Regulation of *Drosophila melanogaster* pro-apoptotic gene *hid*

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Abstract

Key decisions one makes in a lifetime include whether and how often to reproduce, what role to play in the community and, under certain conditions, whether to live or die. Similar decisions are also made at the level of cells: whether to divide, what fate to assume in the multicellular context of metazoan development and, under certain conditions, whether to live or to die. The pro-apoptotic gene *hid* plays an important role in the execution of cell death in *Drosophila*. Here, we review the various levels of control that exist to regulate Hid according to the life-or-death choice of a cell.

Keywords

Drosophila; Apoptosis; *hid*

Drosophila Hid belongs to a family of four pro-apoptotic proteins, Hid (Head involution defective), Grim, Reaper and Sickle, which are collectively known as RHG proteins (Reviewed in [1–3]). RHG proteins act by binding to and neutralizing IAPs (Inhibitor of Apoptosis Proteins) and also by lowering the level of the latter. This results in caspase activation and apoptosis (Fig. 1). Binding to IAPs is mediated by an N-terminal IAP-binding motif (IBM). Outside the IMB domain of RHG proteins, limited sequence similarities have been noted within a "Grim Helix 3" motif of ~15 amino acids and a "Trp-block" of ~30 amino acids that includes the GH3 motif [1, 4–6]. The GH3 domain of Grim is required for its apoptotic function and can induce apoptosis when overexpressed in *Drosophila* cell culture [4]. The regions of Rpr and Hid that contain the GH3 domains are important for localization of these proteins to the mitochondria and to induce cell killing [7–9]. The ability to bind and antagonize IAPs to induce apoptosis is shared between *Drosophila* RHG proteins and their mammalian counterparts, Smac (Second Mitochondria-derived Activator of Caspases)/DIABLO and Omi/HtrA2 proteins. Ectopic expression of Drosophila RHG genes can induce apoptosis in *Drosophila* and in mammalian cell culture (reviewed in [1]).

The genes encoding the RHG proteins are linked at a single ~300 kb locus on chromosome III. Homozygotes of a chromosomal deletion H99 that removes *rpr*, *hid* and *grim* lack most cell death that occurs during normal *Drosophila* development and die as embryos [10]. While *rpr* and *grim* are expressed only in cells that are destined to die, *hid* mRNA is expressed in more cells than are destined to die (for example [11, 12]). Thus, in addition to transcriptional regulation, post-transcriptional repression of *hid* may play a role in keeping *hid*-expressing cells alive. This review focuses on published studies that illustrate different mechanisms by which *hid* is regulated.

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Developmental regulation of *hid*

Hid expression, sub-cellular localization and activity are regulated during embryogenesis, larval development and metamorphosis. In these contexts, Hid is regulated by EGFR/RAS signaling, Hippo tumor suppressor pathway, microRNAs and the hormone Ecdysone.

Cell–cell interaction in the regulation of hid transcript levels

During *Drosophila* development, receptor tyrosine kinase (RTK) signaling through EGFR represses *hid* to allow the survival of pupal retinal cells, larval eye imaginal disc cells and embryonic midline glia [13–17]. In each case, short range cell–cell signaling via EGFR allows the survival of immediate neighbors while more distant cells are culled by apoptosis, thus allowing for precise regulation of cell number. Genetic analysis places EGFR acting upstream of *hid* to prevent apoptosis. Studies during embryogenesis and eye development point to possible mechanisms by which EGFR inhibits *hid* [17–19] (Fig. 1a, b). EGFR signaling results in the activation of RAS, which can signal through a number of different effectors. Of these, genetic evidence implicates RAF/MAPK and PI3 Kinase/Akt, but not Rel, as downstream effectors of RAS in inhibition of Hid [17–19]. RAF/MAPK appears to inhibit *hid* at both transcript and post-translational levels. Gain of function mutations in RAS or RAF and overexpression of Pointed, a transcription factor targeted by RAS/MAPK, result in reduced *hid* mRNA levels in the embryo [18]. It is not known how PI3 Kinase/Akt repress *hid* during eye development, but there is a precedent for a transcriptional repression mechanism in the case of UV irradiation where RAS represses *hid* transcription by acting through PI3-Kinase and Akt to inhibit Foxo [20] ([c] in Fig. 1).

