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Genetic characterization of two gain-of-function alleles of the effector caspase DrICE in *Drosophila*

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Caspases are the executioners of apoptosis. Although much is known about their physiological roles and structures, detailed analyses of missense mutations of caspases are lacking. As mutations within caspases are identified in various human diseases, the study of caspase mutants will help to elucidate how caspases interact with other components of the apoptosis pathway and how they may contribute to disease. DrICE is the major effector caspase in *Drosophila* required for developmental and stress-induced cell death. Here, we report the isolation and characterization of six *de novo drICE* mutants, all of which carry point mutations affecting amino acids conserved among caspases in various species. These six mutants behave as recessive loss-of-function mutants in a homozygous condition. Surprisingly, however, two of the newly isolated *drICE* alleles are gain-of-function mutants in a heterozygous condition, although they are loss-of-function mutants homozygously. Interestingly, they only behave as gain-of-function mutants in the presence of an apoptotic signal. These two alleles carry missense mutations affecting conserved amino acids in close proximity to the catalytic cysteine residue. This is the first time that viable gain-of-function alleles of caspases are described in any intact organism and provides a significant exception to the expectation that mutations of conserved amino acids always abolish the pro-apoptotic activity of caspases. We discuss models about how these mutations cause the gain-of-function character of these alleles.
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Apoptosis is a major form of programmed cell death.[1] The core apoptotic machinery is evolutionary conserved with caspases as the fundamental components.[1–3] Caspases are specific cysteine proteases that are produced as inactive zymogens composed of an N-terminal pro-domain, a large subunit region with the catalytic cysteine residue, and a small subunit region at the carboxyl end.[2,4] Depending on their structures and functions, caspases are grouped into initiator and effector caspases.[2,3] Initiator caspases possess long pro-domains, which facilitate the recruitment of initiator caspases into cell death signaling complexes for activation.[2,3,5] Effector caspases are activated by initiator caspase complexes through proteolytic processing, cleaving off the pro-domain and separating the large and small subunits. The active effector caspase is a tetramer composed of two large and two small subunits and contains two catalytic sites.[2,3] Activated effector caspases cleave many protein targets to trigger the physiological and morphological changes characteristic of apoptosis. In mammals, apoptotic initiator caspases are Caspase-8, -9, -2 and -10, and effector caspases involved in apoptosis are Caspase-3, -7 and -6.[2,3] Of the seven *Drosophila* caspases, only the initiator caspase Dronc (Caspase-9-like) and the effector caspases DrICE and Dcp-1 (Caspase-3-like) have been implicated in apoptosis in imaginal discs.[1,2,6]

Caspase activation is tightly regulated in surviving cells. Inhibitor of apoptosis proteins (IAPs) directly bind to and inhibit processed caspases.[7–9] The best-characterized IAPs are mammalian XIAP and *Drosophila* IAP1 (DIAP1).[10,11] In cells committed to apoptosis, IAP-mediated inhibition of caspases is counteracted by IAP antagonists. Specifically, the IAP antagonists encoded by *reaper*, *hid* and *grim* (*RHG*)[12–15] in *Drosophila* trigger proteolytic degradation of DIAP1[16–20] which releases caspases from DIAP1 inhibition and triggers apoptosis. The overexpression of the *RHG* genes in the fly eye using the eye-specific *GMR* promoter causes an eye ablation phenotype due to massive apoptosis (see, for example, *GMR-hid* in Figure 1a).[12–14] In fact, mutants of *diap1*, *dronc* and *drICE* genes were isolated in genetic screens searching for modifiers of the eye ablation phenotypes caused by *reaper* or *hid* overexpression.[21–27] Mammalian IAP antagonists are Smac/Diablo and HtrA2/Omi, which function similarly to the RHG proteins.[28–31] Both IAPs and IAP antagonists are under tight control by various mechanisms to ensure proper regulation of caspase activity.[1,6,11,32]

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Abbreviations: ALPS, autoimmune lymphoproliferative syndrome; APF, after puparium formation; Ced-3, cell death defective 3; CyO, curly of Oster; Dcp-1, death caspase 1; DIAP1, death-associated inhibitor of apoptosis 1; DNA, desoxyribonucleic acid; drICE, death-related ICE (interleukin converting enzyme); dronc, death regulator Nedd2-like caspase; EMS, ethyl methanesulfonate; Ey, eyeless; Flp, flippase; FRT82B, flippase recombination target at 82B; GFP, green fluorescent protein; Gh, GMR-hid; Hid, head involution defective; GMR, glass multimer reporter; HtrA2, high-temperature-required protein A2; IAP, inhibitor of apoptosis protein; PCR, polymerase chain reaction; R8, photoreceptor 8; RHG, reaper, hid, grim; Smac/Diablo, second mitochondria-derived activator of caspases/direct IAP binding protein with low pI; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Ubi, ubiquitous; wt, wild-type; XIAP, X-linked inhibitor of apoptosis

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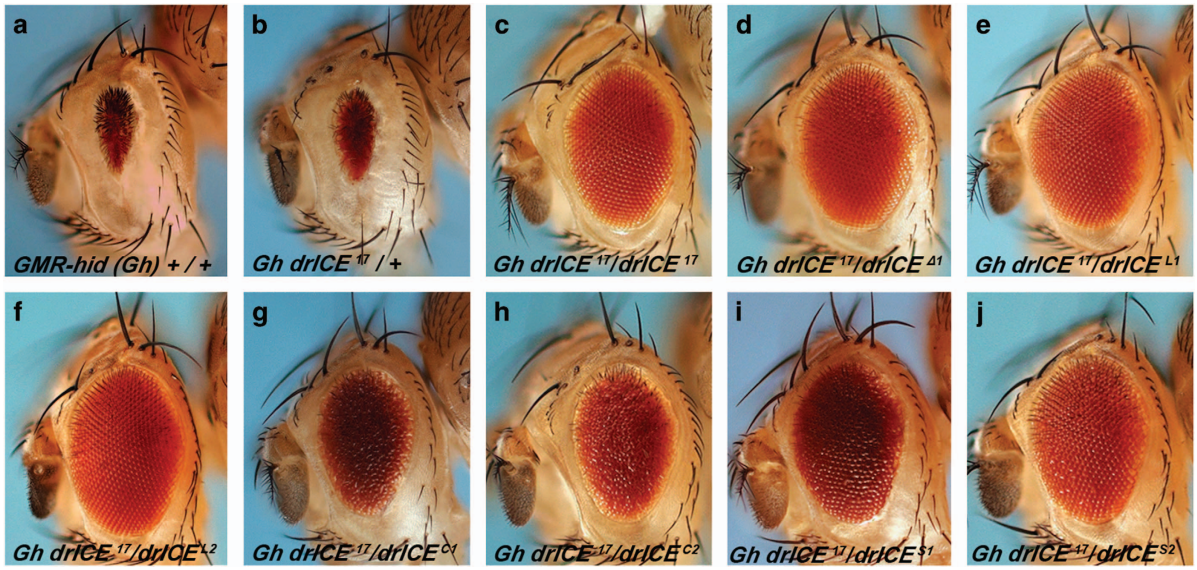

