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Evolutionary analyses of caspase-8 and its paralogs: Deep origins of the apoptotic signaling pathways

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Summary

Although *Caenorhabditis* and *Drosophila* proved invaluable in unraveling the molecular mechanisms of apoptosis, it is now clear that these animals are of limited value for understanding the evolution of apoptotic systems. Whereas data from these invertebrates led to the assumption that the extrinsic apoptotic pathway is restricted to vertebrates, recent data from cnidarians and sponges indicate that this pathway predates bilaterian origins. Here we review the phylogenetic distribution of caspase-8, the initiator caspase of the extrinsic apoptotic pathway, its paralogs and other components of the network. The ancestral *caspase-8* gave rise to four paralogs early in vertebrate evolution, and these have been maintained in many tetrapods. However, eutherians have lost *caspase-18* and myomorph rodents have lost *caspase-10*, these losses suggesting functional redundancy amongst caspase-8 paralogs. The apoptotic network of the eumetazoan ancestor appears to have been complex and vertebrate like, and is only now being revealed by studying simple animals.

Introduction

Cell death is a widespread phenomenon that has been extensively documented in protozoans, fungi and plants as well as animals [1-4]. Although several distinct types of cell death process are now recognized, including necrosis, pyroptosis and autophagy, the form known as apoptosis appears to be unique to, but ubiquitous amongst, members of the animal kingdom (metazoans). Within the Metazoa, apoptosis is important for tissue morphogenesis during development and for maintaining homeostasis in adulthood [5-8], but is also induced for protection of the organism by removing all cells damaged by disease, aging, infection, genetic mutation and exposure to toxic agents [9]. A corollary of this wide range of critical functions is that dysregulation of apoptosis underlies a wide spectrum of human disorders, including cancer and autoimmune diseases, as well as neurodegenerative diseases, ischemic diseases and viral infections [10, 11]. As apoptosis is a regulated type of cell death, involving genetically encoded molecular machinery, it is synonymous with the phrase programmed cell death. Apoptosis is defined as a caspase-dependent variant of regulated cell death [9, 12], with well defined morphological and biochemical correlates. Apoptotic cells typically undergo shrinkage, nuclear condensation, membrane blebbing and cellular and nuclear fragmentation into membrane-bound apoptotic bodies. Biochemical manifestations of apoptosis include exposure of phosphatidylserine on the surface of the plasma membrane, which essentially acts as an "eat me" signal, leading to phagocytosis of apoptotic cells by macrophages or other phagocytes. The first evidence for the genetic basis of apoptosis came from the elegant studies of the nematode *Caenorhabditis elegans*, which provided evidence for a core regulatory pathway (**Fig. 1**) [13, 14]. Intensive efforts over the last quarter of a century, particularly in the model animals *C. elegans*, the fruit fly *Drosophila melanogaster* and mouse *Mus musculus,* have led to a much deeper understanding of the molecular mechanisms that regulate apoptosis (Fig. 1) [8, 12]. Comparisons between the apoptotic systems of these three animals highlight the conservation of the molecular mechanisms regulating programmed cell death during evolution. Furthermore, recent studies on cnidarians [15, 16] and sponges [17, 18], phyla that diverged in the Cambrian or earlier, imply that apoptotic cell death pathways have very early origins.

Caspases (<u>C</u>ysteine-dependent <u>asp</u>artyl-specific prote<u>ases</u>) are central players in apoptotic pathways, and belong to a family of cysteine proteases that recognize specific

tetrapeptide motifs and cleave after the aspartate residue in their substrates. Caspases are essential for the initiation and execution of apoptosis, as well as for the processing and maturation of the inflammatory cytokines [19], but are also involved in cellular proliferation and differentiation [20]. Members of the caspase family have been characterized from a range of vertebrates and invertebrates, but have been most extensively investigated in mammals. In man (*Homo sapiens*), 12 caspases (caspases-1 to -10, caspase-12 and caspase-14) have been identified [21], one of which - caspase-8 (CASP8) - was initially identified as an apoptotic initiator caspase. The CASP8 protein contains tandem death effector domain (DED) motifs in its amino-terminal prodomain as well as the active CASc (Caspase, interleukin-1 β converting enzyme homologues) protease domain at the carboxyl terminus. CASP8 is critically involved in what is known as the extrinsic apoptotic signaling pathway in mammals [22], which is triggered by stimulation of death receptors (DRs) located at the cell-surface (Fig. 1). Six types of DRs, known as TNFR1 (Tumor Necrosis Factor Receptor type-1), Fas, DR3, DR4 (alias: TNF-Related Apoptosis-Inducing Ligand Receptor 1, TRAIL-R1), DR5 (TRAIL-R2) and DR6, all of which contain a death domain (DD) in the cytoplasmic region, have been identified in mammals. Four of the DRs (TNFR1, Fas, DR4 and DR5) are able to activate CASP8 upon ligand binding, leading to apoptotic signal transduction. Of the death receptor pathways, that involving Fas has been most thoroughly characterized [23, 24]. Once Fas is activated by oligomerization, a DED- and DD-containing adaptor molecule known as FADD (Fas-associated death domain protein; sometimes known as MORT1) is recruited to its cytoplasmic region via homophilic interactions mediated by DDs. CASP8 associates in turn with FADD via interactions between DED motifs. Within the death-inducing signaling complex (DISC) formed by these three molecules [25], CASP8 undergoes both dimerization and cleavage to generate its active form that is then released to the cytosol, where it cleaves downstream molecules such as the apoptotic effector, caspase-3 (CASP3) [26, 27]. Thus, CASP8 is a critical component of the extrinsic apoptotic pathway.