A recent study implicates the Hpo tumor suppressor pathway in developmental programmed cell death (PCD) in the eye ([d] in Fig. 1). PCD during eye development specifically removes inter-ommatidial cells of the eye disc that are initially produced in excess via mitotic proliferation and later culled during the pupal stages. These are the same cells that rely on EGFR signaling to survive. In mutants in the Hpo pathway, inter-ommatidial cells show a reduction in *hid* transcript levels and fail to undergo PCD. Thus Hpo normally acts to promote *hid* expression [21], which is then opposed by EGF signaling. How Hpo promotes *hid* expression remains to be determined, but *bantam* miRNA may provide a link. Hpo signaling is known to repress *ban* [22, 23], while *ban* is known to inhibit Hid expression as discussed below.

hid transcripts are also induced upon cell competition during organ formation. Cell competition occurs when cells of different growth rates (due to difference in ribosomal protein gene dosage) or cells expressing different levels of the proto-oncogene homolog MYC are juxtaposed. In the wing imaginal disc, the faster growing or higher MYC expressing 'winners' cause cell death in the 'losers' by inducing *hid* transcripts [24]. Cell competition requires p53 and the initiator caspase Dronc, but exactly how these activities result in *hid* transcript accumulation in loser cells remains to be determined.

RBF1 and E2F limit hid expression in a context-dependent manner ([e] in Fig. 1)

The *Drosophila* genome encodes two homologs of the transcription factor E2F, E2F1 and E2F2 (reviewed in [25, 26]). Each E2F protein must form a complex with a Dp co-factor to bind DNA with high affinity. There is a single Dp homolog in *Drosophila*. Genetic analysis indicates that E2F represses *hid* transcription [27, 28]. Mutations in Dp, which are expected to disable both E2F1 and E2F2, result in elevated *hid* mRNA. This phenotype is restricted to the narrow band of 'Zone of Non-Proliferating Cells' at the dorsal/ventral boundary of wing imaginal discs. In *Drosophila* S2 cells, E2F1 binds to the *hid* enhancer and represses *hid* expression while E2F2 has a relatively minor effect. The *hid* enhancer contains E2F

consensus sequences that are occupied by E2F1 and, to a lesser extent, E2F2 in Chromatin IP experiments in eye-antennal discs [29]. These results suggest that E2F1 in complex with Dp, rather than E2F2, represses *hid* in the ZNC. The relationship between *hid* and E2F/Dp may be different, however, outside the ZNC; *hid* expression appears normal outside the ZNC in the wing disc and in extracts of eye discs in Dp mutants.

RB proteins bind to E2F/Dp complexes and repress transcription. Of the two *Drosophila* RB-like proteins, RBF1 associates with E2F1 and RBF2 associates with E2F2 [30, 31]. Genetic evidence indicates that RBF1 cooperates with E2F to repress *hid.* RBF1 mutants show elevated *hid* mRNA in extracts of whole eye discs or in mutant clones [29]. A *hid* mutant was isolated in a forward genetic screen for suppressors of apoptosis in RBF1 mutants. A transcriptional reporter linked to the *hid* enhancer shows elevated expression in *rbf1* mutants throughout eye and wing imaginal discs. These findings support a model wherein RBF1, via E2F binding sites in the *hid* enhancer, limits *hid* expression in eye and wing discs. This model remains to be reconciled with the above-described findings that Dp mutants express *hid* normally in the eye disc and in the wing disc outside the ZNC [27, 28]. Nonetheless, the emerging view from these studies is that where E2F1/Dp or RBF1 are found to have an effect on *hid* expression, the effect is inhibitory. The role of the second *Drosophila* RBF homolog, RBF2, in *hid* expression has not been addressed.

Regulation of Hid protein expression

The 3′UTR of *hid* is over 2 kilo-bases long and mediates the repression of Hid protein expression by at least two different mechanisms. In embryos from *nanos* mutant mothers, more primordial germ cells (called "pole cells") accumulate Hid protein and undergo apoptosis in a *hid*-dependent manner. *nos* encodes an RNA binding protein. The 3′UTR of *hid* includes a Nos response element (NRE). Deletion of the NRE allows Hid protein accumulation and apoptosis in the pole cells of embryos from wild type mothers. These data suggest a model where Nos normally binds to *hid* 3′UTR to repress Hid translation, thereby preventing apoptosis in the future germline [32] ([f] in Fig. 1).