Figure 1 Recovery of new *drICE* alleles as recessive suppressors of *GMR-hid*. (**a**) *GMR-hid*-induced eye ablation phenotype. (**b**) Heterozygosity of $drICE^{17}$ does not dominantly modify (suppress) the *GMR-hid (Gh)* eye ablation phenotype. (**c**) $drICE^{17}$homozygously suppresses the *GMR-hid* (*Gh*) eye ablation phenotype. Genotype: *GMR-hid* $drICE^{17}$(*Gh* $drICE^{17}$)/$drICE^{17}$. (**d**) Loss-of-heterozygosity of $drICE^{17}$ by $drICE^{\Delta 1}$ suppresses *GMR-hid (Gh)*. Genotype: *GMR-hid* $drICE^{17}$/$drICE^{\Delta 1}$. (**e–j**) $drICE^{L1}$, $drICE^{L2}$, $drICE^{C1}$, $drICE^{C2}$, $drICE^{S1}$ and $drICE^{S2}$ suppress the *GMR-hid (Gh)* eye ablation phenotype in trans to $drICE^{17}$. Genotypes: *GMR-hid* $drICE^{17}$/*FRT82B* $drICE^{X}$. x denotes the *drICE* allele. See also Supplementary Figure S1

Given the pivotal roles of apoptosis in development and tissue homeostasis, it is not surprising that deregulation of caspases has been implicated in various pathological conditions, including neurodegeneration, autoimmune diseases and cancers.[33] Mutations in *Caspase-10* and *-8* are found in autoimmune lymphoproliferative syndrome (ALPS) and ALPS-related disorders.[34] Mutations and polymorphisms of *Caspase-8*, *-9*, *-3* and *-7* have been implicated in various cancers.[35,36] Although some of these mutations disrupt the apoptotic activity of the affected caspase in cell culture studies,[37–41] detailed understanding of how these mutations affect the function and expression of the caspases is scarce. It is thus of great biological and clinical significance to isolate more caspase mutants, especially the ones carrying point mutations, and to analyze their behaviors *in vivo*.

The *Drosophila* model system provides a great venue to answer such inquiries, given the conservation of caspase genes and the well-established genetic techniques to isolate and characterize mutations in genes of interest. DrICE and Dcp-1 are the effector caspase orthologs of mammalian Caspase-3 and Caspase-7 (ref. 42, 43; reviewed in ref. 2,6). Although Dcp-1 has a crucial role in nurse cell death during mid-oogenesis, DrICE is required for apoptosis in most developmental and irradiation-induced cell death.[25,44,45] Four *drICE* mutants have been reported so far, only one of which, $drICE^{17}$, is caused by a missense mutation.[25,44,46] $drICE^{\Delta 1}$, $drICE^{\Delta 1E4}$ and $drICE^{\Delta 2C8}$ were generated through imprecise P-element excisions, through which the entire *drICE* open-reading frame was removed to produce null alleles.[44,46] $drICE^{17}$ carries an N to Y mutation at residue 116 and was isolated in an EMS mutagenesis screen as a recessive suppressor of the small eye phenotype induced by *GMR-hid*. This missense mutation leads to decreased protein stability and behaves as a strong hypomorphic allele.[25] These alleles have improved our understanding of the essential roles of *drICE* in developmental, stress-induced and *RHG*-induced cell death;[25,44,46] however, they have not provided information on how various amino-acid substitutions affect the behavior of the caspase protein.

In this study, we describe the isolation and characterization of six new EMS-induced recessive loss-of-function alleles of *drICE*, all of which affect highly conserved amino-acid residues. Four of these alleles produce unstable DrICE proteins. However, the remaining two alleles behave genetically differently. Although they were isolated as loss-of-function alleles homozygously, they surprisingly display gain-of-function characteristics in the presence of a wild-type copy of *drICE*, that is, in a heterozygous condition. Interestingly, they only behave as gain-of-function mutants in the presence of an apoptotic signal. Molecularly, the mutations in these alleles change conserved residues in close proximity to the catalytic Cys residue. We discuss models about how these mutations cause the gain-of-function character of these alleles.

Results

Isolation of *de novo drICE* mutant alleles. Ectopic expression of *hid* under the control of the eye-specific *GMR* promoter causes an eye ablation phenotype owing to excessive cell death (Figure 1a).[14] This eye ablation phenotype has been used in EMS mutagenesis screens to isolate mutants of genes involved in apoptosis. To isolate additional *drICE* mutant alleles to broaden our understanding of DrICE function in cell death, we performed an allele screen. The existing *drICE* allele, $drICE^{17}$, does not suppress the *GMR-hid*-induced eye ablation phenotype in a dominant or heterozygous manner (Figure 1b). Only homozygously

