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

For labeling dead cells with TUNEL assay, ApopTag® Red In Situ Apoptosis Detection Kit (Cat #S7165) was used according to manufacturer's instruction. Briefly, dissected eye discs were fixed for 20 minutes at room temperature followed by three washes with 1X PBS. Then the samples were incubated in equilibration buffer for 1 minute followed by incubation in reaction buffer (TdT enzyme; ratio 7:3) at 37 °C for 1 hour. TdT reaction mix was replaced with stop buffer (diluted 1:34 in dH2O) and incubated for 10 min at room temperature. Samples were washed three times with 1X PBS, 5 min per wash; and incubated with anti-digoxigenin antibody solution (diluted 31:34 in blocking solution) for 30 min at room temperature. The samples were then washed three times in 1X PBS, 5 min per wash. For the subsequent antibody staining, the samples were blocked in PBST (1X PBS + 0.5% Triton-X) for 30 min, and incubated with primary antibodies in PBST overnight at 4 °C. The samples were next washed with PBST and incubated for 2 h with secondary antibodies in PBST, and then again washed with PBST, followed by PBS wash and samples were mounted in mounting media.

Statistical analysis

Statistical analysis of clone size data reported in Figur 1N is described in the figure legend.

Prediction of caspase cleavage sites

Caspase cleavage sites were predicted for Delta using Cascleave 2.0(Wang et al., 2014). Cascleave 2.0 was set to a medium stringency threshold for prediction of cleavage sites.

Results

Caspase activity causes growth defects in *emc* mutants

To determine the contribution of caspases to growth inhibition in *emc* mutant cells, we used the FLP/FRT system (Xu & Rubin, 1993) to generate clones of *emc* mutant cells that were unable to activate caspases normally. We achieved this by homozygously deleting the linked *reaper* (*rpr*), *head involution defective* (*hid*) and *grim* genes using *Df(3L)H99*. Rpr, Hid and Grim proteins promote Diap1 degradation in response to apoptotic stimuli and allow activation of initiator caspases such as *dronc* to start apoptosis(Yoo et al., 2002). By removing all three pro-apoptotic proteins, *Df(3L)H99* affects both apoptotic and non-apoptotic caspase functions in *Drosophila* (White et al., 1994; Tapadia & Gautam, 2011). Thus, clones of *emc Df(3L)H99* mutant cells should be defective for caspase activation. In addition, we also made clones of *emc* mutant cells further mutated for *dronc*, which encodes the main initiator caspase in *Drosophila* that is necessary for most developmental apoptosis (C. J. Hawkins et al., 2000; Meier, Silke, Leevers, & Evan, 2000; Quinn et al., 2000). Dronc contributes to non-apoptotic caspase functions as well, so *emc dronc* mutant cells should also show reduced non-apoptotic and well as apoptotic caspase functions.

In comparison to neutral clones, which grew equivalently to their twin-spot controls, *emc* mutant clones showed greatly reduced growth in eye or wing imaginal discs, as described previously (**Figures 1A-D**) (Garcia-Alonso & Garcia-Bellido, 1988; Bhattacharya & Baker, 2011; Andrade-Zapata & Baonza, 2014). By contrast, *emc* mutant clones that were also mutant for *dronc*, or *emc Df(3L)H99* clones that lacked the *rpr*, *grim* and *hid* genes, grew more like their twin spot controls in both eye and wing (**Figures 1E-H**). Deletion of the *rpr*, *hid* and *grim* genes restored growth indistinguishable from the wild type to *emc* clones, although mutation of *dronc* rescued less completely (see **Figure 1N** for quantification). Neither homozygosity for *dronc*, nor deletion of *rpr*, *grim* and *hid*, affected growth of otherwise wild type imaginal disc clones (**Figure 1I-L**).

Previous studies of *emc* phenotypes have often used Minute genetic backgrounds (ie heterozygosity for mutations in *Rp* genes) to retard growth and enhance the size of the *emc* mutant clones(Bhattacharya & Baker, 2009). When *emc Df(3L)H99* clones were induced in the *M(3)67C* background, the clones took over almost the entire disc, leaving only a few *M(3)67C* heterozygous cells remaining (**Figure 1M**).

These results indicate that the growth disadvantage of *emc* mutant imaginal disc clones is mostly attributable to cell death genes. Preventing caspase activation, either by mutating the main initiator caspase, or by preventing Diap1 turnover, partially or completed restored normal growth. Our data did not support a previous suggestion that *dronc* was required for normal wing disc growth(Verghese, Bedi, & Kango-Singh, 2012).

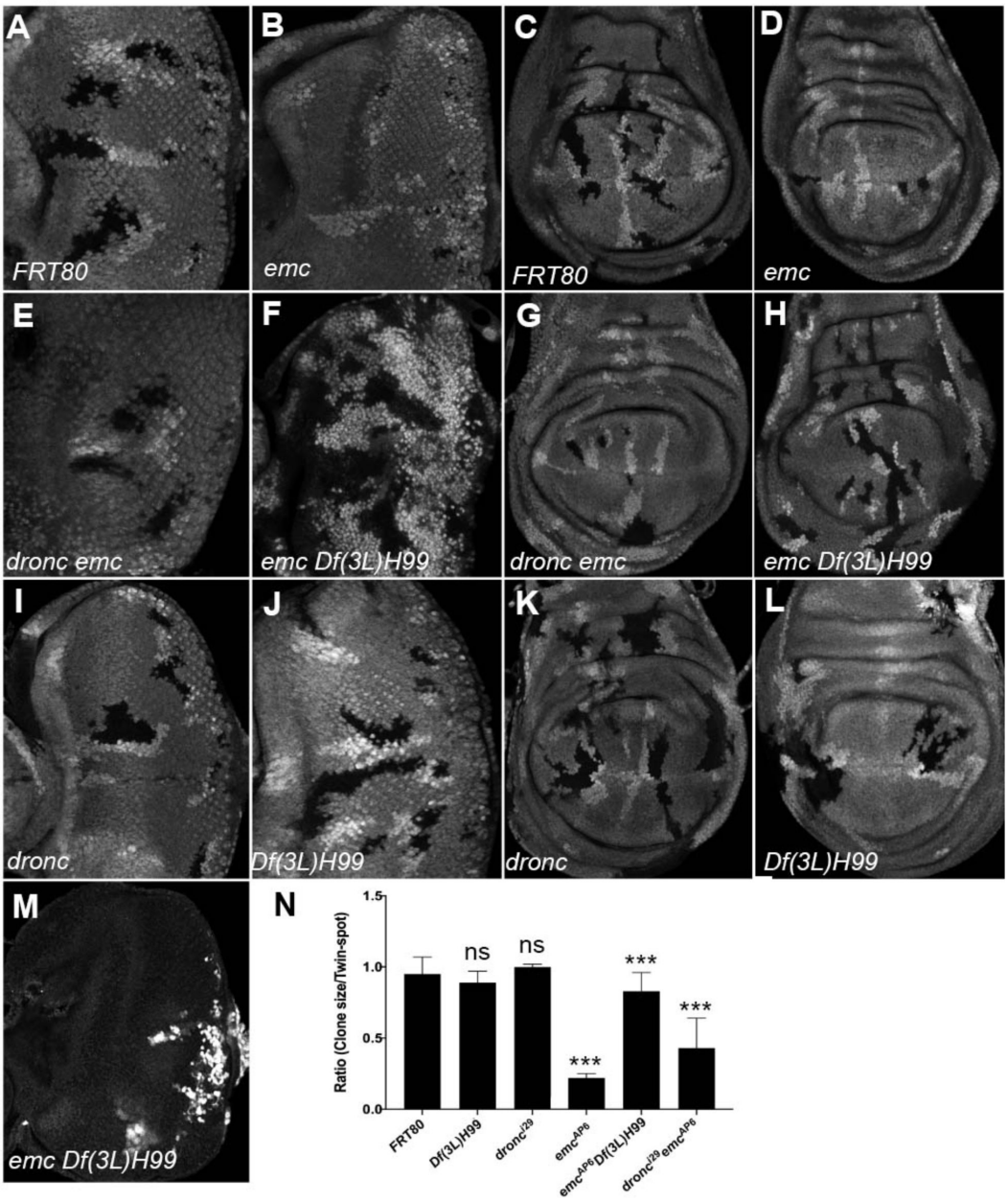

Figure 1

Df(3L)H99 rescues growth of *emc* mutant (A-L) Control and mutant clones in eye and wing imaginal disc were induced at the end of first instar and are associated with sibling twin-spot clones marked by two copies of the GFP marker (brighter grey) that serves as internal control for growth. Clones are labeled by the absence of GFP. (M) *emc H99* clones induced in the minute background (N) Quantification of the ratio of clone size to twin-spot measured in wing imaginal discs and standard deviations. After log- transformation of clone/twin-spot ratios to ensure normality, one way ANOVA rejected the null hypothesis that these results are the same ($p=2.57 \times 10^{-10}$). The Holm correction for multiple comparison was used to identify significant differences between all pairs of samples. Whereas clone/twin-spot ratios for *Df(3L)H99* and *dronc*^{i29} did not differ significantly from the FRT80 control ($p>0.05$), that for *emc* homozygous clones was significantly different ($p=4.95 \times 10^{-8}$). Ratios for both *emc Df(3L)H99* and *emc dronc* clones differed significantly from *emc* clones ($p=2.19 \times 10^{-8}$ and 4.89×10^{-4}, respectively). Clone/twin-spot ratios for *emc dronc* clones also differed significantly from *dronc* clones ($p=0.044$) and from *Df(3L)H99* clones ($p=0.048$). For columns 2-4, *** denotes significant difference from the FRT80 control ($p<0.001$), NS denotes no significant difference. For columns 5-6, *** denotes significant difference from the *emc* clones.Genotypes: (A and C) *ywhsF;;FRT80/[UbiGFP]FRT80* (B and D) *ywhsF;;emc*^{AP6}*FRT80/[Ubi- GFP]FRT80* (E and G) *ywhsF;;dronc*^{i29}*emc*^{AP6}em*FRT80/[UbiGFP]FRT80* (F and H) *ywhsF;;emc*^{AP6} *Df(3L)H99 FRT80/[UbiGFP]FRT80* (I and K) *ywhsF;;dronc*^{i29} *FRT80/[UbiGFP]FRT80* (J and L) *ywhsF;;Df(3L)H99FRT80/[UbiGFP]FRT80* (M) *ywhsF; emc*^{AP6} *Df(3L)H99 FRT80/[UbiGFP] M(3)67C FRT80*.

Emc regulates furrow progression through non-apoptotic caspase activity

Emc mutations have multiple effects on the eye imaginal disc, although only a few aspects of retinal differentiation depend on proneural bHLH genes. The proneural gene *atonal* is required, along with *da*, for the specification of R8 photoreceptor precursors in the morphogenetic furrow that found each ommatidial cluster in the larval eye disc(Jarman, Grell, Ackerman, Jan, & Jan, 1994; Brown et al., 1996). Later, during pupal development, proneural genes of the Achaete-Scute gene Complex (AS-C), along with *da*, are required for the specification of the interommatidial bristles(Cadigan, Jou, & Nusse, 2002). All the other cell types develop independently of proneural bHLH genes, and most of them develop independently of *da* (Jimenez & Campos-Ortega, 1987; Brown et al., 1996).

In *emc* mutant clones, retinal differentiation begins precociously, associated with more rapid transit of the morphogenetic furrow across the disc (**Figure 2A**, B)(Brown et al., 1995; Bhattacharya & Baker, 2011, 2012). By contrast, we found that 75% of the time, the morphogenetic furrow progressed normally through *emc dronc* double mutant clones (**Figure 2C**). Eye discs containing *emc Df(3L)H99* double mutant clones always appeared completely normal (**Figure 2D**). Both *dronc* and *Df(3L)H99* mutant clones also showed normal furrow progression in the absence of *emc* mutations (**Figure 2 Figure Supplement 1A,B**).