In addition to Nos, several miRNAs have been implicated in limiting Hid expression via sequences in the 3′UTR ([g, h] in Fig. 1). Embryos in which miR-6 and miR-2/13 have been neutralized with antisense oligo injection display widespread apoptosis, in part due to the elevated levels of Hid protein. Increased expression after miR-6 depletion in embryos is also seen for a GFP reporter with *hid* 3′UTR [33]. Similar neutralization of *bantam* miRNA in embryos results in elevated apoptosis in the embryo and smaller imaginal discs in the larvae [33]. These results are consistent with the phenotype of *ban* mutants and the effects of *ban* overexpression, which indicate that *ban* promotes cell proliferation while inhibiting apoptosis [34, 35]. A *ban* target that is relevant for apoptosis is Hid. Ectopic *ban* also represses apoptosis caused by overexpression of Hid or a Hid mutant refractory to phosphorylation and inhibition by MAPK, without altering *hid* transcript levels; thus repression of *hid* by *ban* is post-transcriptional, at least under overexpression conditions [35]. The *hid* 3′UTR contains five putative *ban* target sites. Ectopic *ban* represses a GFP reporter in which GFP coding sequences are followed by the *hid* 3′UTR. Furthermore, this repression is dependent on the presence of *ban* target sites in the *hid* 3′UTR.

ban has also been found to repress *hid* in two other contexts: after exposure to ionizing radiation (IR) and in mutants in the *Drosophila* Rb homolog, RBF1. We discuss RBF1 mutants here and regulation after IR exposure in a later section. In the eye imaginal discs of RBF1 mutants, *hid* mRNA is elevated throughout the disc, but Hid protein and apoptosis are elevated only near the Morphogenetic Furrow (MF), a narrow column of cells in which photo-receptor differentiation commences [29]. Simultaneously mutating *ban* allows

apoptosis to occur in RBF1 mutant clones that are far from the MF. Thus *ban* may limit Hid protein accumulation in eye imaginal cells.

The primary mechanism of action of miRNAs in animal cells was initially thought to be translational repression of the target mRNAs, but this view is being modified as the targets are also found to become destabilized [36]. In the regulation of *hid* by miRNAs, relative contribution of translational repression and mRNA destabilization remains to be investigated.

Regulation of Hid expression in the embryo may also occur at the level of the translational machinery itself [37]. During apoptosis and upon heat shock, cap-dependent translation is diminished. However, *hid* mRNA associates with polysome fractions after heat shock, suggesting that *hid* can be translated in a cap-independent manner in response to cellular stress. The *hid* 5′UTR contains an internal ribosome entry site (IRES). Mutants in the cap-binding protein eIF4E have increased apoptosis. These data suggest a model wherein *hid* translation is repressed via the mRNA cap, but the repression is bypassed in response to stress by cap-independent translation of *hid*.

Sub-cellular localization of Hid

Once expressed, Hid protein may be limited in its activity by phosphorylation and the requirement for mitochondrial localization. Hid contains at least two functional domains; an N-terminal domain for binding to IAPs and a C-terminal mitochondrial localization domain. Hid co-localizes with mitochondrial markers when expressed in human cells [38]. In *Drosophila* eye imaginal discs, Hid protein levels are elevated in RBF1 mutants as discussed above [29]. Elevated Hid is peri-nuclear, suggestive of mitochondrial localization. Elevated Hid is diffused throughout the cytoplasm if cells are double mutants for RBF1 and the initiator caspase, Dronc. This suggests that caspase activity is required for mitochondrial localization of Hid protein. The mechanistic basis for this requirement remains to be understood.

The requirement for Dronc in mitochondrial localization of Hid can explain the finding that caspase activity is needed for mitochondrial fragmentation that results from Hid overexpression in S2 cells. In S2 cells, Hid co-localizes with Cytochrome C and, when overexpressed, induces mitochondrial fragmentation and Cytochrome C release [9]. Caspase activity, although insufficient on its own, is necessary for mitochondrial fragmentation or Cytochrome C release. These results agree with the requirement for Dronc in Hid localization in the eye disc, suggesting that caspase activity is needed for mitochondrial localization of Hid in both S2 cells and larval imaginal discs. Deletion of 20 amino acids from the C-terminus, which includes a GH3 motif [6], compromised Hid's ability to co-localize with Cytochrome C, to disrupt the mitochondria and to induce caspase activation.

Taken together, these results suggest that caspase activity is necessary to localize Hid to the mitochondria and that mitochondrial localization of Hid allows efficient mitochondrial fragmentation and caspase activation.