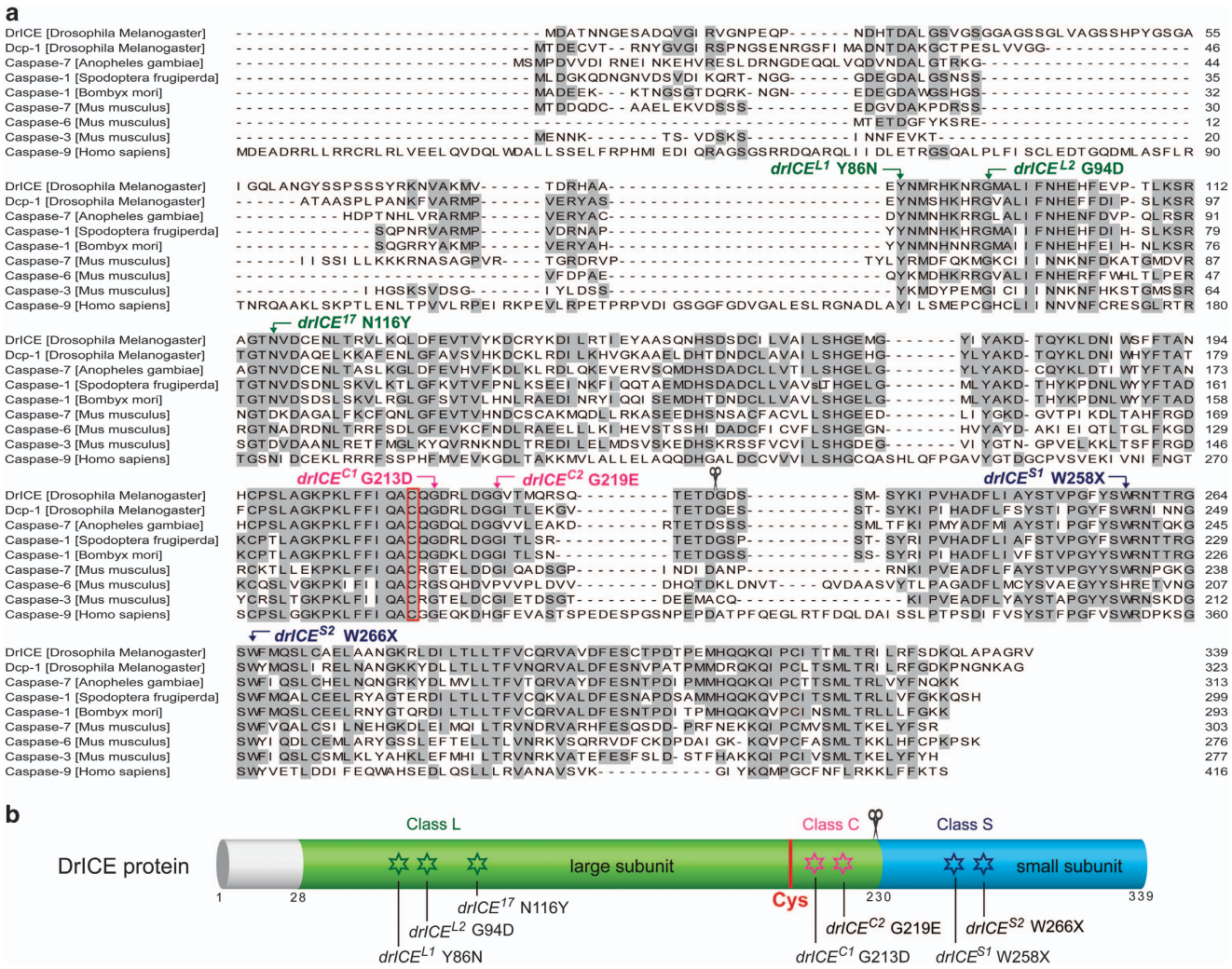

Figure 2 The new *drICE* alleles affect highly conserved amino acids. (**a**) Alignment of various effector caspases in insects, mammals and humans. $drICE^{L1}$ and $drICE^{L2}$ carry Y86N and G94D missense mutations, respectively. $drICE^{C1}$ and $drICE^{C2}$ mutants bear G213D and G219E missense mutations, respectively. $drICE^{S1}$ and $drICE^{S2}$ carry nonsense mutations at W258 and W266, respectively. As previously published, $drICE^{17}$changes N116 to Y.[25] (**b**) Schematic outline of DrICE protein and the relative locations of the mutations in *DrICE*. Class L alleles carry missense mutations affecting conserved amino acids in the large subunit, which includes $drICE^{L1}$, $drICE^{L2}$ and $drICE^{17}$. Class S alleles are $drICE^{S1}$ and $drICE^{S2}$, both of which carry nonsense mutations in the small subunit. The class C group is composed of $drICE^{C1}$ and $drICE^{C2}$, both of which carry missense mutation affecting amino acids in the vicinity of the catalytic cysteine residue

(Figure 1c), or in trans to a *drICE* deficiency ($drICE^{\Delta 1}$) (Figure 1d), can suppression of *GMR-hid* be recorded. Therefore, to isolate new alleles of *drICE*, we took advantage of the fact that loss-of-heterozygosity of $drICE^{17}$ causes strong suppression of *GMR-hid*. In an EMS mutagenesis screen, we screened 40 000 F1 progeny and recovered six suppressors, namely *L1, L2, C1, C2, S1* and *S2*, which suppressed *GMR-hid* only in trans to $drICE^{17}$ (Figure 1e–j) and are therefore good candidates for new *drICE* alleles. In addition, the six mutants are homozygously viable with wing clearance defects (data not shown) as observed in $drICE^{17}$ and $drICE^{\Delta 1}$ alleles.[25,44]

Molecular characterization of the novel *drICE* alleles. Through DNA sequencing, we identified in each of these six alleles point mutations affecting amino acids highly conserved among effector caspases in various species (Figure 2a). $drICE^{L1}$ and $drICE^{L2}$ contain missense mutations of amino acids located in the large subunit, changing Y86 to N and G94 to D, respectively. $drICE^{S1}$ and $drICE^{S2}$ bear nonsense mutations in the small subunit at positions 258 and 266, respectively, and produce carboxyl-end truncated *drICE* proteins. $drICE^{C1}$ and $drICE^{C2}$ carry missense mutations of amino acids located near the catalytic cysteine, C211, changing G213 to D and G219 to E, respectively. We refer to the alleles affecting the large subunit as class L alleles (*L1* and *L2*), except the ones located close to the catalytic cysteine residue, which we refer to as class C alleles (*C1* and *C2*). Mutants affecting the small subunit are class S alleles (*S1* and *S2*) (Figure 2b).

DrICE protein levels are markedly decreased in class L and class S mutants, but not in class C alleles. To assess how these novel *drICE* mutations affect DrICE function, we

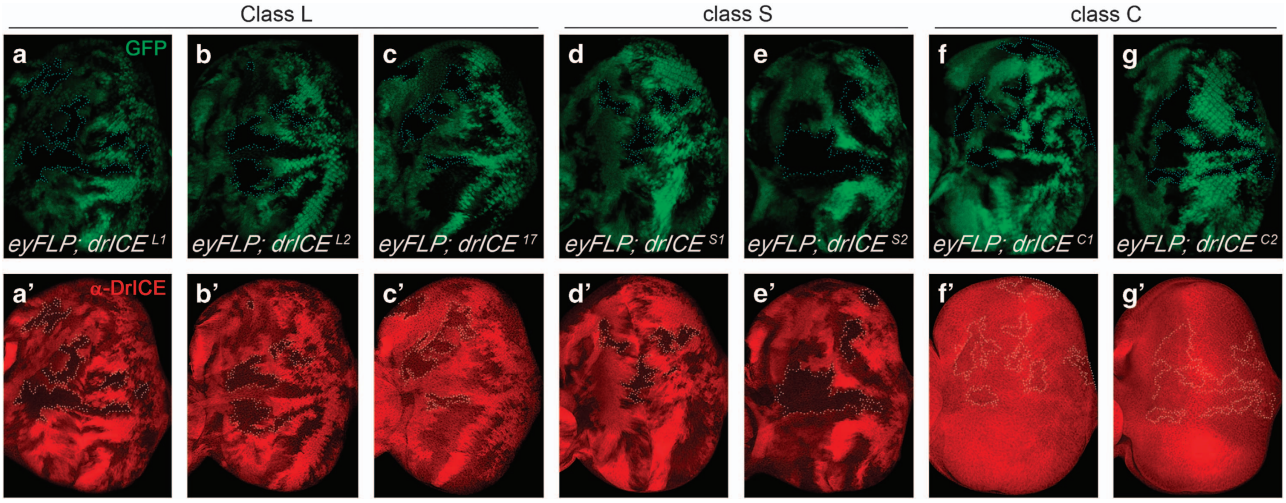

Figure 3 DrICE protein distribution in *drICE* mutant clones. Confocal scannings of immunofluorescence images of third instar larval mosaic eye imaginal discs are shown. (**a**–**g**) Mutant *drICE* clones for the indicated alleles are marked by the absence of GFP (green). (**a**'–**g**') anti-DrICE labeling is shown in red. (**a–e**) Class L and S alleles produce little or unstable DrICE protein. Please note that the anti-DrICE labeling in (**a**'–**e**') matches the outline of the GFP labeling in (**a**–**e**), which marks wild-type or heterozygous tissue. Genotypes: *ey-FLP; FRT82B drICE^{X} /FRT82B ubi-GFP* with x indicating the mutant *drICE* allele. (**f, f', g, g'**) DrICE protein levels are not changed or only slightly decreased in class C mutant clones. Genotypes: (**f**, **f'**) *ey-FLP; FRT82B drICE^{C1}/FRT82B ubi-GFP*; (**g**, **g'**) *ey-FLP; FRT82B drICE^{C2}/FRT82B ubi-GFP*

examined the stability of the mutant DrICE proteins. Mosaic eye imaginal discs of the *drICE* alleles from third instar larvae were labeled with an anti-DrICE polyclonal antibody.[47] Levels of DrICE protein decreased markedly in mutant clones of class L and class S alleles (Figure 3a'–e'), similar to *drICE^{17}*, which was previously shown to encode an unstable DrICE protein (ref. 25). These results suggest that class L and class S alleles produce little or unstable DrICE protein.

In contrast, class C alleles behave differently. Although mutant clones of *drICE^{C1}* contain slightly decreased protein levels, *drICE^{C2}* produces normal DrICE protein levels comparable to wild-type tissues (Figure 3f' and g'). To verify that the class C alleles indeed encode recessive loss-of-function alleles, we examined if they affect normal developmental cell death during eye development. At 28 h after puparium formation (APF), a wave of apoptosis removes all excess interommatidial cells.[48–50] This developmental cell death is lost in mutant clones of both class C alleles (Figure 4a' and b'), suggesting that class C alleles are recessive loss-of-function mutants. Therefore, because these two alleles produce normal DrICE protein levels, they affect DrICE activity independently of protein stability. Given that they have mutations in highly conserved residues close to the catalytic cysteine residue (Figure 2), it is likely that these mutants have reduced catalytic activity.