Not only the position of the morphogenetic furrow, but also the overall pattern of retinal differentiation revealed by labeling for Senseless, which is specific for R8 photoreceptor cells, and Elav, which labels all neuronal photoreceptor cells, appeared so normal in these genotypes that we deemed it necessary to confirm that the supposedly *emc dronc* mutant and *emc Df(3L)H99* mutant clones were indeed mutated for *emc*. This was confirmed by lack of staining by an antibody against Emc protein, similar to plain *emc* mutant clones that did affect the morphogenetic furrow (**Figure 2 Figure supplement 1C-E**). We also found that *emc dronc* and *emc Df(3L)H99* clones upregulated Da protein expression to a comparable degree to plain *emc* mutant clones (**Figure 2 Figure Supplement 1C-E**)(Bhattacharya & Baker, 2011). This further confirmed the absence of *emc* function from *emc dronc* mutant and *emc Df(3L)H99* genotypes, and also showed that cell death pathways were not required for the regulation of Da protein levels by *emc*.

We have previously shown that *diap1* transcription is reduced in *emc* mutant clones, and in Da-overexpressing cells, due to Yki inhibition downstream of *ex* (Wang & Baker, 2015b). In eye imaginal discs, Diap1 protein was normally present uniformly (**Figure 3A**). Diap1 protein levels were cell-autonomously reduced in *emc* mutant clones (**Figure 3B**). A comparable reduction was also seen in *emc Df(3L)H99* clones, (**Figure 3C**). Because *emc Df(3L)H99* clones developed normally, this suggested Diap1 protein levels were upstream of the cell death pathway in affecting morphogenetic furrow speed, and that *rpr*, *grim*, and *hid* activities were required downstream of *diap1*. To test the role of Diap1 directly, *diap1* was over-expressed in the posterior eye by transcription under GMR-Gal4 control. This rescued morphogenetic furrow speed to normal in *emc* clones (**Figure 3D**). We also saw restoration of morphogenetic furrow speed in *emc* clones that expressed Baculovirus P35 under GMR-Gal4 control (**Figure 3E**).

Baculovirus P35 encodes a caspase pseudo-substrate that inhibits all *Drosophila* caspases except Dronc(Hay, Wolff, & Rubin, 1994; Xue & Horvitz, 1995; C.J. Hawkins et al., 2000; Meier et al., 2000). The *emc* clones expressing p35 also lacked cell death (**Supplemental Figure 2D**). The restoration of morphogenetic furrow speed by Diap1, a *dronc* antagonist, as well as by the caspase inhibitor P35, suggest that the effect of morphogenetic furrow acceleration in *emc* mutant clones is due to the caspase cascade.

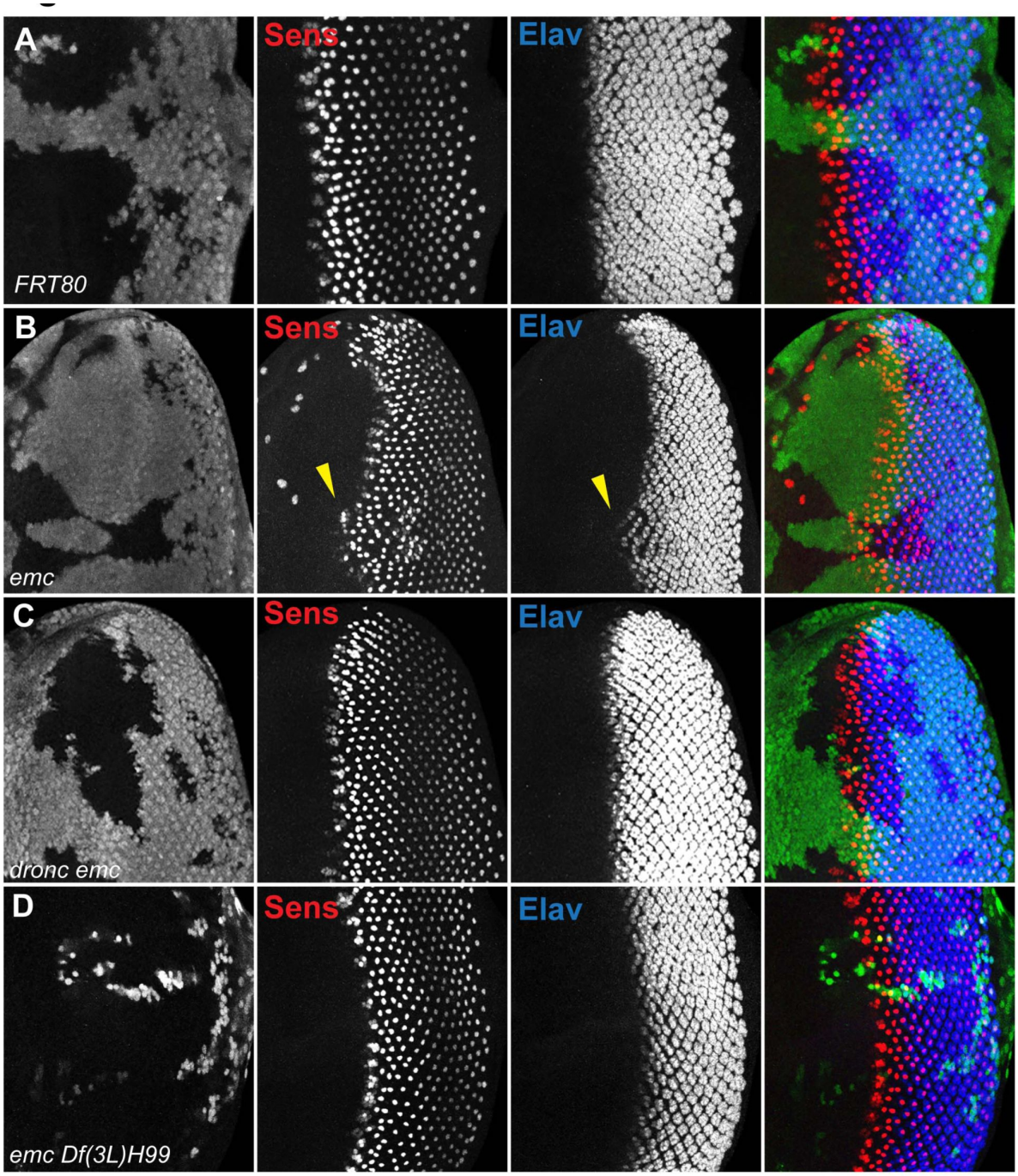

Figure 2

Morphogenetic furrow progression is affected by cell death pathways In all panels, the differentiating neurons are marked by Elav (in blue), and mutant cells are identified by the absence of GFP expression (in green). A) The wave of retinal differentiation from posterior to anterior (right to left), marked here by Senseless expression in R8photoreceptor cells (red) is normal in FRT80 control clones. B) *emc* null cell lacking GFP shows acceleration of retinal differentiation marked by yellow arrow. C) In contrast, retinal differentiation proceeds at the same pace in *emc dronc* double mutant clones as in nearby wild type regions in 75% of the eye discs. D) *emc H99* double mutants show a stronger suppression of the acceleration of retinal differentiation (compare panel B). Genotypes: (A) *ywhsF;;FRT80/[UbiGFP] M(3)67C FRT80* (B) *ywhsF;;emc^{AP6}FRT80/[Ubi-GFP] M(3)67CFRT80* (C) *ywhsF;;dronc^{i29}emc^{AP6} FRT80/[UbiGFP] M(3)67C FRT80* (n=12)(D) *ywhsF;;emc^{AP6} Df(3L)H99 FRT80/[UbiGFP] M(3)67C FRT80*.

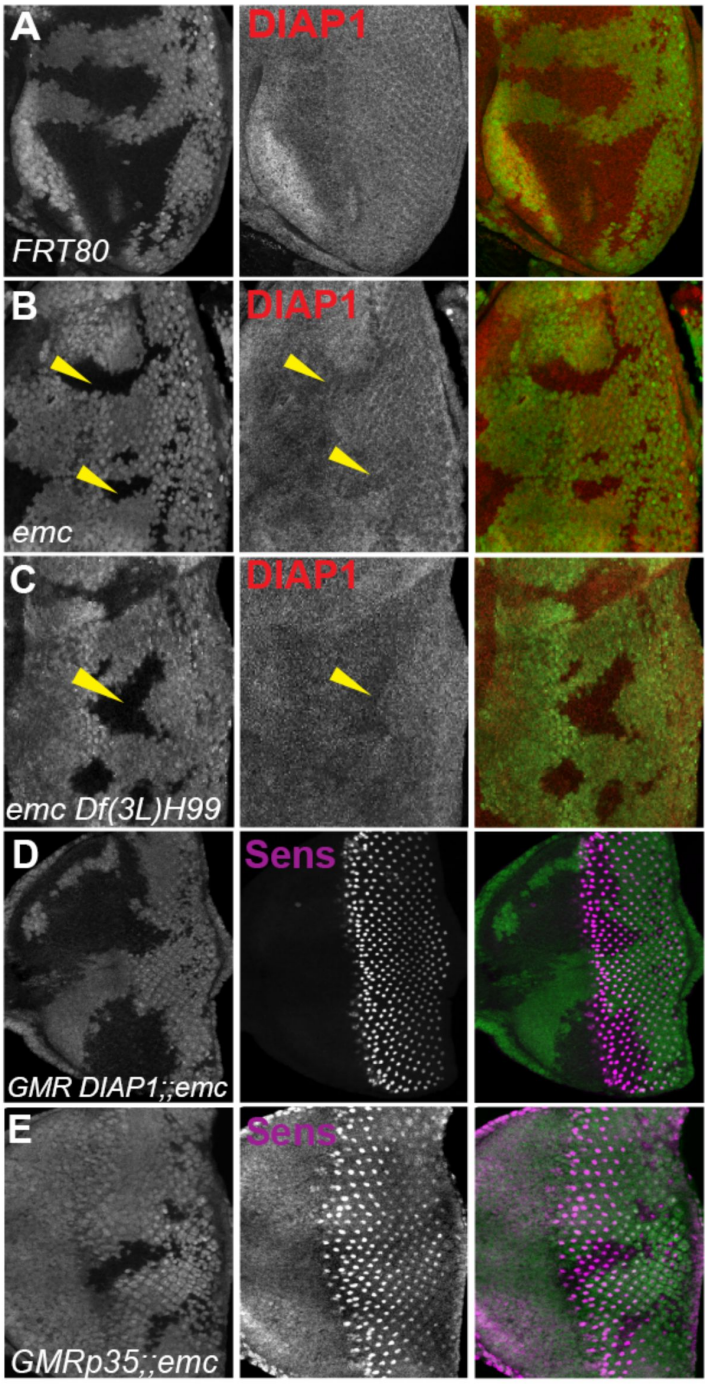

Figure 3

Diap1 expression and function in *emc* mutant clones In mosaic eye discs with (A) FRT80 clones, there is no change in Diap1 levels within and outside the clone. However, both (B) *emc* mutant clones and (C) *emc H99* clones show reduced Diap1 levels. (D) In GMR-DIAP1 eye disc mutant for emc furrow progression is normal and is marked by Senseless staining. Similarly, in GMR-p35 eye disc mutant for emc also show normal furrow progression (E). Genotypes: (A) *ywhsF;;FRT80/[UbiGFP] M(3)67C FRT80* (B) *ywhsF;;emc^{AP6}FRT80/[Ubi-GFP] M(3)67C FRT80* (C) *ywhsF;;emc^{AP6} Df(3L)H99 FRT80/[UbiGFP] M(3)67C FRT80* (D) ywhsF/GMR-DIAP1;;*emc^{AP6}FRT80/[Ubi-GFP] M(3)67C FRT80*. (E) ywhsF/GMR-p35;;*emc^{AP6}FRT80/[Ubi-GFP] M(3)67C FRT80*.