Phosphorylation by MAPK ([b] in Fig. 1)

Genetic analysis identified EGFR signaling through RAS as an inhibitor of Hid function. We discussed in preceding paragraphs how RAS might act through MAPK and PI3K-Akt axis to suppress Hid transcript levels. Additional data indicate that RAS suppresses Hid by MAPK-dependent phosphorylation in embryos and during eye development. Hid contains 5 MAPK consensus sequences. Mutating either three or all five of these sites, to generate '3A' or '5A' mutants, respectively, renders Hid more efficient at killing S2 cells and refractory to rescue by constitutively active RAS or MAPK [19]. Constitutively active RAS or MAPK are

also less able to rescue the small eye phenotype produced by ectopic expression of Hid-3A and 5A mutants than by wild type Hid. Repression of Hid by phosphorylation also operates in the midline glia of the embryo where MAPK is required for cell survival in response to survival signals from the neighboring axon [17]. MAPK becomes dispensable for midline glia cell survival in *hid* mutants, suggesting that the key requirement for MAPK is to repress *hid*. Ectopic expression of the Hid-5A mutant in the wild type background phenocopied MAPK mutants and resulted in the ablation of midline glial cells. Hid-5A mutant is expected to be refractory to phosphorylation by MAPK. Therefore, the role of MAPK in glial cell survival is likely to be through phosphorylation and inhibition of Hid.

Hid expression in response to ecdysone (^i in Fig. 1)

Metamorphosis accompanies extensive death of polyploid larval cells such as those in the salivary gland. *hid* transcripts increase in dying cells in response to the steroid hormone Ecdysone that acts through its receptor, EcR. EcR can bind to and directly activate the transcription of several loci. EcR's ability to induce *hid* transcription, however, is likely to be indirect because it requires new protein synthesis; *hid* induction by Ecdysone is abolished upon treatment with a protein synthesis inhibitor, cycloheximide [39].

In sum, during *Drosophila* development, tumor suppressor homolog Hpo induces the expression of *hid* to cull extra cells in the final stages of eye development while Ecdysone induces *hid* to kill larval cells during metamorphosis. In addition, cap-independent translation may induce Hid expression in response to heat stress. On the other hand, *hid* is antagonized by pro-survival signaling through EGFR/RAS, by miRNAs and by E2F1/RBF1. What is still missing from this picture is the mechanistic understanding of how *hid* transcription is regulated during normal embryo and larval development. As the name implies, *head involution defective* is required for proper sculpting of the embryonic head through PCD. During embryogenesis, *hid* transcripts are found in a dynamic and complex pattern that overlaps significantly but not entirely with regions of cell death [11]. We have yet to fully understand how transcription of *hid* is regulated to produce this pattern during embryogenesis.

Regulation of *hid* in response to DNA damage

Heterozygotes of a chromosomal deficiency, H99, that removes *rpr, hid* and *grim* are viable and are able to undergo developmental cell death. H99 heterozygotes, however, are unable to induce apoptosis efficiently in response to external stimuli such as IR. Thus, the ability to induce apoptosis beyond the normal developmental level is sensitive to the dosage of pro-apoptotic genes [40]. More specifically, damaged-induced apoptosis appears sensitive to *hid* gene dosage. Heterozygotes of strong loss-of-function alleles of *hid* are also viable but are unable to induce apoptosis in response to external stimuli such as IR, suggesting that the level of *hid* gene products determines whether a cell lives or dies in response to damage [40]. In contrast, mutants with reduced *rpr* can still induce apoptosis in response to IR [28]. The importance of *skl* and *grim* in damage-induced apoptosis remains to be addressed. We discuss here mechanisms that are known to regulate *hid* expression or activity after exposure to radiation.

Changes in hid expression after radiation exposure

hid transcript levels increase after exposure to IR or UV, with the maximal increase ranging from 1.7- to 4-fold [28, 40, 41]. The increase in *hid* transcripts after IR exposure in embryos and larvae requires p53 and its presumptive activator, Chk2 kinase (^j in Fig. 1). Despite the requirement for p53 in inducing *hid*, p53 has not been shown to directly activate

transcription from the *hid* promoter. In contrast, p53 has been shown to bind consensus sequences in the *rpr* promoter and activate transcription after IR exposure [42].