Class C alleles affect substrate binding. From a structural perspective, it is quite clear why these mutations (G213D and G219E) lead to inactivation of DrICE when they are homozygous. In caspases generally, the substrate-binding groove loops 2, 3 and 4 from one half of the tetramer interact with loop 2′ from the opposite half of the tetramer to form an ordered substrate-binding groove (Figure 5a). Loops 2 and 2′ form a lock that holds loops 3 and 4 into the proper conformation. If the loop 2/loop 2′ interaction is lost, loops 3 and 4 become disordered and the enzyme is unable to bind substrate (Figure 5c). Based on examination of the DrICE structure (3SIP),[51] residues G213 and G219 are both contained within loop 2 of DrICE. Neither of these positions can accommodate any residue larger than a glycine (Figure 5b) and the G213D and G219E mutations are predicted to inhibit critical interactions between loops 2 and 2'. G213 forms an exceptionally tight contact with the backbone of F256 on loop 3, the loop that forms the base of the substrate-binding groove (Figure 5b). Substitution of G213 with aspartate disrupts the contact with F256 and prevents the tight association of loops 2 and 2′, which supports substrate binding. G219, also on loop 2, interacts with the side chain of V241, which is contained on loop 2′ (Figure 5b). Substitution of G219 with any rotomers of glutamate results in steric clash with adjacent residues. This steric clash would be sufficient to prevent loop ordering and substrate binding (Figure 5c), explaining the loss of enzymatic activity of these two mutants in a homozygous condition.

Class C *drICE* alleles dominantly enhance *GMR-reaper*. The new *drICE* alleles were recovered as recessive suppressors of *GMR-hid* (Figure 1). They are also recessive suppressors of *GMR-reaper* (Supplementary Figure S1). Surprisingly, however, in the course of this analysis, we noted that the two class C alleles—when heterozygous in trans to a wild-type (*drICE^{+}*) allele—dominantly enhanced *GMR-reaper* (Figure 6b,c and d), whereas class L and class S alleles weakly suppress it (Figure 6a; Supplementary Figure S2). Class C alleles also appear to act as dominant enhancers of *GMR-hid* (Supplementary Figure S3).

One possibility by which class C alleles dominantly enhance *GMR-reaper* is through increased caspase activity.

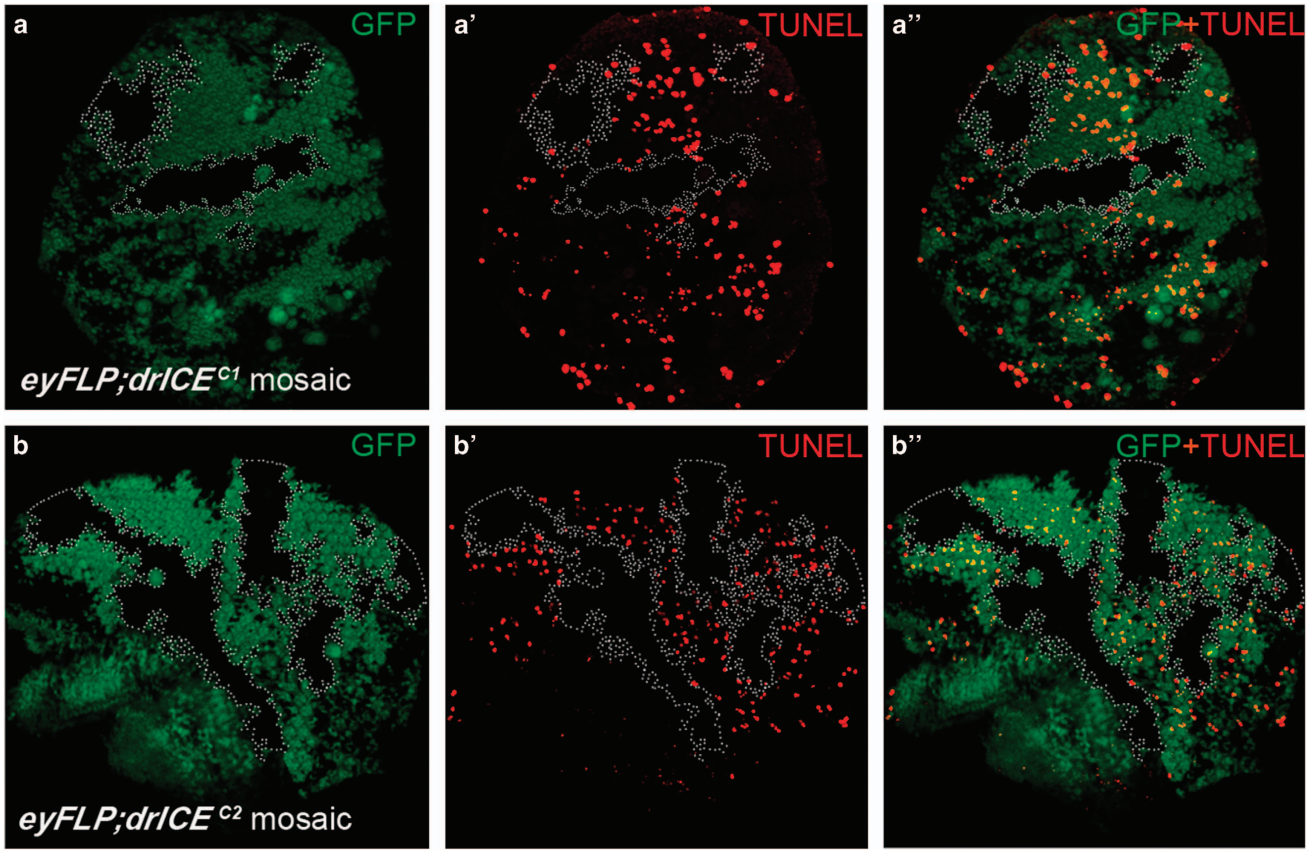

Figure 4 Developmental cell death in the pupal retina is absent in mutant clones of the class C alleles. *drICE*^{C1} (**a**) and *drICE*^{C2} (**b**) mosaic pupal eye discs 28 h after puparium formation (APF). *drICE* mutant clones are marked by absence of GFP (see dashed lines as examples). (**a',a'',b',b''**) TUNEL labeling indicates the absence of developmental cell death in C class *drICE* mutant clones

To examine this possibility, we performed fluorometric caspase assays with head extracts from *GMR-reaper* animals heterozygous for various *drICE* alleles. Consistently, although the loss-of-function alleles *drICE*^{L2} and *drICE*^{S1} have lost significant caspase activity, the class C allele *drICE*^{C1} displayed increased caspase activity compared with *GMR-reaper* alone (Figure 6e). Furthermore, more TUNEL-positive signals are observed in *GMR-reaper* eye imaginal discs heterozygous for class C alleles compared with *GMR-reaper* in wild-type or heterozygous class L background (Figure 6f–j). Therefore, the dominant enhancement of the eye ablation phenotype of *GMR-reaper* by class C alleles is indeed due to increased cell death.

To further characterize the new *drICE* alleles, we examined DIAP1 degradation triggered by reaper.[16–20] In *GMR-reaper* eye imaginal discs, DIAP1 degradation is very prominent in cells immediately posterior to the column of R8 photoreceptor neurons, which are very resistant to apoptosis[52] and where DIAP1 is not degraded (Figure 7a and b; R8 photoreceptor columns are marked by arrows; the zones of DIAP1 degradation by asterisks (*)). Interestingly, reduced DrICE activity in heterozygous loss-of-function alleles partially protects DIAP1 from reaper-induced degradation (Figure 7c and d), which is consistent with the suppression of *GMR-reaper* by these alleles (Figure 6a). In contrast, the gain-of-function alleles *drICE*^{C1} and *drICE*^{C2} fail to protect DIAP1 from reaper-induced degradation (asterisks in Figure 6e and f). These findings suggest that the complex interaction between reaper, DIAP1 and DrICE is affected by the class C alleles.