To test whether reduced Diap1 expression promoted apoptosis and thereby accelerated the morphogenetic furrow, we assessed apoptosis levels by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of eye discs containing mutant clones. We found almost no cell death posterior to the furrow in *emc* clones (**Supplemental Figure 2B**), and cell death in *emc* clones anterior to the furrow was comparable to controls and less than that of cells surrounding the clones (**Supplemental Figure 2A**). Some cell death is expected outside *emc* or control clones, as these cells have the *M* background that is itself associated with an increase in apoptosis(Coelho et al., 2005; Li & Baker, 2007; Kale, Li, Lee, & Baker, 2015). To test whether apoptosis in the eye disc would be sufficient to promote morphogenetic furrow progression, we generated mosaic clones for a *pineapple eye (pie)* mutation. These *pie* mutant cells have an elevated rate of apoptosis in imaginal discs, but not sufficient to prevent *pie* homozygous clones surviving late into larval and even adult life (Shi, Stampas, Zapata, & Baker, 2003). The rate of morphogenetic furrow progression was unaffected in eye discs containing *pie* clones (**Supplemental Figure 2C**). Because no excess cell death was detected in *emc* clones, and cell death was insufficient to accelerate the morphogenetic furrow in otherwise wild type eye discs, *emc* clones might be affected by a non-apoptotic caspase activity.

Wingless and Dpp signaling are unaffected by *emc*

To understand how caspases could affect the speed of the morphogenetic furrow, we analyzed pathways known to contribute. Hedgehog (Hh) and Decapentaplegic (Dpp) signaling drive this differentiation wave, along with a contribution from Notch signaling (U. Heberlein, T. Wolff, & G. M. Rubin, 1993; Ma, Zhou, Beachy, & Moses, 1993; Borod & Heberlein, 1998; Baonza & Freeman, 2001b; Fu & Baker, 2003). A negative regulator of morphogenetic furrow progression is Wingless (Wg), which is expressed at the dorsal and ventral eye disc margins (Maurel-Zaffran & Treisman, 2000; Lee & Treisman, 2002).

Because we found that *ex* mutations affected thoracic bristle patterning through a caspase-dependent non-apoptotic effect on Wg signaling (Wang & Baker, 2019), we looked first to see whether *emc* mutations reduced Wg signaling in the eye. We used Frizzled-3 RFP (Fz3- RFP) as a reporter (Sato, Kojima, Ui-Tei, Miyata, & Saigo, 1999). In control eye discs, the Fz3- RFP recapitulates the pattern of endogenous Wg signaling activity at the wing margins (**Figure 4 figure supplement 1**) (Treisman & Rubin, 1995). Frizzled-3 RFP expression was not changed in *emc* clones (**Figure 4A**). We then used a mutation in *naked cuticle* (*nkd*), encoding a negative feedback regulator of Wg signaling, to modulate Wg signaling(Zeng et al., 2000; Chang, Chang, Barolo, & Cadigan, 2008). If the morphogenetic furrow was accelerated in *emc* mutant clones due to reduced Wg signaling, more normal development should occur in *emc nkd* clones. The morphogenetic furrow was still accelerated in *emc nkd* clones, however (**Figure 4B**). These results provided no evidence that Wg signaling was the relevant *emc* target in the eye.

We also examined Dpp signaling, since ectopic Dpp signaling is sufficient to accelerate the morphogenetic furrow(Pignoni & Zipursky, 1997). The pattern of pMad, a readout of Dpp signaling, was identical in *emc* mutant and control clones spanning the morphogenetic furrow (**Figure 4C**, D). Thus, Dpp signaling did not seem altered by *emc* mutants either.

Hedgehog pathway

Hedgehog signaling is a key mover of the morphogenetic furrow(U. Heberlein, T. Wolff, & G.M. Rubin, 1993; Ma et al., 1993; Treisman, 2013). Elevated Hh signaling is sufficient to accelerate the morphogenetic furrow(Heberlein, Singh, Luk, & Donohoe, 1995; Ma & Moses, 1995). Notably, it has been suggested previously that *emc* mutations affect the morphogenetic furrow by activating Hedgehog signaling, because *emc* mutant cells accumulate Ci protein (Spratford & Kumar, 2013). Full-length Ci protein (Ci155) is targeted to the proteosome by Cul1 for processing

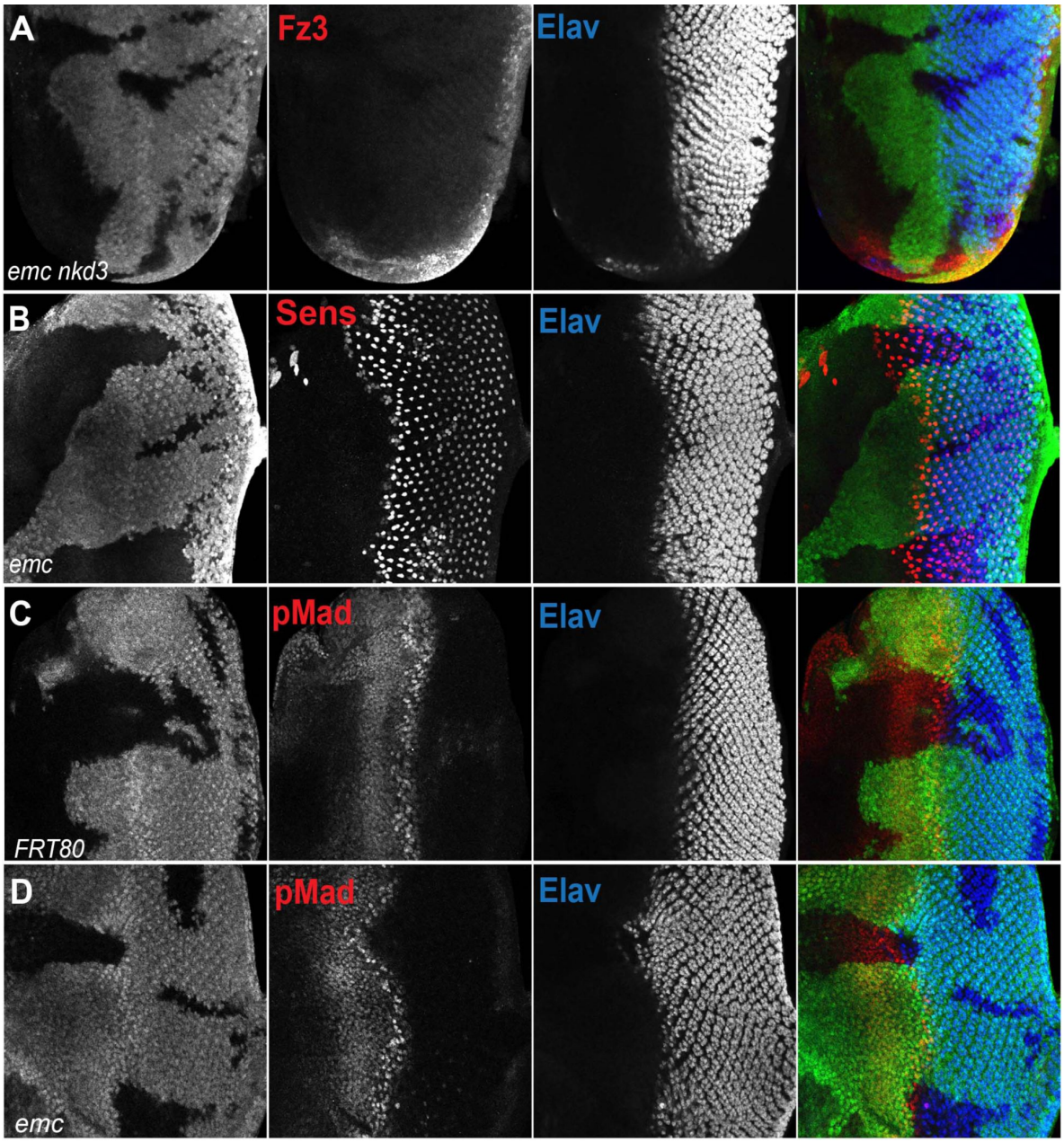

Figure 4

Wingless and Dpp signaling are not caspase targets (A) No reduction in the Wg signaling reporter Fz3-RFP was detectable in emc clones. See **Figure 4 Figure Supplement 1** for Fz3-RFP expression in the wild type. (B) Retinal differentiation is accelerated in *emc nkd*^{3} double mutant clones like in *emc* clones. Senseless expression in R8 photoreceptor cells and Elav staining in differentiating photoreceptors are shown. (C) p-Mad accumulates around the morphogenetic furrow in eye discs containing control clones(Vrailas & Moses, 2006; Firth, Bhattacharya, & Baker, 2010) (E) Except for the advanced progression, p-Mad levels were unchanged in *emc* clones. Genotypes: (A) Fz3-RFP/+; *emc*^{AP6}*FRT80/[Ubi-GFP] M(3)67C FRT80* (B) *ywhsF;;emc*^{AP6}*nkd*^{3}*FRT80/[Ubi-GFP] M(3)67C FRT80* (C) *ywhsF;;FRT80/[UbiGFP] M(3)67C FRT80* (D) *ywhsF;;emc*^{AP6}*FRT80/[Ubi-GFP] M(3)67C FRT80*.

into a transcriptional repressor protein Ci75(Aza-Blanc, Ramirez-Weber, Laget, Schwartz, & Kornberg, 1997). By inhibiting this processing, Hh prevents repression of target genes by Ci75 and promotes transcriptional activation downstream of Ci155.

Accordingly, Ci155 accumulation is a feature of cells receiving Hh signals(Motzny & Holmgren, 1995).

We confirmed that *emc* mutant cells contain higher levels of Ci155, as reported previously (**Figure 5A**)(Spratford & Kumar, 2013). Ci155 was elevated in *emc dronc* clones (**Figure 5B**) but reduced to wild-type levels in *emc Df(3L)H99* clones (**Figure 5C**, **Figure 5 figure supplement 1**). Thus, Ci155 levels did not correlate perfectly with behavior of the morphogenetic furrow.

We noticed that Ci155 levels were elevated in *emc* mutant clones in the posterior, differentiating eye disc, as well as in and ahead of the morphogenetic furrow (**Figure 5A**). This is significant, because Ci155 is not affected by Hh-dependent Cul1 processing posterior to the morphogenetic furrow (Ou, Lin, Chen, & Chien, 2002; Baker, Bhattacharya, & Firth, 2009).

Ci155 accumulation posterior to the furrow suggests a Hh-independent mechanism.

To test whether Ci155 accumulation in *emc* clones indicates elevated Hh target signaling, we looked at the transmembrane receptor Patched (Ptc) which is a transcriptional target of Hh signaling, acting in a negative feedback loop(Hepker, Wang, Motzny, Holmgren, & Orenic, 1997). We saw no changes in Ptc protein levels in either *emc* or *emc Df(3L)H99* clones compared to controls, questioning the notion that Hh signaling was altered by *emc* (**Figure 5 figure supplement 2**).

To test definitively whether Ci155 is responsible for accelerating the furrow in *emc* clones, we generated *emc ci* double mutant clones. To achieve this, a genomic transgene that rescues ci^{94} flies to adulthood (Little, Garcia-Garcia, Sul, & Kalderon, 2020), was introduced into chromosome arm 3L where it is linked to the wild type *emc* locus, so that mitotic recombination in the ci^{94}null background leads to *emc ci* double mutant clones. For unknown reasons, *emc ci* double mutant clones were small and difficult to obtain, even in the Minute background. In all the *emc ci* double mutant clones we found that spanned the morphogenetic furrow; eye differentiation was still accelerated (**Figure 5D**). These data might not exclude a quantitative contribution of Ci to more rapid morphogenetic furrow progression in the absence of *emc* but are sufficient to show that *emc* regulates the speed of the morphogenetic furrow through at least one other caspase target.