In mammals, a second apoptotic pathway has also been extensively characterized. The intrinsic, or mitochondria-dependent, apoptotic pathway is initiated by intracellular stimuli (Fig. 1), and here the mitochondria and Bcl-2 family members are involved in apoptotic signal transduction. Pro-apoptotic members of the Bcl-2 family, Bax and Bak, induce cytochrome c (Cyt-c) release from the mitochondria, resulting in the assembly of a complex

called the apoptosome, which is composed of Cyt-c, ATP, apoptotic peptidase-activating factor 1 (APAF-1) and an initiator caspase, caspase-9 (CASP9). Within the complex, APAF-1 and CASP9 interact via caspase recruitment domains (CARDs) present in each molecule, resulting in CASP9 activation and its release, whereupon it targets the effector protein CASP3.

Two model invertebrates, the nematode (*C. elegans*) and fruit fly (*D. melanogaster*), have been particularly useful in unraveling many of the general principles of apoptosis [14, 28]. However, one key difference between these model invertebrates and mammals is the apparent absence of an extrinsic apoptotic pathway in either worm or fly. Neither *casp8* nor *fadd* genes, which encode essential components of the extrinsic pathway, are present in the *C. elegans* genome [29] and, although a casp8 ortholog (Dredd) is present in *D. melanogaster,* it functions primarily in innate immunity [30]. On the basis of the data from fly and worm, until recently it was considered likely that the extrinsic pathway was restricted to vertebrates. However, identification of most of the components required for extrinsic signal transduction in some other invertebrates has led to a re-evaluation of this evolutionary scenario. Recent data, particularly from phyla that diverged early in animal evolution, imply that both extrinsic and intrinsic pathways have deep origins, and that the ancestral apoptotic network may have been complex and vertebrate-like [29, 31, 32].

Gains and losses of CASP8-related molecules during vertebrate evolution

In the human genome, the *CASP8* gene and the paralogous genes, *caspase-10* (*CASP10*) and *CASP8- and FADD-like apoptosis regulator* (*CFLAR*; gene product, c-FLIP) are clustered in the same region of chromosome 2 (**Fig. 2** and **Supplementary Fig. S1**) [33-35]. The three corresponding proteins have the same domain structure – tandem DED motifs in the prodomain and a CASc protease domain; like CASP8, the CASP10 protein has protease activity [33], whereas an amino acid substitution in the active site of CASc has rendered the c-FLIP protein inactive [36]. The lack of protease activity enables c-FLIP to act as a regulator for CASP8 activation in the extrinsic signal transduction pathway [37]. Interestingly, depending on the specific isoforms or relative levels of the c-FLIP protein expressed, c-FLIP can act as either a negative or positive regulator [38]. Although an alternatively spliced short form of c-FLIP, c-FLIP(S), universally acts as a negative regulator of CASP8, low levels of the full-length long form of c-FLIP, c-FLIP(L), can

activate CASP8 whereas high levels of c-FLIP(L) prevent CASP8 activation (further detailed in [37]). Whereas in mammals, genes encoding CASP8, CASP10, and c-FLIP are clustered, in the chicken genome, an additional paralog is present in the corresponding cluster [39]. The *caspase-18* (*CASP18*) gene is located between the *CASP8* and *CASP10* loci in the chicken genome (Fig. 2 and Supplementary Fig. S1B). The clustering of the *CASP8*, *CASP10*, *CASP18*, and *CFLAR* genes, together with the fact that the corresponding proteins have the same domain structure (two DEDs and a CASc/CASc*; *:inactive), led us to propose that these four genes arose early in vertebrate evolution as a result of duplication events acting on an ancestral *CASP8* gene [39]. In the course of evolution to bony fish such as zebrafish and stickleback, furthermore, chromosomal segregation has substantially reorganized these genes, and a novel (fish-specific) *CASP8-like* gene, *card-casp8*, occurs adjacent to the *casp8* locus (Fig. 2 and Supplementary Fig. S1A) [39].