We also examined if the class C alleles behaved as gain-of-function alleles in the absence of an apoptotic signal such as *GMR-reaper*, and thus may cause inappropriate cell loss using the developing retina as a model. At 42 h APF, the retina forms a highly regular lattice with a constant number of cells[48–50] (Supplementary Figure S4). Inappropriate cell loss results in disruption of lattice symmetry and is easy to score. However, for both class C alleles, we did not detect any irregularity in the appearance of the lattice that may indicate inappropriate cell loss (Supplementary Figure S4). This analysis suggests that the class C mutations do not cause premature and inappropriate activation of DrICE in the absence of an apoptotic signal.

Taken together, the class C alleles exert a very complex genetic behavior. Homozygously mutant *drICE*^{C1} and *drICE*^{C2} are recessive loss-of-function alleles and produce mutant DrICE proteins with impaired substrate binding. However, in the presence of functional DrICE protein encoded from the *drICE*$^{+}$ allele, they trigger more cell death than normal and enhance the *GMR-reaper* eye ablation phenotype. Thus, in a heterozygous condition they behave as gain-of-function alleles.

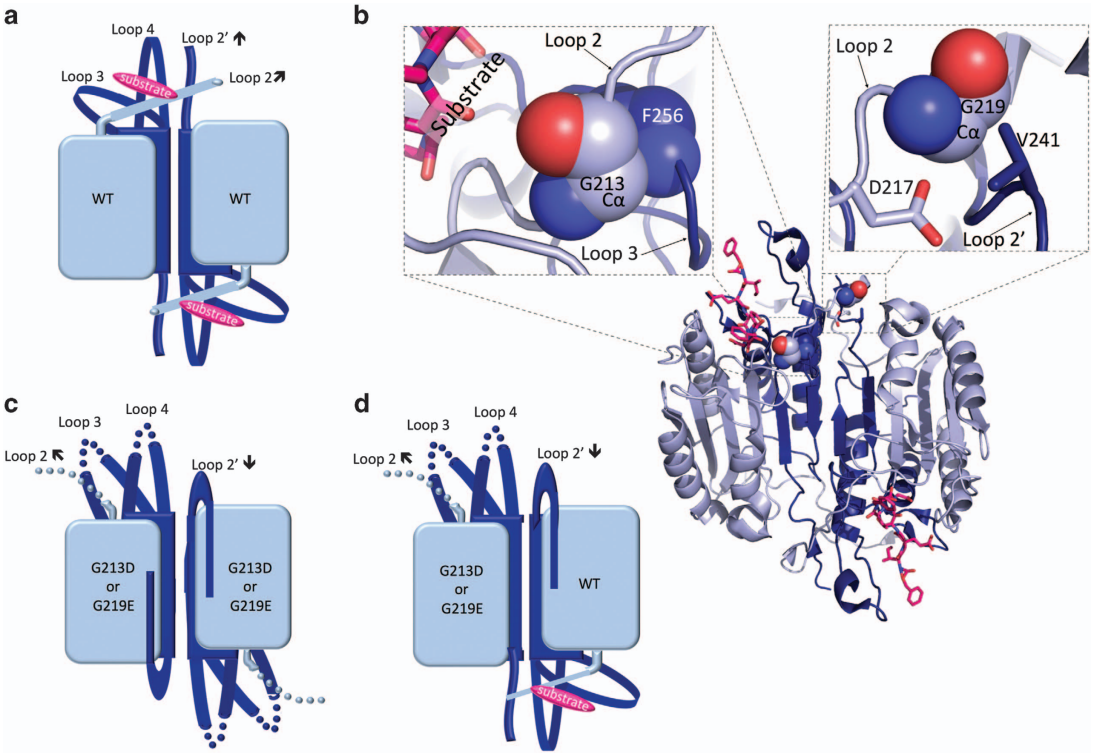

Figure 5 Loop position explains loss of activity in homozygous class C mutants. (**a**) Shown is a schematic drawing of a wild-type DrICE tetramer. The two large subunits of the tetramer are illustrated in light blue, the small subunits in dark blue behind the large subunits. The substrate-binding groove loops 2, 3 and 4 from one half of the tetramer and 2′ from the other half are indicated. The position of substrate (pink rods) is modeled based on superposition of DrICE with the highest available resolution caspase-3 structure (2CNN).[67,68] The substrate binds in the same region as the inhibitory region of the DIAP1 present in the DrICE (3SIP) structure.[51] (**b**) Based on examination of the DrICE structure (3SIP), residues G213 and G219 are both contained within loop 2 of DrICE. G213 forms an exceptionally tight contact with the backbone of F256 on loop 3, the loop that forms the base of the substrate-binding groove. G219 interacts with the side chain of V241, which is contained on loop 2'. At both positions, 213 and 219, any residue larger than glycine would sterically clash and lead to destabilization or distortion of the active form of the enzyme. The G213D and G219E mutations are predicted to prevent the tight association of loops 2 and 2', which supports substrate binding. (**c**) A homozygous class C DrICE tetramer. Steric clash of G213D and G219E with residues on the L2' loop prevents the necessary interactions between loops 2 and 2' and thus prevents the proper conformation of all four of the substrate-binding groove loops (compare with **a**). (**d**) Wild-type/class C DrICE heterotetramer contains one functional and one non-functional DrICE dimer (compare with **a**)

Discussion

In this study, we report the isolation and characterization of six new mutants of the *Drosophila* effector caspase *drICE*, the ortholog of mammalian caspase-3. According to the types and locations of the mutations in these six new *drICE* alleles, the expression levels of the mutant DrICE proteins and the genetic interactions with *GMR-hid* or *GMR-reaper,* we have grouped these mutants into three classes. Class L and S alleles carry point mutations in the large and small subunits, respectively (Figure 2b). In addition, according to this classification criterion, $drICE^{17}$(N116Y) also belongs to the class L group. Both class L and class S alleles affect the stability of their DrICE protein products and hence have reduced caspase activity. Overall, they are recessive loss-of-function *drICE* alleles.

The class C alleles $drICE^{C1}$ and $drICE^{C2}$ carry missense mutations in conserved amino acids (G213D and G219E) in close proximity of the catalytic cysteine (C211) residue. In contrast to class L and class S alleles, these mutations do not markedly impact DrICE protein stability. Most importantly, although these alleles act as loss-of-function alleles homozygously, in the presence of a wild-type $drICE^{+}$ allele (i.e., heterozygously), they can enhance cell death induced by *reaper* expression, suggesting that they can function as gain-of-function alleles. However, our analysis suggests that class C mutants only behave as gain-of-function alleles in an apoptotic background such as *GMR-reaper*, whereas they do not cause spontaneous activation of DrICE in the absence of apoptotic signals. Therefore, they can only act as gain-of-function mutants after proteolytic processing.