Delta expression is a target of caspases

The remaining signaling pathway that contributes to morphogenetic furrow movement is Notch. Specifically, only cells where Notch signaling is active are competent to initiate retinal differentiation in response to Dpp (Baonza & Freeman, 2001a; Fu & Baker, 2003). Accordingly, ectopic expression of Delta, the transmembrane ligand for Notch, is sufficient to accelerate retinal differentiation anterior to the morphogenetic furrow by expanding the effective range of Dpp signaling (Baonza & Freeman, 2001a; Li & Baker, 2001).

To test whether *emc* restrains Notch, we examined the bHLH proteins of the E(spl)-C, widely characterized targets of the Notch pathway (Bray, 2006). We used mAb323 to detect up to five E(spl) bHLH proteins with Notch-dependent expression (Jennings, Preiss, Delidakis, & Bray, 1994). E(spl) protein expression, and hence Notch signaling, was higher in the morphogenetic furrow in *emc* clones than in wild type cells (**Figure 6A**). By contrast, E(spl) protein expression was normal in *emc dronc* clones (**Figure 6B**).

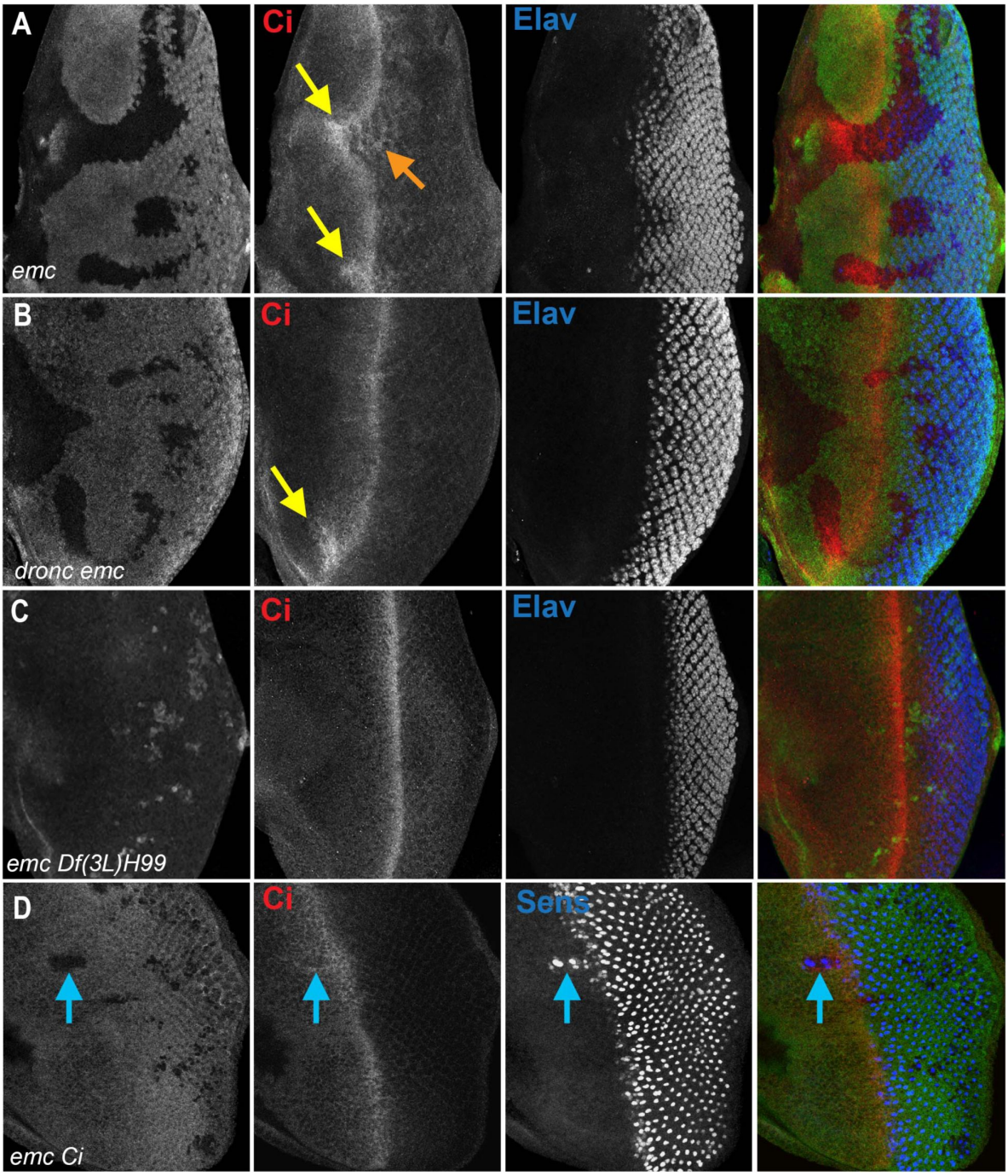

Figure 5

Ci expression and function in *emc* mutant clones A) Ci is elevated within *emc* mutant cells (yellow arrows). This was also true posterior to the morphogenetic furrow (orange arrow) B) Higher Ci was also seen in *emc dronc* mutant cells, even when the morphogenetic furrow progressed normally, as indicated by Elav staining. C) Ci levels were completely normal in *emc H99* clones. D) *emc* and *ci* doubly-mutant clone lacking GFP shows accelerated retinal differentiation (blue arrow). Genotypes: (A) *ywhsF;;emc^{AP6}FRT80/[Ubi-GFP] M(3)67C FRT80* (B) *ywhsF;;dronc^{i29}emc^{AP6} FRT80/[UbiGFP] M(3)67C FRT80* (C) *ywhsF;;emc^{AP6} Df(3L)H99 FRT80/[UbiGFP] M(3)67C FRT80* (D) *ywhsF;;emc^{AP6}FRT80/[Ubi-GFP] ci^{+} M(3)67C FRT80;ci[94]/ci[94]*.

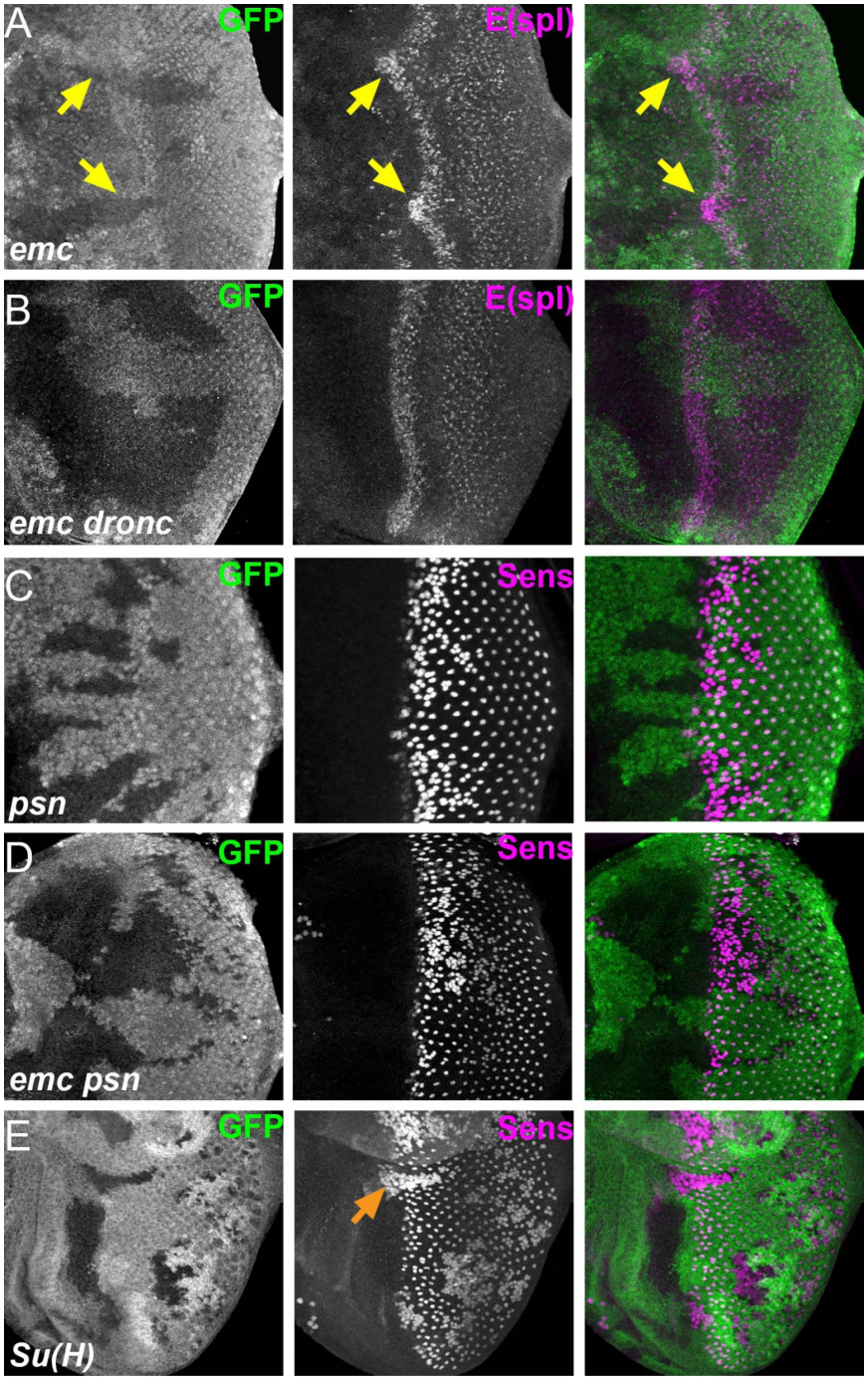

Figure 6

Non-apoptotic caspase signaling targets Notch pathway by promoting Dl expression (A) E(spl), an important N target for lateral inhibition, is elevated in the morphogenetic furrow region of *emc* clones (yellow arrows). (B) *emc dronc* clones have normal levels of E(spl) protein.(C) Senseless staining shows a neurogenic phenotype in *psn* mutant clones, due to reduced Notch signaling. Morphogenetic furrow progression is unaffected. (D) A neurogenic phenotype was also observed in *emc psn* clones, along with normal furrow progression. (E) *Su(H)* mutant clones identified by absence of GFP labeling show a strong neurogenic phenotype as well as advanced retinal differentiation (orange arrow) (Li & Baker, 2001). The cell-autonomous effect results in a discontinuity at the borders of *Su(H)* clones, where differentiation outside the clones lags that within clones (orange arrow) Genotypes: (A) *ywhsF;;emc^{AP6} FRT80/[Ubi-GFP] M(3)67C FRT80* (B) *ywhsF;; dronc^{i29}emc^{AP6} FRT80/[UbiGFP] M(3)67C FRT80* (C) *ywhsF/+;;psn^{V1} FRT80/[Ubi-GFP]M(3)67CFRT80* (D) *ywhsF/+;; emc^{AP6} psn^{V1} FRT80/[Ubi- GFP]M(3)67CFRT80* ((E) *ywhsF; Su(H)^{D47}*FRT40/FRT40*[UbiGFP]*.