The ability of cells to induce *rpr* and *hid* transcripts in response to IR is also subject to epigenetic regulation during embryogenesis. An IRER (Irradiation-Responsive Enhancer Region) resides upstream of the *rpr* gene and ~250 kb away from the *hid* gene [43]. Deletions that removed the IRER also abolished the IR-induced induction of *hid* and *rpr* transcripts. Interestingly, *grim*, which lies between *hid* and *rpr,* is not even induced by IR, suggesting that higher-order chromosome arrangements may allow the IRER to regulate *hid*. The requirement for IRER in the induction of *skl* by IR remains to be investigated.

As embryogenesis progresses beyond stage 12, the IRER acquires a more 'closed' chromatin state. Concomitantly, the induction of *rpr* and *hid* expression or apoptosis in response to IR becomes less robust [43]. Histone deacetylase 3 (HDAC3) and several other chromatin remodelers are required for the onset of changes in chromatin structure and accompanying reduction in IR-responsiveness of *hid* and *rpr* transcript levels ([k] in Fig. 1). This mode of epigenetic regulation may not be limited to embryos; mutants in HDAC3, but not HDAC1, show elevated *hid* mRNA expression and elevated spontaneous apoptosis in larval imaginal discs [44].

Expression of *rpr* and *hid* may also be linked in other ways. Overexpression of *rpr* in wing imaginal discs results in apoptosis but cells can be prevented from dying by co-expression of the viral caspase inhibitor, p35. *hid* transcripts are induced in such 'undead' cells but the mechanism remains to be investigated [45].

Drosophila imaginal discs also undergo p53-independent apoptosis after exposure to IR [41]. In this case, *hid* is induced 18 h after IR exposure in p53 null mutants, as opposed to 2–4 h after exposure to similar IR doses in wild type. What mediates *hid* induction in response to IR in p53 mutants remains to be investigated.

hid transcripts also increase in response to UV exposure, but the mechanism appears to be different for this radiation type ([c] in Fig. 1). Although p53 plays a critical role in inducing *hid* mRNA and plays a pro-death role after IR exposure, it has a pro-repair and thus a protective role after UV-C exposure [46]. Instead, it is through the actions of Jun N-terminal Kinase (JNK) signaling and transcription factors, Foxo and AP-1 (*Drosophila* Fos), that *hid* transcript levels increase in UV-irradiated pupal retina [20]. JNK mutants fail to increase *hid* mRNA levels and to undergo apoptosis in pupal retina after UV irradiation. Ectopic expression of nuclear Foxo or an active form of JNK in eye and wing imaginal discs induces *hid* mRNA. Induction of *hid* by active JNK is reduced upon reduction of *foxo* gene dosage. The pro-apoptotic action of Foxo is opposed by RTKs (EGFR and IGFR) acting through Akt ([a] in Fig. 1). RTK signaling also provides a pro-survival function by repressing *hid* during normal eye development as discussed in a preceding paragraph.

Regulators of hid under normal conditions are important for radiation-induced apoptosis

Two factors known to regulate *hid* under normal growth conditions also alter the ability of cells to undergo apoptosis. First, mutants in Dp show elevated *hid* transcripts in the ZNC region of wing imaginal discs as described in a preceding paragraph. The ZNC of mutants in Dp or its partner E2F1 undergoes apoptosis more readily after IR exposure [27]. Second, *ban* miRNA represses Hid protein accumulation under normal growth conditions. *ban* mutant larvae undergo more IR-induced apoptosis and are more sensitive to killing by IR. IR sensitivity of *ban* mutants is rescued by a reduction in the *hid* gene dosage, suggesting that *hid* is an important target of *ban* in radiation responses [47]. These results are consistent with the finding that the reduction of *hid* gene dosage by half can reduce IR-induced

apoptosis. In other words, altering the level of *hid* gene products under normal growth conditions can alter the ability to cells to undergo apoptosis in response to DNA damage.

Transcription factor association at the *hid* locus

It is important to note that changes in *hid* transcript level could result from changes in transcriptional regulation at the *hid* promoter, changes in *hid* mRNA turnover or both. Many studies described here examined the level of *hid* transcripts and did not differentiate between transcriptional changes and altered mRNA stability. Association of a protein with the *hid* locus would be one indication that regulation occurs through a direct effect on *hid* transcription. We list below known instances of transcription factor association at the *hid* locus.