The underlying mechanisms of the apoptotic enhancement by heterozygous class C alleles are less clear. In the few cases where certain mutants behave as recessive loss-of-function and dominant gain-of-function alleles, dimerization is the underlying cause of this different genetic behavior. For example, certain mutations of the *Toll* receptor are recessive loss-of-function alleles, but are gain-of-function owing to physical interaction with the wild-type allele.[53,54] Given that two processed DrICE molecules form an enzymatically active tetramer with two catalytic sites,[2,3] one can speculate that class C mutant subunits exert a dominant effect via interaction with a wild-type DrICE molecule. Statistically, only 50% of the DrICE tetramers in the cell are composed of a wild-type and a class C subunit (referred to as wt/class C heterotetramer; Figure 5d). The remaining 50% are either wild-type (Figure 5a)

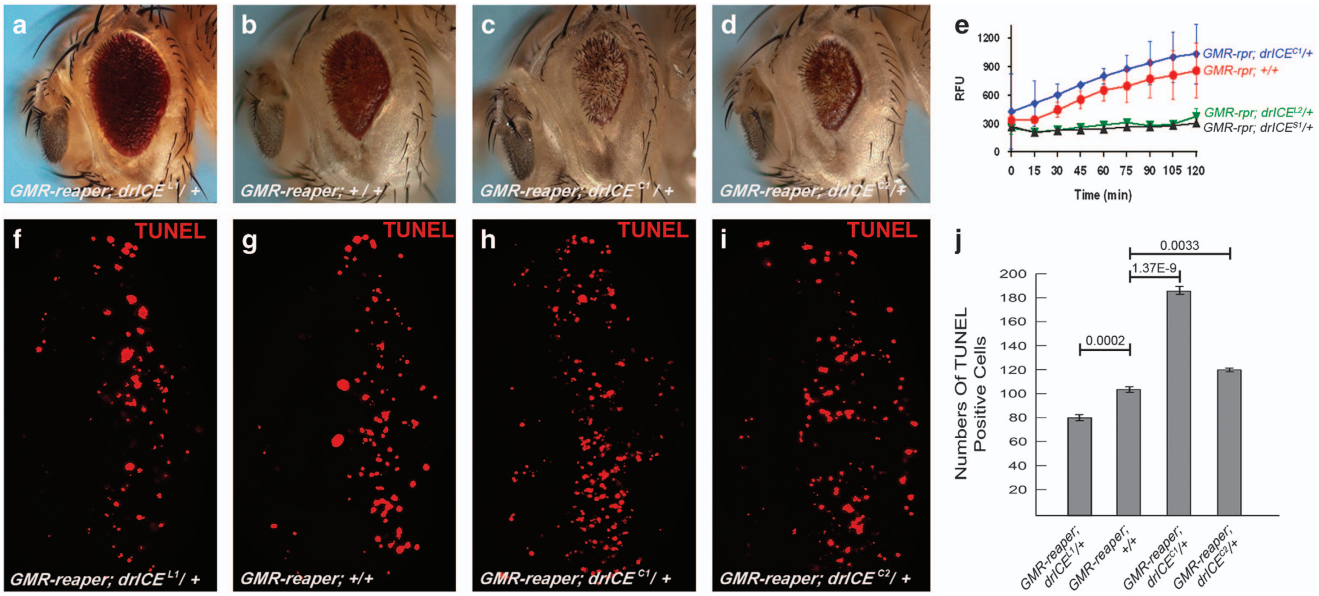

Figure 6 Class C alleles enhance the *GMR-reaper*-induced eye ablation phenotype. (**a**, **b**) The class L allele *drICE*^{L1} suppresses the *GMR-reaper*-induced eye ablation phenotype (**a**) compared with *GMR-reaper* alone. (**b**) More examples for the effect of class L and class S alleles on *GMR-reaper* are shown in Supplementary Figure S2. (**c, d**) The class C alleles *drICE*^{C1} and *drICE*^{C2} dominantly enhance the *GMR-reaper*-induced eye ablation phenotype. (**e**) Fluorometric caspases assays with head extracts from flies of the indicated genotype. Substrate (DEVD-AMC) cleavage activity of head extracts is presented as relative fluorescence units (RFU) with change over time. (**f–i**) TUNEL labelings of *GMR-reaper* eye discs in wild-type or heterozygous *drICE* mutant background. Although the class L allele *drICE*^{L1}heterozygously decreases the number of TUNEL-positive nuclei compared with *GMR-reaper* alone (**f**), class C alleles increase it (**g**, **h**). (**j**) Statistical summary of (**f–i**). The two-tailored student's *t*-test was used. *P*-values are indicated. Genotypes: (**a**, **e**) CyO,*2xGMR-reaper/+; FRT82B drICE*^{L1} / +. (**b**, **f**) CyO,*2xGMR-reaper/+; FRT82B/* +. (**c**, **g**) CyO,*2xGMR-reaper/+; FRT82B drICE*^{C1} / +. (**d**, **h**) CyO,*2xGMR-reaper/+; FRT82B drICE*^{C2}/+. See also Supplementary Figures S2 and S3

or class C tetramers (Figure 5c), the latter of which has no enzymatic activity. Thus, the wt/class C heterotetramers actually have more activity as the data imply because they also compensate for the loss of activity of the class C tetramers.

How can we explain the gain-of-function activity of the wt/class C heterotetramers? Because of the loss of loop organization and substrate binding in the class C subunit (Figure 5), it is unlikely that the presence of the wild-type subunit in the wt/class C heterotetramer 'rescues' the defect in the class C subunit. It is more likely that an upstream regulatory mechanism is deregulated by the wt/class C heterotetramer. There are several possibilities. For example, because IAPs require effector caspases for inhibitory activity,[55,56] it may be possible that a wt/class C heterotetramer does not produce as much active DIAP1 compared with a wild-type tetramer. Specifically, the N terminus of DIAP1 is an intramolecular inhibitor of DIAP1, keeping DIAP1 in an auto-inhibited conformation, unable to bind and inhibit DrICE.[51,57–59] After caspase cleavage at D20 (likely by DrICE), the inhibitory N terminus is removed and now activated DIAP1 can bind to DrICE and inhibit it. Because DIAP1 is a substrate of DrICE before it becomes an inhibitor, the wt/class C heterotetramer would produce less-active DIAP1 and thus would be less-efficiently inhibited.

Another possible model includes cooperativity of the binding of DIAP1 to the two active sites of the DrICE tetramer. Because the inhibitory region of DIAP1 (pink rod in Figure 5d) binds to the substrate-binding domain of DrICE present in the 3SIP structure,[51] DIAP1 does not bind to the mutant catalytic site of the class C subunit. If there is cooperativity of the binding of DIAP1 to the two catalytic sites of DrICE, the failure of DIAP1 to bind to the class C catalytic side may affect the binding of DIAP1 to the catalytic site of the wild-type subunit. Thus, the catalytic site of the wild-type subunit would be free from IAP inhibition, which should lead to increased activity only in the heterozygous condition.

Finally, we also take into account that class C alleles are only dominantly active in the presence of an apoptotic signal such as reaper (Figure 6,Supplementary Figure S4). Reaper and DrICE compete for binding to the BIR1 domain of DIAP1.[47] If reaper is in excess, it triggers degradation of DIAP1 (Figure 7b) and thus apoptosis.[16–20] Therefore, owing to the failure of DIAP1 to bind to the class C subunit, the wt/class C heterotetramer may shift the equilibrium towards binding of DIAP1 to reaper such that DIAP1 is more efficiently degraded by reaper, resulting in higher enzymatic activity of the wt/class C heterotetramer. Consistent with this notion, reduced DrICE activity in heterozygous loss-of-function alleles partially protects DIAP1 from reaper-induced degradation (Figure 7c and d), whereas the class C alleles of DrICE fail to protect DIAP1 from degradation (Figure 7e and f), suggestive of decreased binding of the wt/class C heterotetramer to DIAP1.

These models to explain the gain-of-function behaviors of the class C alleles are not mutually exclusive and other models may be possible, too.