To test whether elevated Notch signaling was required to accelerate the morphogenetic furrow in *emc* mutants, we examined *emc psn* double mutant clones. Presenilin (Psn) is the enzymatic component of γ-secretase that releases the intracellular domain of Notch during active Notch signaling, and the *psn* gene is linked to *emc*. Loss of *psn* function leads to a Notch loss of function phenotype (Struhl & Greenwald, 1999; Ye, Lukinova, & Fortini, 1999). Accordingly, *psn* clones lead to a neurogenic phenotype in the eye, without affecting the progression of the morphogenetic furrow (**Figure 6C**)(Li & Baker, 2001). The position of the morphogenetic furrow was also not affected in *emc psn* clones (**Figure 6D**). Thus, the furrow was not accelerated in *emc* mutant clones also defective for Notch signaling.

These two results together indicated that *emc* mutants promoted Notch signaling in the morphogenetic furrow, acting through caspase signaling on a step prior to γ-secretase cleavage of the intracellular domain of Notch. Accordingly, we decided to check Delta (Dl) protein levels.

We found that Dl protein levels were clearly elevated cell-autonomously throughout *emc* clones, both posterior and anterior to the furrow (**Figure 7B**). Importantly, this includes the region just ahead of the morphogenetic furrow that lacks Delta expression in normal development(Parks, Turner, & Muskavitch, 1995; Baker & Yu, 1998)(**Figure 7B**). By contrast, levels of Dl protein in *emc dronc* clones and *emc Df(3L)H99* clones were similar to those of wild type controls (**Figure 7A,C**,D). This indicated that Dl protein is a target of caspases, directly or indirectly.

Because Dl activates Notch signaling cell non-autonomously, we wondered whether the effect of *emc* mutant clones was cell-autonomous. We note that, in all the experiments reported here, and in all the previous studies of *emc* mutant clones affecting morphogenetic furrow movement, the morphogenetic furrow is maintained as a continuous groove across the eye disc (Brown et al., 1995; Bhattacharya & Baker, 2011, 2012; Spratford & Kumar, 2013). That is, the advanced front of retinal differentiation within *emc* clones is always smoothly continuous with the normal morphogenetic furrow outside the clones, implying a progressive gradual increase in morphogenetic furrow speed near the lateral edges of *emc* mutant clones (eg **Figures 2B**, 4B, 4D, 5A, 6A). Clones of *Su(H)* null mutants, which act cell-autonomously, provide a contrasting example. Complete loss of *Su(H)* accelerates the morphogenetic furrow due to loss of default Su(H) repression of Notch targets(Li & Baker, 2001). The boundaries of *Su(H)* null clones exhibit a clear discontinuity between the rates of differentiation within and outside the clones (**Figure 6E**). This difference between *Su(H)* and *emc* mutant clones is consistent with the idea that *emc* affects morphogenetic furrow progression non-autonomously.

Specific ommatidial cell fates are regulated by caspases in *emc* mutants

Because the general pattern of neurogenesis revealed by pan-neuronal anti-Elav staining appeared so normal in *emc dronc* and *emc Df(3L)H99* clones (**Figure 2C,D**), we examined whether effects of *emc* on particular retinal cell fates was caspase dependent. Emc is also required for R7 differentiation and for timely onset of cone cell differentiation(Bhattacharya & Baker, 2009), two cell fate decisions that also depend on Notch signaling(Cooper & Bray, 2000; Flores et al., 2000; Tomlinson & Struhl, 2001; Treisman, 2013). These cell fates are normally independent of *da*, but like imaginal disc cell growth and morphogenetic furrow progression, ectopic *da* activity perturbs them in *emc* mutants(Reddy et al.; Brown et al., 1996).

Clones of *emc* mutant cells lack R7 photoreceptor cells (**Figure 8A,B**)(Bhattacharya & Baker, 2009). R7 differentiation was restored to 90% of ommatidia in *emc Df(3L)H99* clones (**Figure 8C**). As shown before, *emc* mutant clones delayed Cone Cell differentiation by 2-3 columns (corresponding to a delay of 3-6 h)(Bhattacharya & Baker, 2009)(**Figure 8D,E**). We found no

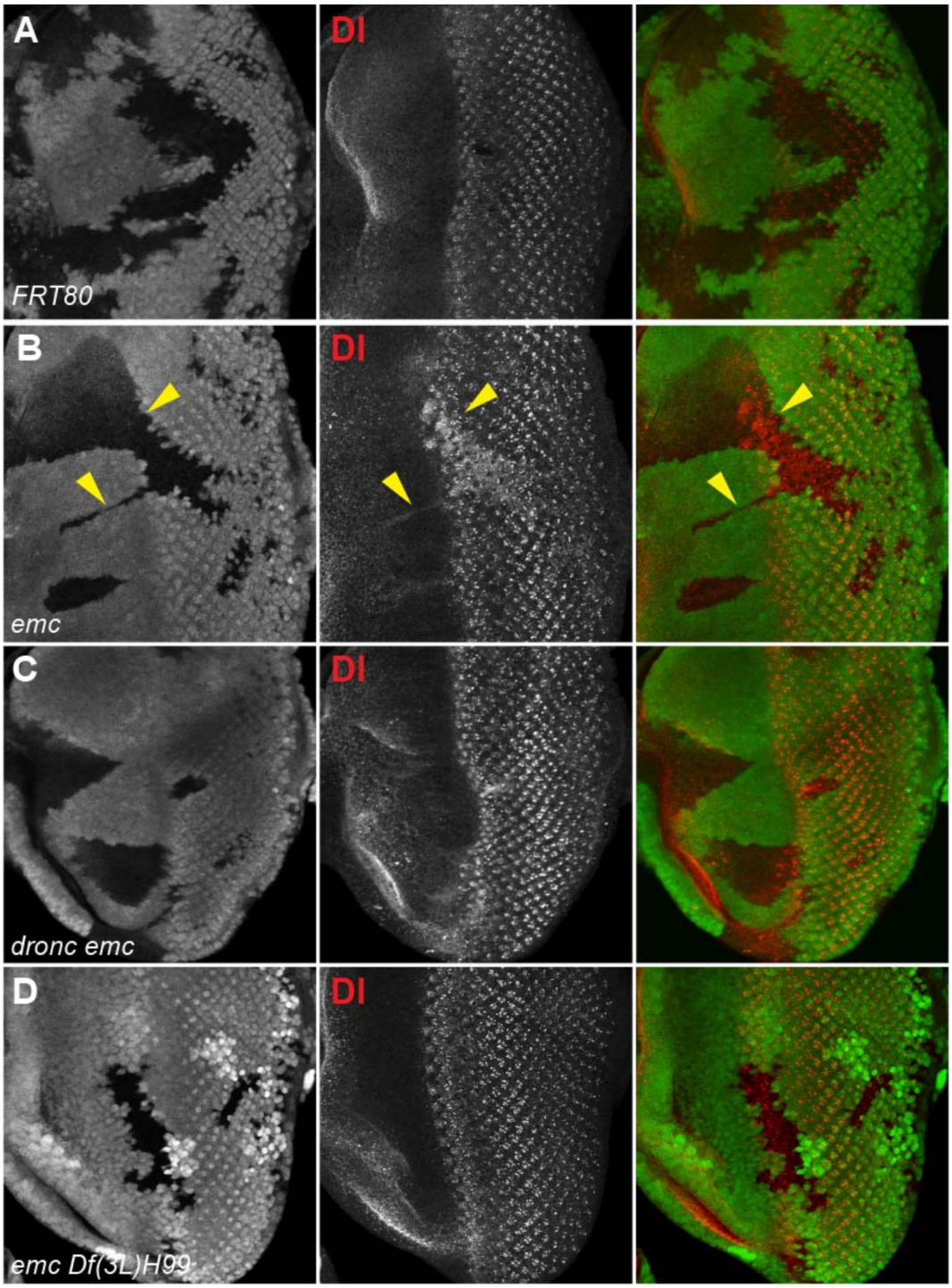

Figure 7

Caspase regulation of Delta levels (A) Normal levels of Delta protein are seen in FRT80 clones as compared to elevated Delta levels in (B) *emc* clones whereas that phenotype is reversed in (D) *emc H99* clones. However, as seen in (C) *dronc emc* clones show high Delta levels. Genotypes: (A) *ywhsF;;FRT80/[UbiGFP] M(3)67C FRT80* (B) *ywhsF;;emc^{AP6}FRT80/[Ubi-GFP] M(3)67C FRT80* (C) *ywhsF;;dronc^{i29}emc^{AP6} FRT80/[UbiGFP] M(3)67C FRT80* (D) *ywhsF;;emc^{AP6} Df(3L)H99 FRT80/[UbiGFP]FRT80*.

delay in cone cell differentiation in most *emc Df(3L)H99* clones (**Figure 8F**). These results indicated that caspase activity contributes to the R7 and cone cell differentiation defects that are also characteristic of *emc* mutants.

Discussion

The Inhibitor of DNA binding (Id) proteins are important regulators of differentiation(Massari & Murre, 2000; Ling et al., 2014; Wang & Baker, 2015a; Roschger & Cabrele, 2017; Singh et al., 2022). They are well known to antagonize proneural basic helix- loop-helix (bHLH) proteins (Benezra et al., 1990; Ellis et al., 1990; Garrell & Modolell, 1990; Cabrera et al., 1994; Ellis, 1994; Norton, 2000). It has been uncertain whether this is their only function, as in *Drosophila* it is clear that *emc* mutations affect processes that are independent of proneural genes. Here, we report that multiple effects of *emc* mutations that occur independently of proneural bHLH genes, and are due to the loss of restraint on *da* function in *emc* mutant cells, are caused by caspase-dependent non-apoptotic processes that result from reduced expression of Diap1 protein.

One known effect of *emc* is that it restrains the *Drosophila* E protein Da. Da is expressed ubiquitously and is the obligate heterodimer partner of proneural bHLH proteins in neurogenesis.

In undifferentiated progenitor cells, most or all Da is thought to be sequestered in inactive heterodimers with Emc(Li & Baker, 2018). In *emc* mutant cells, Da levels rise and become functional, potentially as homodimers(Bhattacharya & Baker, 2011).

In the context of imaginal disc cell growth, *emc* mutant cells experience *da*-dependent over-expression of the *ex* gene(Wang & Baker, 2015b, 2018). Although most study of *ex* has focused on its role in growth control through the SHW pathway, *ex* was recently shown to influence patterning through Yki-dependent transcription of *diap1*, an important regulator of cell death pathways. This was in the developing thorax, where *ex* mutations affect bristle patterning through *diap1* and a non-apoptotic effect of caspases on Wg signaling(Wang & Baker, 2019).

This observation led us to explore whether aspects of the *emc* phenotype could reflect reduced *diap1* expression and elevated caspase activity.

Remarkably, we found that all four features of the *emc* phenotype we examined depended on the caspase-mediated cell death pathway. The normal growth of imaginal disc cells, normal speed of the morphogenetic furrow, specification of R7 photoreceptor cells, and timing of non- neuronal cone cell development were all restored when caspase activation was prevented, which was achieved by inactivating the initiator caspase Dronc, deleting the proapoptotic *rpr*, *grim*, and *hid* genes, over-expressing *diap1*, or over-expressing baculovirus p35. These appeared to be non-apoptotic effects, because no elevation in apoptosis was apparent in *emc* mutant cells, because generating apoptosis by another means did not mimic the effects, and because the effects were mediated by elevated Dl protein expression in cells that were not apoptotic. They appeared to be effects of caspase activity, because they depended in part on *dronc*, an initiator caspase that cleaves and activates effector caspases, because they were suppressed by baculovirus p35, a caspase inhibitor that is a caspase pseudo-substrate, and because they were suppressed by Diap1, a ubiquitin ligase that inhibits caspases and targets them for degradation. The effects may depend on the full caspase cascade of initiator and effector caspases, because sensitivity to p35 indicates that caspases other than Dronc are necessary, and because mutating dronc alone gave lesser degrees of rescue than deleting *rpr*, *grim*, and *hid*. This is consistent with many other studies that describe the pathways for apoptotic and non-apoptotic caspase functions as similar(Kanuka et al., 2005; Kuranaga & Miura, 2007; Aram et al., 2017; Nakajima & Kuranaga, 2017; Baena-Lopez et al., 2018; Su, 2020; Colon-Plaza & Su, 2022).