E2F, Foxo and Fos have been shown to bind to the *hid* locus. Three E2F consensus sites have been reported, at −1.4 kb, −165 bp and +2.2 kb relative to *hid* transcription [27, 29]. In ChIP assays, the −1.4 kb site associates with E2F1 in S2 cells and with E2F1 and, to a lesser extent, E2F2 in eye-antennal discs. In reporter assays in S2 cells and in eye and wing imaginal discs, this site appears to mediate repression of *hid* transcription. The −165 bp site also associates with E2F1 in S2 cells in ChIP assays, but to a lesser extent than does the −1.4 kb site. The +2.2 kb site is not found to associate with E2F1 or E2F2 [29]. The functional significance of −165 bp or +2.2 kb sites remains to be investigated.

The first intron of *hid* contains several Foxo and AP-1 consensus sites that are occupied by the respective transcription factors in ChIP assays of S2 cells [20]. Therefore, regulation of *hid* by these proteins in UV-irradiated retina may be via direct transcriptional regulation.

Conclusions

There are as many ways to activate Hid as there are to repress it. Developmental signals, hormonal changes during metamorphosis and cell–cell competition can increase *hid* transcripts. Exposure to genotoxins causes further induction of *hid* transcripts via p53 or JNK. Pro-apoptotic activities that promote *hid* transcript accumulation are counterbalanced by pro-survival activities that repress *hid* not only at the transcriptional level but also at post-transcriptional and post-translational levels. These include E2F1/RBF1 under normal growth conditions, epigenetic modification of the enhancer, RAS/MAPK and PI3 K/Akt pathways in response to RTK signaling, and miRNAs. The balance between pro-apoptotic and pro-survival activities operating within a cell could produce a read-out in terms of Hid activity. The accumulation of Hid activity above a certain level would result in cell death. Interconnectivity between *hid*-activating and *hid*-inhibiting activities could further provide feedback loops to control apoptosis. For example, in larval wing discs, IR exposure results in p53-dependent *hid* transcript accumulation as well as p53-dependent *ban* activation [47]. *ban* represses *hid* post-transcriptionally, thus negatively feeding back on p53-dependent activation and limiting apoptosis in the disc. It is through understanding mechanisms that regulate Hid in the context of each other that we may understand how cells reach life-or-death decisions.

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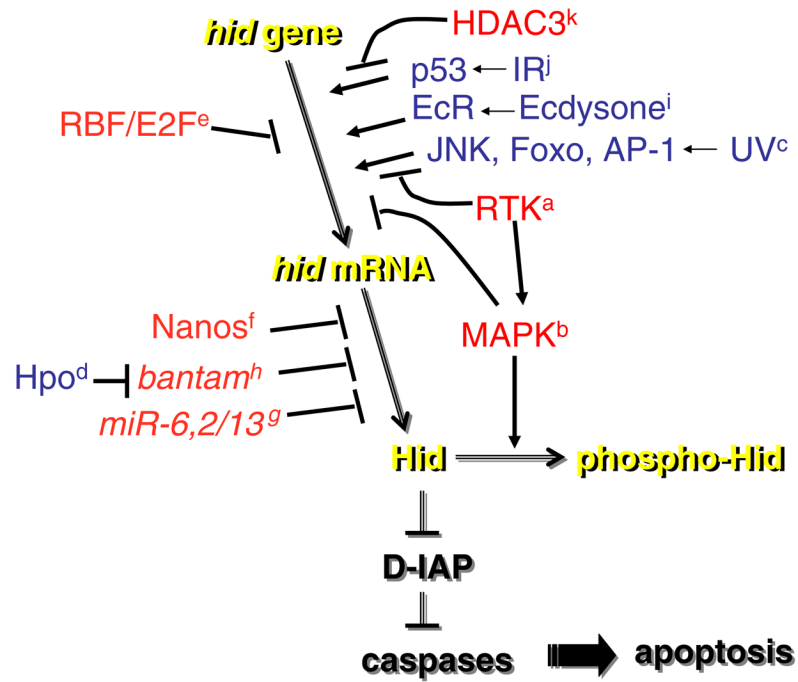
Fig. 1.

A summary of mechanisms that regulate *hid*. Hid neutralizes *Drosophila* IAP to cause caspase activation and apoptosis. Multiple mechanisms regulate *hid* expression. We have not distinguished between direct and indirect action of activators (*blue*) or repressors (*red*) in the diagram. Regulation of mitochondrial localization and regulation of Hid where the mechanism is not understood are not depicted. See text for details and reference to the *superscript* on each mechanism. (Color figure online)