To our knowledge, this is the first time that viable gain-of-function alleles of caspases are described in any intact organism and provides a significant exception to the

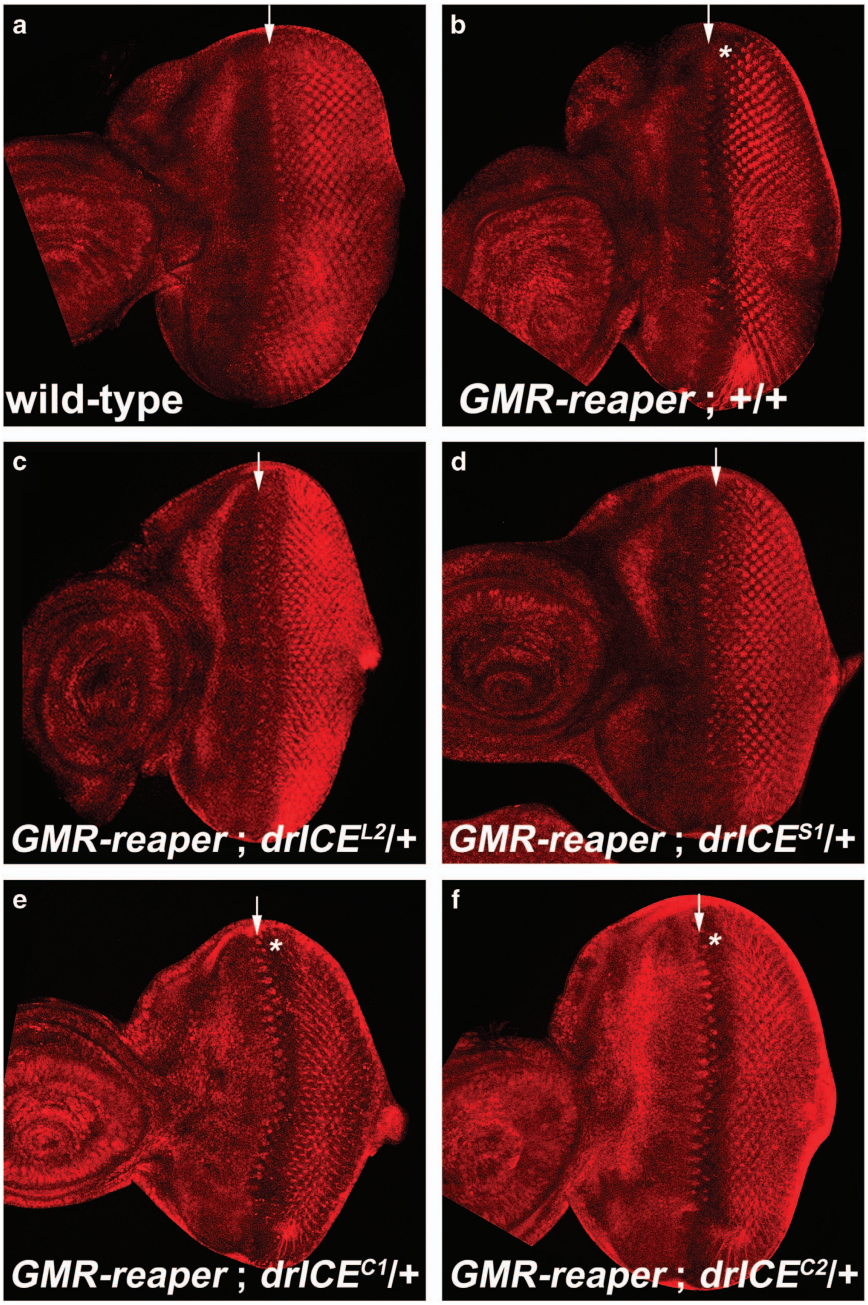

Figure 7 Reaper-induced DIAP1 degradation is differently influenced by *drICE* alleles. The white arrow indicates the column of R8 photoreceptor neurons. The zone of DIAP1 degradation is marked by the asterisk. Posterior is to the right. (**a**) Anti-DIAP1 labeling of a wild-type eye imaginal disc. (**b**) Anti-DIAP1 labeling of a *GMR-reaper* eye imaginal disc. Although DIAP1 protein persist in R8 photoreceptor neurons (white arrow), the cells immediately posterior to it strongly degrade DIAP1 (**c, d**). The loss-of-function alleles *drICE*^{L2} and *drICE*^{S1} partially protect DIAP1 from reaper-induced degradation. The zone of DIAP1 degradation is partially restored (**e, f**). The gain-of-function alleles *drICE*^{C1} and *drICE*^{C2} fail to protect DIAP1 from reaper-induced degradation

expectation that mutations of conserved amino acids always abolish the pro-apoptotic activity of caspases. Because the affected residues of the class C alleles are conserved in other caspases (Figure 2a), it would be of great interest to generate and test such mutations in other caspases to examine if it is a universal phenomenon. In *Caenorhabditis elegans*, a large collection of *ced-3* mutants has been characterized, but no gain-of-function alleles have been reported.[60] Interestingly, one *ced-3* allele, *n2433*, substitutes G360 with S. This glycine residue corresponds to G213 in DrICE, which is changed to D in *drICE*^{C1} (Figure 2). However, in contrast to *drICE*^{C1}, *n2433* behaves as a dominant negative.[60] It is unclear, if these different genetic behaviors are due to the different amino-acid substitutions (S in *n2433* vs D in *drICE*^{C1}) or to intrinsic differences between Ced-3 and DrICE.

In mice, the *Melody* mutation in Caspase-3 substitutes the catalytic C with a S residue. This mutation also behaves as a dominant negative in a heterozygous condition.[61] The only

mutation in Caspase-3 that confers constitutively active properties to Caspase-3 is the V266E substitution, which was characterized by *in vitro* mutagenesis.[62,63] This mutation increases Caspase-3 activity 60-fold. V266 is located in the dimer interface of the small subunit and the V266E mutation promotes dimerization of Caspase-3 without proteolytic processing.[63] V266 is not conserved in DrICE or other caspases (Figure 2), but even if it was, it is unlikely that such a mutation can be recovered *in vivo* as it will cause dominant lethality due to excessive apoptosis.

The allele-specific enhancement of apoptotic activity by caspase mutants is also of clinical significance. For example, it might be of interest to determine whether such dominant mutations of caspases have a role in the pathogenesis and development of neurodegenerative disorders, for which the underlying mechanism is apoptotic neuron loss. Sequence analysis of caspase genes in these patients will help in answering this possibility. Conversely, it would also be of interest to design and screen for drugs that would slightly twist the conformation of caspase tetramers to increase their enzymatic activity and induce cell death in tumors.

Materials and Methods

Isolation and identification of *drICE* mutant alleles. An isogenized *FRT82B* stock was used for EMS mutagenesis and also used as a control for genetic analyses. *FRT82B* males were treated with 25 mM EMS in 5% sucrose solution for 24 h. After recovery for 3 h, they were crossed to *GMR-hid drICE*^{17} females, and incubated at 25 °C. In all, 40 000 F1 progeny were screened for suppression of the small eye phenotype of *GMR-hid drICE*^{17}. Six mutants were identified as *de novo drICE* alleles by genetic analysis and DNA sequencing as described in the Results section.

Fly stocks and genetics. The following mutant and transgenic fly stocks were used: drICE^{L1}; drICE^{L2}; drICE^{C1}; drICE^{C2}; drICE^{S1}; drICE^{S2} (this study); drICE^{17} (ref. 25); drICE$^{\Delta 1}$ (ref. 44); GMR-hid;[14] CyO,2xGMR-reaper;[12] ey-FLP, FRT82B ubi-GFP;[64] GMR-hid ey-FLP, FRT82B ubi-GFP.[25] GMR-hid drICE^{17} is a recombinant chromosome carrying a GMR-hid transgene and drICE^{17}. Genetic mosaics for immunohistochemical analysis were obtained by crossing the FRT82B drICE alleles with ey-FLP; FRT82B drICE^{-}/FRT82B ubi-GFP.

DNA sequencing. Genomic DNA from homozygous mutant flies or trans-heterozygous over *drICE*$^{\Delta 1}$flies was isolated and PCR-amplified using *drICE*-specific primers. PCR fragments were sequenced by Sanger sequencing. The single amino-acid code was used.