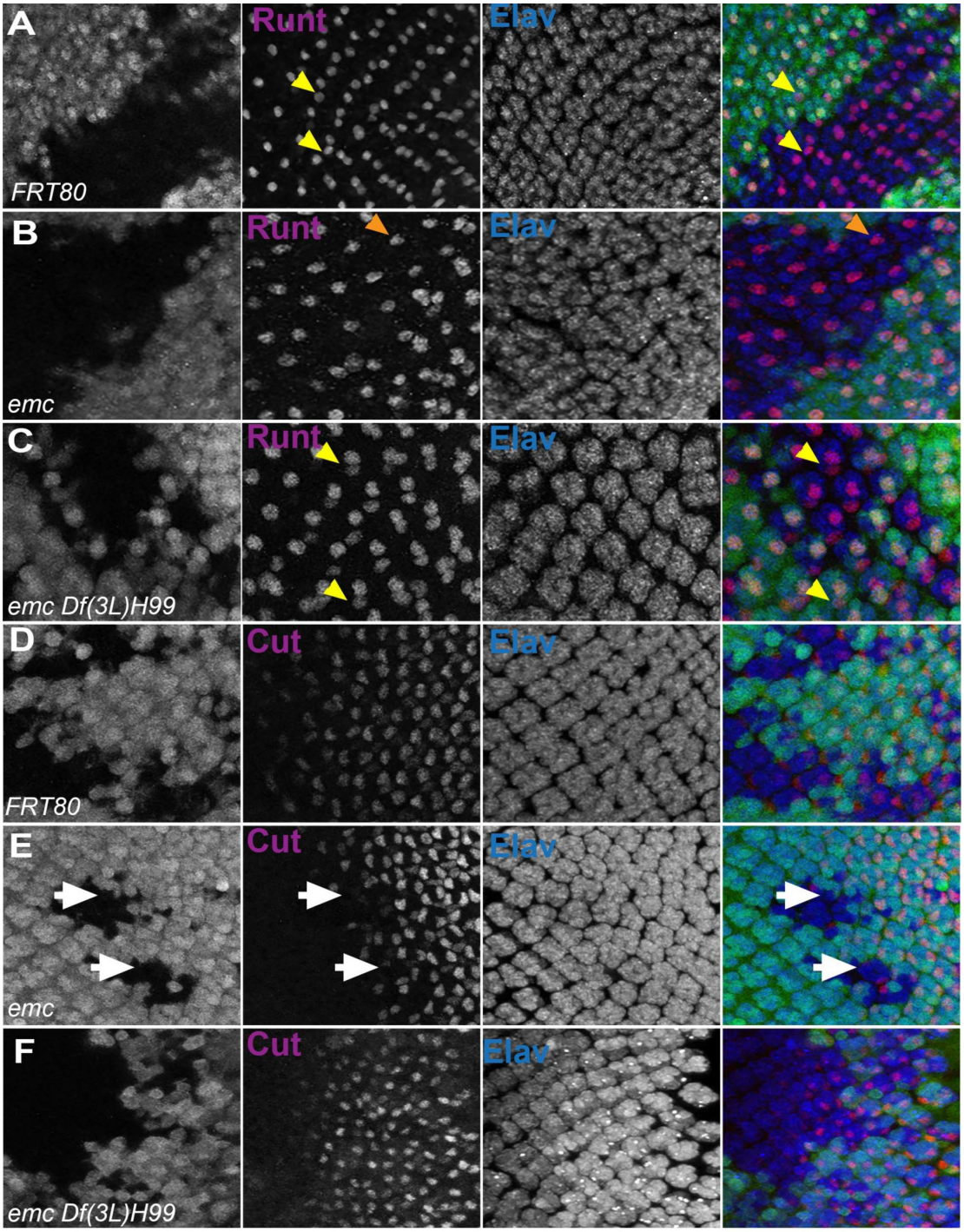

Figure 8

Caspases contribute to R7 photoreceptor and cone cell defects in *emc* mutants In all panels *emc* mutant cells are marked by the absence of GFP expression (in green) and photoreceptor neurons are marked by Elav in blue. (A) Runt (in red) is expressed in R7 and R8 (yellow arrowhead) photoreceptor cells inside and outside the clone in FRT80 controls. (B) Inside *emc* clone, Runt expression is lost from R7 cells, while expression in R8 cells remains unaffected (red arrow). (C) However, inside the *emc H99* clones, Runt is expressed in both R7 and R8 cells. (D) Cut (in red) is expressed in cone cells in FRT80 controls. (E) Inside *emc* clone, cut is delayed. (F) However, inside the *emc H99* clones, cut staining is not delayed. Genotypes: (A, D) *ywhsF;;FRT80/[UbiGFP] M(3)67C FRT80* (B, E) *ywhsF;;emc^{AP6}FRT80/[Ubi-GFP] M(3)67C FRT80* (C, F) *ywhsF;;emc^{AP6} Df(3L)H99 FRT80/[UbiGFP] M(3)67C FRT80*.

Whereas non-apoptotic caspase activity promotes Wg signaling during specification of thoracic bristles(Kanuka et al., 2005), in the *Drosophila* eye the non-apoptotic caspase target was Notch signaling, due to elevated Delta protein levels. We do not know whether Delta is the direct caspase target. The Delta protein sequence contains multiple predicted caspase target sites, as do many proteins (Wang et al., 2014). Only one candidate site lies in the intracellular domain, where it would potentially be accessible to caspases (**Figure 9B**). Truncation of the Dl intracellular domain is usually associated with loss of Dl function, not stabilization and enhanced function, however (Daskalaki et al., 2011). Another possibility is that a regulator of Dl expression or stability and function is the direct caspase target.

Notably, *emc* mutations accelerate the morphogenetic furrow by enhancing Notch signaling, apparently cell-autonomously, but inhibit R7 and cone cell differentiation through cell-autonomously reduced N signaling(Reddy et al.; Bhattacharya & Baker, 2009). These contrasts may be explained through the dual functions of the Notch ligand Delta in trans- activation and in cis-inhibition (Micchelli, Rulifson, & Blair, 1997; Jacobsen, Brennan,

Martinez-Arias, & Muskavitch, 1998; Li & Baker, 2004; Bray, 2016). The eye disc region ahead of the morphogenetic furrow lacks Delta expression (Parks et al., 1995; Baker & Yu, 1998).

Ectopic Dl expression here is sufficient to activate N non-autonomously and drive morphogenetic furrow progression (Baonza & Freeman, 2001a; Li & Baker, 2001). Within the R7 equivalence group, Delta is expressed early in R1 and R6 photoreceptor precursors, protecting them from N activation through cis-inhibition, and establishing the distinction between R1,6 and R7 precursor cells (Miller, Lyons, & Herman, 2009). Ectopic Dl expression posterior to the morphogenetic furrow has cis-inhibitory effect. Elevated Dl levels within *emc* mutant clones may render potential R7 and cone cell precursors cell-autonomously resistant to N activation, as normally seen in R1/6 precursor cells.

A previous study suggested that *emc* mutations might affect Hh signaling (Spratford & Kumar, 2013). Our data point much more clearly to an effect on Notch signaling. Although the Hh target Ci155 accumulates in *emc* mutant cells, increased Hh signaling is not the only possible cause of this. Ci155 is cytoplasmic, and thought to be only a precursor for a labile nuclear activator molecule(Ohlmeyer & Kalderon, 1998). Preventing activation and nuclear translocation of Ci155 by mutating the kinase *fused* also leads to Ci155 accumulation, without activating Hh signaling. Presumably Ci155 accumulates because it is more stable than its activated derivative(Ohlmeyer & Kalderon, 1998). Significantly, *emc* mutant cells have been reported to over-express the *fused* antagonist *su(fu)*, providing a potential alternative explanation of Ci155 accumulation in *emc* mutant cells(Spratford & Kumar, 2013). Analyzing *emc ci* double mutant cells suggested that *ci* was not required to accelerate the morphogenetic furrow in *emc* mutant clones.

Da protein binds to and potentially regulates hundreds of genes throughout the *Drosophila* genome(Li et al., 2008). Accordingly, Da activity in *emc* mutant cells might be expected to lead to non-specific and pleiotropic effects. Contrary to this expectation, multiple aspects of proneural bHLH-independent *emc* mutant phenotypes have a simple common basis, ie reduced *diap1* expression and elevated caspase activity. Remarkably, mutating *emc* may be the most general way to activate non-apoptotic caspase activities yet discovered.

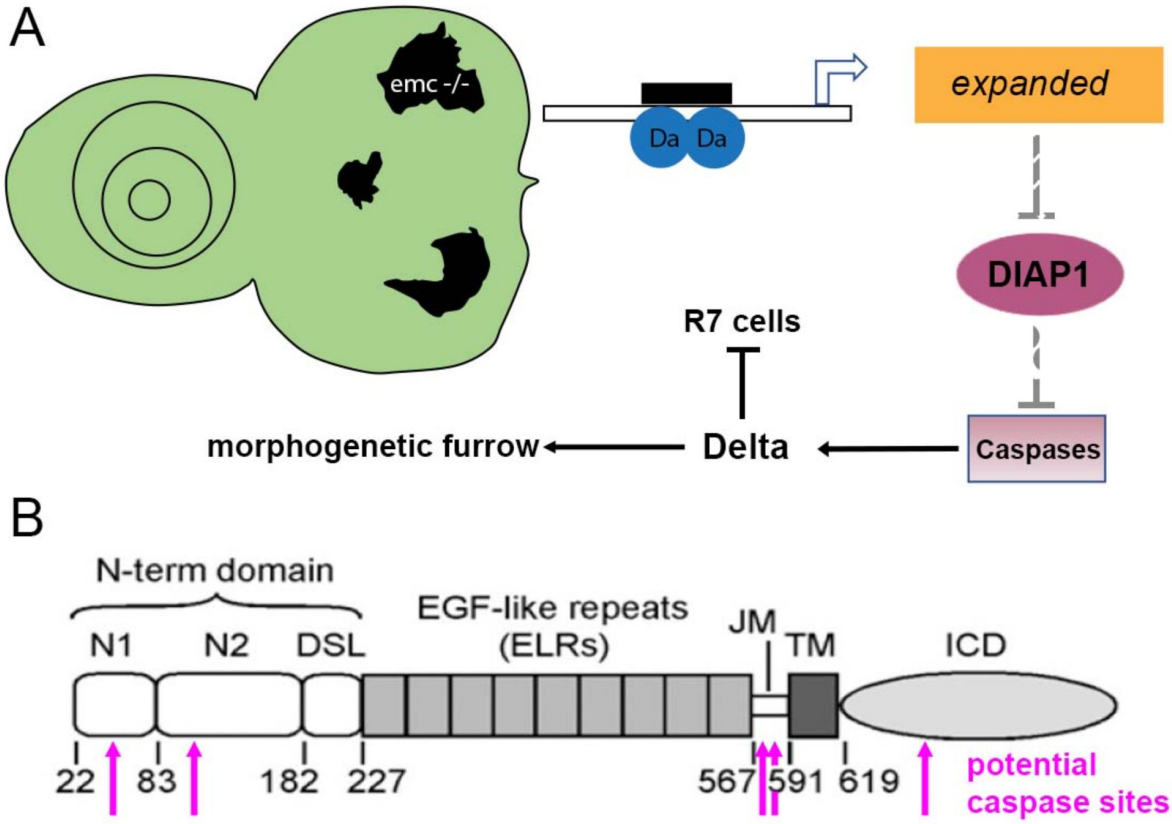

Figure 9

Model of *emc* effects on *Drosophila* eye development.

A) Loss of *emc* allows Da protein to form heterodimers and activate *ex* transcription, increasing SHW pathway activity. SHW activity reduces DIAP1 expression, thereby derepressing caspase activity. In the eye, non- apoptotic caspase activity increases expression of the Notch ligand Delta. Elevated Delta expression accelerates morphogenetic furrow progression, while cis-inhibiting Notch signaling posterior to the morphogenetic furrow, inhibiting R7 cell specification and cone-cell differentiation. B) Like many proteins, Delta has multiple potential caspase cleavage sites, here predicted suing Cascleave 2.0 (Wang et al., 2014). None are high-confidence predictions and only one is in the intracellular domain where caspase access is plausible (position 675, predicted score 0.647). The intracellular domain is required for Delta signaling. Cleavage at position 675 would remove the main ubiquitylation site required for signaling, as well as the binding site for *mindbomb*, so is not anticipated to enhance signaling activity (Daskalaki et al., 2011).

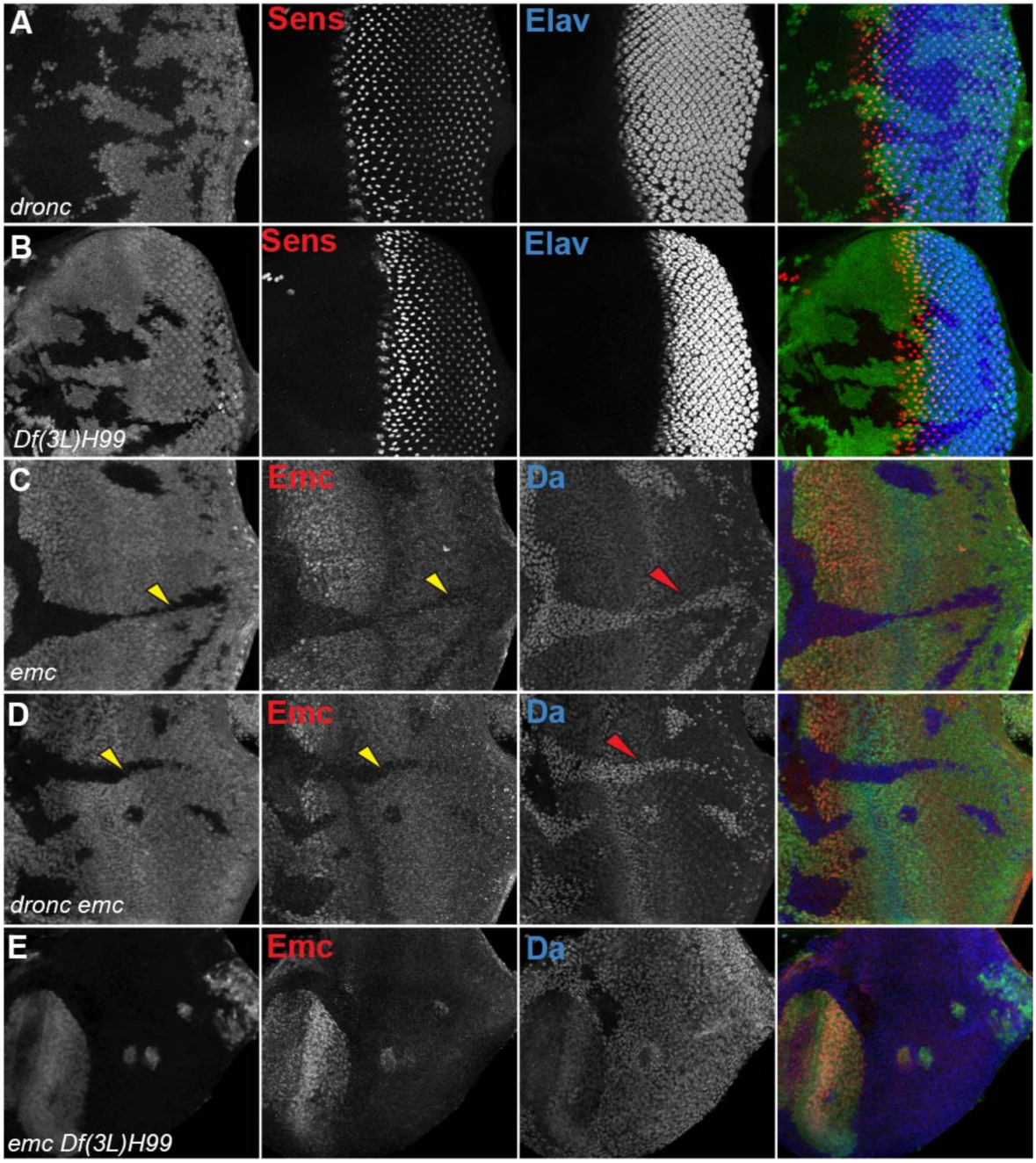

Figure 2 Figure Supplement 1

Morphogenetic furrow progression in *emc* mutant cells In eye imaginal discs, normal furrow progression was observed in (A) *dronc* $^{-/-}$ and (B) *Df(3L)H99* homozygous clones, indicated by Senseless and Elav staining. Homozygous mutant clones of *emc*$^{-/-}$ (C) lacking GFP expression, have no Emc staining (yellow arrowhead) and higher Da (red arrowhead). At the morphogenetic furrow as shown by yellow arrow, Emc expression goes down and Da goes up. Similar results were observed in (D) *dronc*$^{-/-}$ *emc*$^{-/-}$ clones and (E) *emc*$^{-/-}$ *Df(3L) H99* $^{-/-}$ clones confirm that these are *emc* null clones. Genotypes: (A) *ywhsF;;dronc*^{i29}*FRT80/[UbiGFP] M(3)67C FRT80* (B) *ywhsF;; Df(3L)H99 FRT80/[UbiGFP] M(3)67C FRT80*. (C) *ywhsF;;emc*^{AP6}*FRT80/[UbiGFP] M(3)67C FRT80* (D) *ywhsF;;dronc*^{i29}*emc*^{AP6} *FRT80/[UbiGFP] M(3)67C FRT80* (E) *ywhsF;;emc*^{AP6} *Df(3L)H99 FRT80/[UbiGFP] M(3)67C FRT80*.

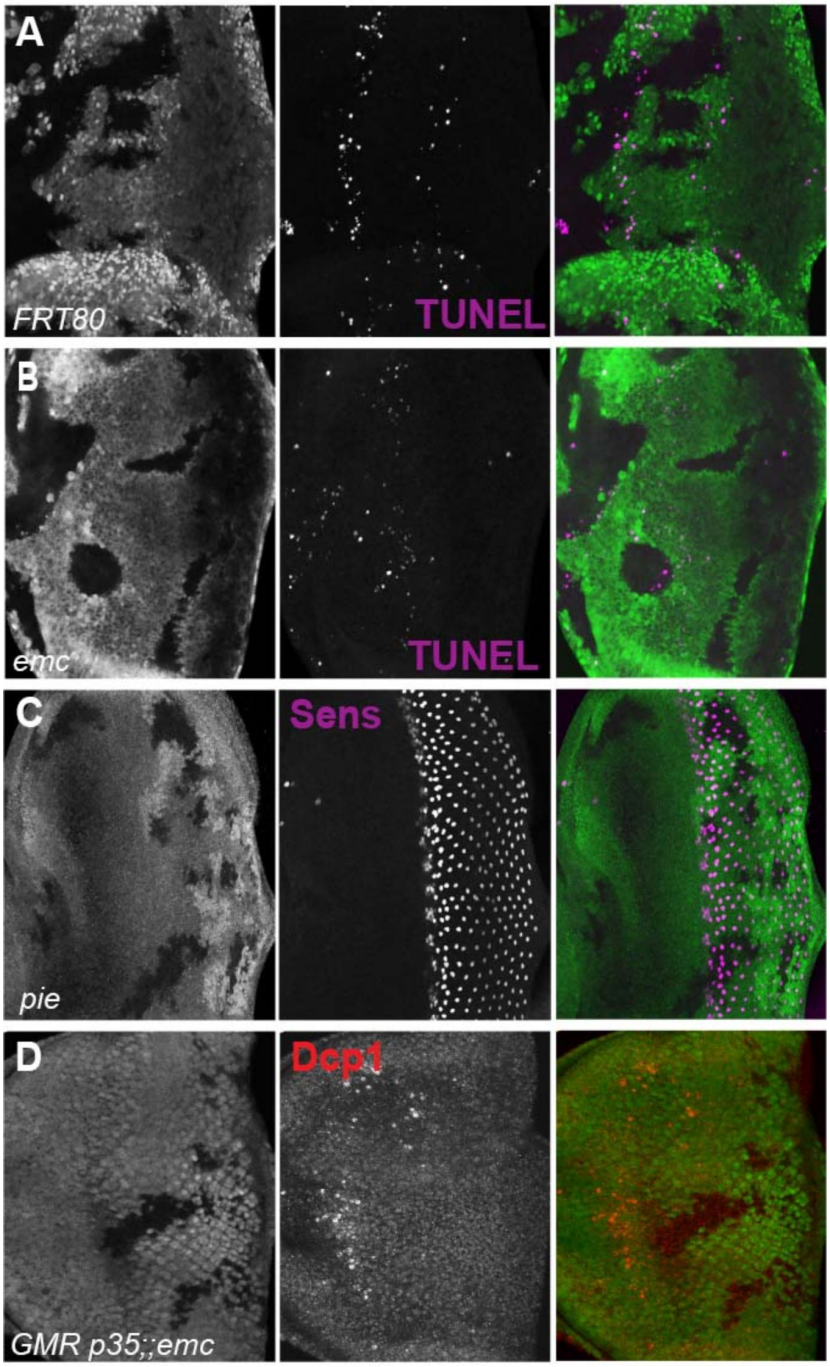

Figure 2 Figure Supplement 2

Apoptosis and furrow progression

Representative images of eye imaginal discs stained for TUNEL (A) *ywhsF;;FRT80/[UbiGFP] M(3)67C FRT80* (B) *ywhsF;;emc^{AP6}FRT80/[Ubi-GFP] M(3)67C FRT80*. In (C) *pie^{EB3}* homozygous clones, furrow progression occurs at normal speed marked by Senseless staining in R8 photoreceptors. (D) In GMR-p35 eye disc mutant for emc also show lack of Dcp1staining. Genotypes: (A) *ywhsF;;FRT80/[UbiGFP] M(3)67C FRT80* (B) *ywhsF;;emc^{AP6}FRT80/[Ubi-GFP] M(3)67CFRT80* (C) ywhsF/+; *pie^{EB3}FRT40/FRT40Alz*. (D) ywhsF/GMR-p35;;*emc^{AP6}FRT80/[Ubi-GFP] M(3)67C FRT80*.

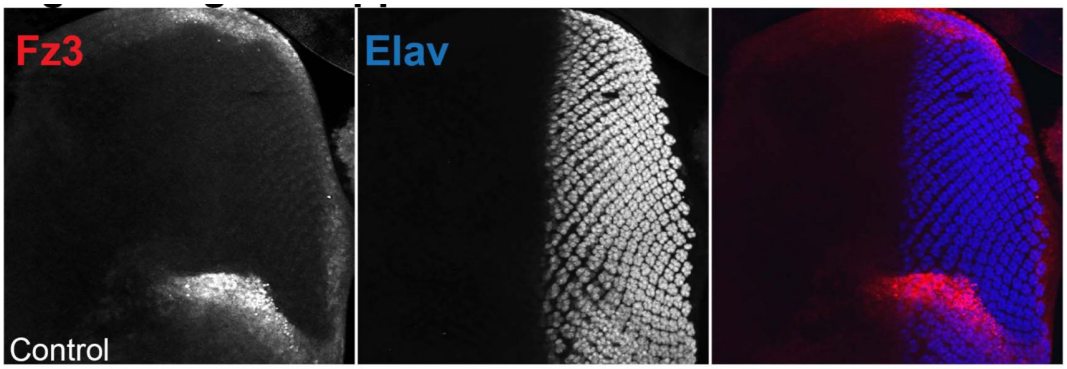

Figure 4 Figure Supplement 1

Fz3-RFP/+ eye disc showing RFP and Elav staining.