Immunohistochemistry. TUNEL and immunohistochemistry were carried out as described.[65] Anti-DrICE antibody (a kind gift of Pascal Meier) was raised in guinea pig[47] and used at a dilution of 1 : 200. Anti-Dlg antibody (a kind gift of Georg Halder) was used at a dilution of 1 : 2000. Anti-DIAP1 (SK14) antibody (a kind gift of Pascal Meier) was raised in guinea pig and used at a concentration of 1 : 400. Cy3- and Cy-5 fluorescent-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). In general, 10–20 eye imaginal discs were analyzed, unless stated otherwise. Fluorescent Images were captured using a Zeiss Axio Imager Z1 with ApoTome technology or an Olympus Optical FV500 confocal microscope.

Quantification of *GMR-reaper*-induced excessive cell death. All TUNEL-positive cells in *GMR-reaper* larval eye discs in various genotypes were counted to indicate how much cell death was induced. For each genotype, 15–17 representative eye discs were counted. Significance was calculated by using unpaired two-tailed Student's *t*-test with a 95% confidence interval.

Fluorometric caspase assays. Fluorometric caspase assays were performed as described with modifications.[66] Adult heads were dissected on ice and lysed with a pestle in caspase assay buffer (50 mM HEPES, pH 7.5; 100 mM NaCl; 1 mM EDTA; 0.1% CHAPS; 10% sucrose; 5 mMDTT; 0.5% TritonX-100; 4% glycerol; 1 × protease inhibitor cocktail (Promega, Madison, WI, USA)) and then sonicated once for 7 s. Protein concentrations of the lysates were determined by Bradford Protein Assay. For each genotype, 40 μg of total protein lysates were used, respectively. Protein lysates and 100 μM of the fluorometric substrate DEVD-AMC (MP Biomedicals, Santa Ana, CA, USA) were combined in a 96-well plate on ice. Reaction volume was brought to 100 μl with caspase assay buffer. Spectrophotometer was used to measure fluorescence (excitation 385 nm and emission 460 nm) at 15 min intervals for 2 h at 37 °C.

Conflict of Interest

The authors declare no conflict of interest.

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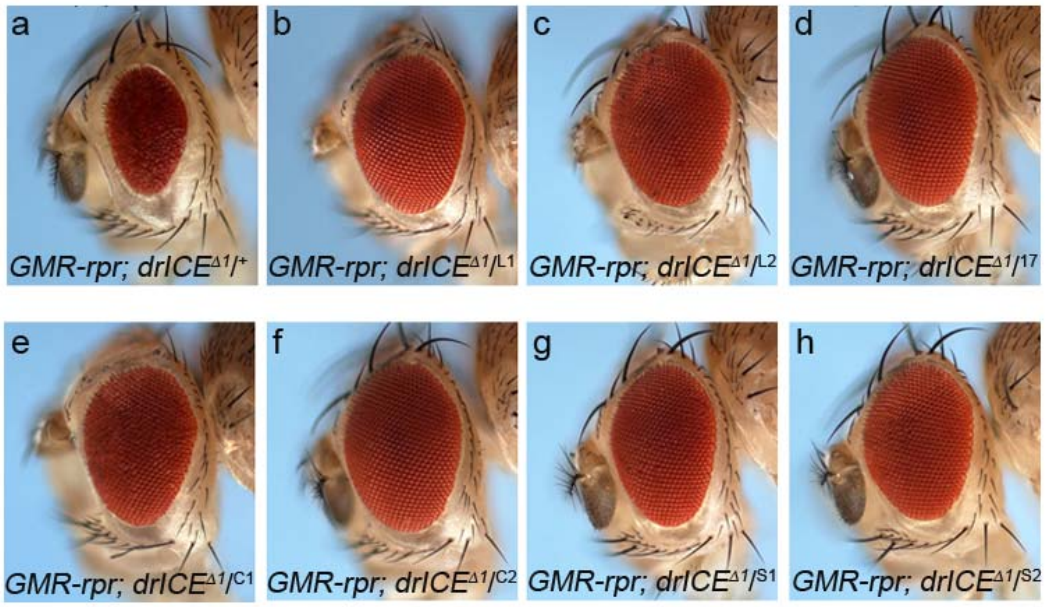
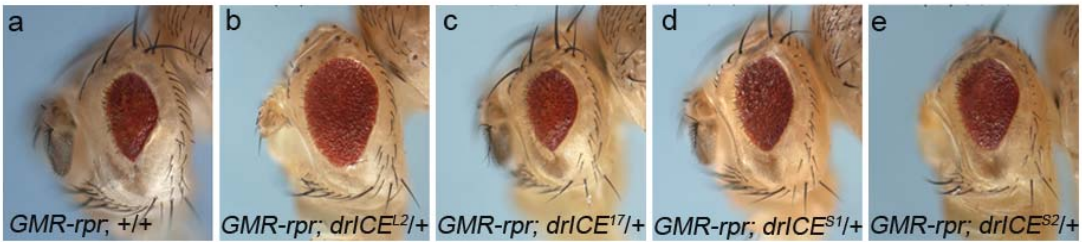
Supplementary Information accompanies this paper on Cell Death and Differentiation website (http://www.nature.com/cdd)

Supplementary Information: Figures S1-S4



Supplemental Figure S1. All *drlCE* mutant alleles suppress *GMR-reaper* when in trans to a *drlCE* deficiency.

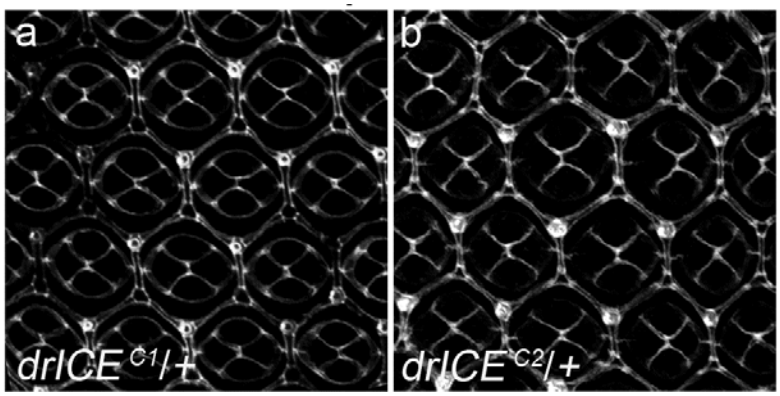
Related to Fig. 1. All newly isolated *drlCE* alleles suppress *GMR-reaper* (*GMR-rpr*) when in trans to a *drlCE* deficiency. Genotypes: *CyO,2xGMR-reaper; drlCE$^{\Delta 1}$ / drlCE^{X}* , with X denoting the *drlCE* allele used.



Supplemental Figure S2. Dominant effects of the *drlCE* alleles on *GMR-reaper*.

Related to Fig. 6. The class L and class S alleles which are not shown in Fig. 6 weakly suppress *GMR-reaper* (*GMR-rpr*) Genotypes are indicated in the panels.

Supplemental Figure S3. Dominant effects of the new *drICE* alleles on *GMR-hid*.
Related to Fig. 6. (**a, b**) The class L allele *drICE*^{L1} slightly suppresses the *GMR-hid*-induced eye ablation phenotype (a) compared to *GMR-hid* (b).
(**c-d**) The class C alleles *drICE*^{C1} and *drICE*^{C2} may weakly enhance the *GMR-hid*-induced eye ablation phenotype.



Supplemental Figure S4. No obvious patterning defects of the retinal lattice in class C alleles.
42hr APF *drICE*^{C1}/+ and *drICE*^{C2}/+ pupal retinae were labeled with anti-Dlg. The patterning of the cells in the pupal retina resembles wild-type pupal discs of the same developmental stage.