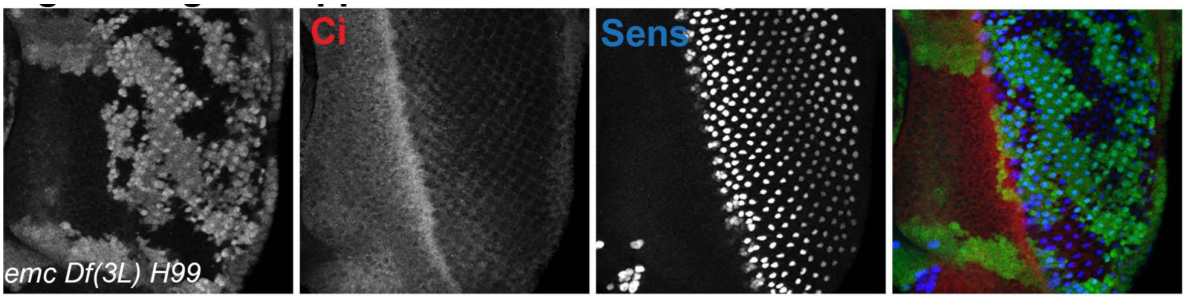

Figure 5 Figure Supplement 1

Ci155 levels when cell death pathways are blocked

Representative image of eye imaginal disc in *emc H99* clones in the non-Minute background, showing similar areas inside and outside clones. Genotype: *ywhsF;; emc^{AP6} Df(3L)H99 FRT80/[UbiGFP]FRT80*

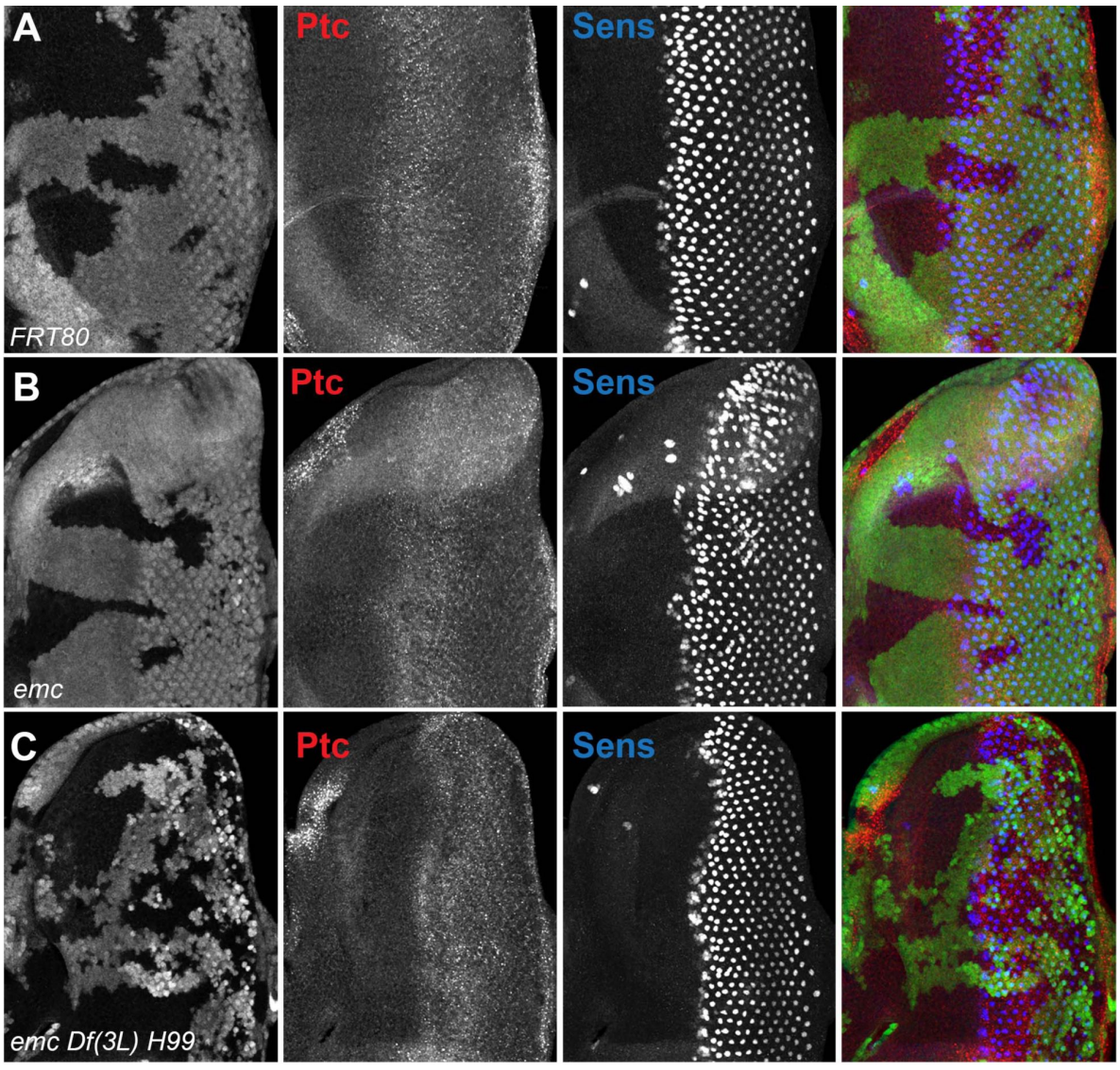

Figure 5 Figure Supplement 2

Patched staining in emc mutants (A) *ywhsF;;FRT80/[UbiGFP] M(3)67C FRT80* (B) *ywhsF;;emc^{AP6}FRT80/[Ubi-GFP] M(3)67C FRT80* (C) *ywhsF;;emc^{AP6} Df(3L)H99 FRT80/[UbiGFP] M(3)67C FRT8*.

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Reviewer #1 (Public Review):

Summary:
The extra macrochaetae (emc) gene encodes the only Inhibitor of DNA binding protein (Id protein) in Drosophila. Its best-known function is to inhibit proneural genes during development. However, the emc mutants also display non-proneural phenotypes. In this manuscript, the authors examined four non-proneural phenotypes of the emc mutants and reported that they are all caused by inappropriate non-apoptotic caspase activity. These non-neuronal phenotypes are: reduced growth of imaginal discs, increased speed of the morphogenetic furrow, and failure to specify R7 photoreceptor neurons and cone cells during eye development. Double mutants between emc and either H99 (which deletes the three pro-apoptotic genes reaper, grim, and hid) or the initiator caspase dronc suppress these mutant phenotypes of emc suggesting that the cell death pathway and caspase activity are mediating these emc phenotypes. In previous work, the authors have shown that emc mutations elevate the expression of ex which activates the SHW pathway (aka the Hippo pathway). One known function of the SHW pathway is to inhibit Yorkie which controls the transcription of the inhibitor of apoptosis, Diap1. Consistently, in emc clones the levels of Diap1 protein are reduced which might explain why caspase activity is increased in emc clones giving rise to the four non-neural phenotypes of emc mutants. However, this increased caspase activity is not causing ectopic apoptosis, hence the authors propose that this is non-apoptotic caspase activity. In the last part of the manuscript, the authors ruled out that Wg, Dpp, and Hh signaling are the target of caspases, but instead identified Notch signaling as the target of caspases, specifically the Notch ligand Delta. Protein levels of Delta are increased in emc clones in an H99- and dronc-dependent manner. The authors conclude that caspase-dependent non-apoptotic signaling underlies multiple roles of emc that are independent of proneural bHLH proteins.

Strengths:
Overall, this is an interesting manuscript and the findings are intriguing. It adds to the growing number of non-apoptotic functions of apoptotic proteins and caspases in particular. The manuscript is well written and the data are usually convincingly presented.

Weaknesses:
1. One major concern I have is the observation by the authors in Figure 3C in which protein levels of Diap1 are still reduced in emc H99 double mutant clones. If Diap1 is still reduced in these clones, shouldn't caspases still be de-repressed? Given that emc H99 double mutants rescue all emc phenotypes examined, the observation that Diap1 levels are still reduced in emc H99 clones is inconsistent with the authors' model. The authors need to address this inconsistency.

2. Are Diap1 protein levels reduced in all emc clones, including clones anterior to the furrow? This is difficult to see in Figure 3B. it is also recommended to look in emc mosaic wing discs.

3. The authors speculate that Delta may be a direct target of caspase cleavage (Figure 9B), but then rule it out for a good reason. However, I assume that the increased protein levels of Delta in emc clones (Figure 7) are the results of increased transcription. In that case, shouldn't caspases control the transcriptional machinery leading to Delta expression?

4. How does caspase activity in emc clones cause reduced growth? Is this also mediated through Delta signaling?

5. Figure 1M: Is there a similar result with emc dronc mosaics?

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Reviewer #2 (Public Review):

Id proteins are thought to function by binding and antagonizing basic helix-loop-helix (bHLH) transcription factors but new findings demonstrate roles for emc including in tissues where no proneural (Drosophila bHLH) genes are known to function. The authors propose a new mechanism for developmental regulation that entails restraining new/novel non-apoptotic functions of apoptotic caspases.

Specifically, the data suggest that loss of emc leads to reduced expression of diap1 and increased apoptotic caspase activity, which does not induce apoptosis but elevates Delta expression to increase N activity and cause developmental defects. Indeed, many of the phenotypes of emc mutant clones can be rescued by a chromosomal deficiency that reduces caspase activation or by mutations in the initiator caspase Dronc. A related manuscript that shows that loss of emc results in increased da, linked previously to diap1 expression, provides supporting data. There is increasing appreciation that apoptotic caspases have non-apoptotic roles. This study adds to the emerging field and should be of interest to readers.

The data, for the most part, support the conclusions but I do have concerns about some of the data and the interpretations that should be addressed.

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Reviewer #3 (Public Review):

The work extends earlier studies on the Drosophila Id protein EMC to uncover a potential pathway that explains several tissue-scale developmental abnormalities in emc mutants. It also describes a non-apoptotic role for caspases in cell biology.

Strengths:
The work adds to an emerging new set of functions for caspases beyond their canonical roles as cell death mediators. This novelty is a major strength as well as its reliance on genetic-based in vivo study. The study will be of interest to those who are curious about caspases in general.

Weaknesses:
The manuscript relies on imaging experiments using genetic mosaic imaginal discs. It is for the most part a qualitative analysis, showing representative samples with a small number of mutant clones in each. Although the senior author has a long track record of using experiments like this to rigorously discover regulatory mechanisms in this system, it is straightforward in 2023 to use Fiji and other image analysis tools to measure fluorescence. Such measurements could be done for all replicate clones of a given genotype as well as genetic control sampling. These could be presented in plots that would not only provide quantitative and statistical measurements, but will be more reader-friendly to those who are not fly people.

Likewise, more details are needed to describe how clone areas were measured in Figure 1. Did they measure each clone and its twin spot, and then calculate the area ratio for each clone and its paired twin spot? This would be the correct way to analyze the data, yielding many independent measurements of the ratio. And doing so would obviate the need to log transform the data which is inexplicable unless they were averaging clones and twins within a disc and making replicates. More explanation is needed and if they indeed averaged, then they need to calculate the ratios pairwise for each clone and twin.

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