To better understand the evolution of *casp8*-related genes and their genomic organization, whole genome data for the lamprey (*Petromyzon marinus*), spotted gar (*Lepisosteus oculatus*) and coelacanth (*Latimeria chalumnae*) were investigated, on the basis that these organisms represent key steps in vertebrate evolution. The coelacanth is the closest living relative to tetrapods and gars are a primitive and ancient type of bony fish. In the coelacanth, the number of CASP8 paralogs and their genomic organization was exactly as in the chicken, but in the spotted gar, the *cflar* gene was not linked to the other three CASP8–related genes (Fig. 2 and Supplementary Fig. S1A). These observations support the hypothesis that genes encoding the four CASP8 paralogs were clustered in the vertebrate common ancestor, whereas the ancestral cluster has become extensively, perhaps progressively, dispersed during teleost evolution (Fig. 2 and Supplementary Fig. S1A). The fish-specific *card-casp8* gene could be either a completely novel gene, or have been derived from CASP18 by domain swapping (i.e. replacing DED motifs with a CARD motif) (Supplementary Fig. S1A).

With the exception of eutherian mammals, a *CASP18* gene is present in all tetrapods so far examined – from coelacanth to marsupials, such as the opossum (*Monodelphis domestica*) [40]. Like CASP8 and CASP10, chicken CASP18 can initiate apoptosis, as is evident from its ability to kill when expressed in cultured mammalian cells (Supplementary Fig. S2). The loss of CASP18 in the case of eutherian mammals (Fig. 2 and Supplementary Fig. S1B) may reflect a degree of functional redundancy amongst these (CASP8, CASP10,

CASP18) genes. Data from two clawed frogs provide some support for this idea; the *CASP18* gene of *Xenopus tropicalis* encodes a protein that lacks a protease domain [29], and the transcript from *Xenopus laevis* with the highest level of similarity to the *X. tropicalis* cDNA likewise encodes only DED motifs [29]. These data suggest that in amphibians the *CASP18* gene may be losing its function, and perhaps this degeneration has been taken a step further in eutherians.

A similar scenario may also apply to the *CASP10* gene during rodent evolution. Although *CASP10* has been conserved from fish to eutherians (Fig. 2 and Supplementary Fig. S1B), in the case of the mouse, this gene has been lost (Fig. 2 and Supplementary Fig. S1C). Not all rodents have lost this gene, however. Within the Order Rodentia, three major subgroups are recognized, Hystricomorpha, Myomorpha and Sciuromorpha. Examination of the genomic regions around the *CASP8* loci of the guinea pig (*Cavia porcellus*; a representative of the Hystricognathi), the squirrel (*Ictidomys tridecemlineatus*; Sciuromorpha) and the rat (like the mouse, a member of the Myomorpha) revealed that, although the *CASP10* gene has been lost from both the mouse and rat (i.e. Myomorpha), this is not the case in either guinea pig or squirrel (i.e. Hystricomorpha and Sciuromorpha; Supplementary Fig. S1C). Given that the major rodent clades are thought to have diverged in the Cretaceous [41], *CASP10* gene loss from mouse and rat occurred relatively recently. Thus, although these four paralogous genes (*CASP8*, *CASP10*, *CASP18* and *CFLAR*) are likely to have arisen early in vertebrate evolution and remain as a cluster in some animals, *CASP18* and in some cases also *CASP10* have been lost during the evolution of mammals. Although the four-gene cluster was present in the ancestral teleost, it has been fragmented during bony fish evolution so that the individual loci are dispersed in "advanced" fish such as the stickleback.

Similarities and differences between human and mouse CASP8

In the mouse, mutations affecting Casp8 function typically result in embryonic lethality (**Fig. 3A**) [42, 43], whereas this is not the case in man even if the *CASP8* gene is mutated to the extent that protease activity is completely lost [44]. To date, there have been no reports of differences between the human and mouse CASP8 proteins with respect to substrate specificity, and the three-dimensional structures of the active forms of these proteins are very similar (Fig. 3B). Although these studies provide no insights into functional

differences between the mouse and human CASP8 proteins, there are hints that post-translational modifications may be a factor. As shown in Fig. 3C and **Table 1**, several studies documented modifications of CASP8 proteins by kinases [45-50] or by E3 ubiquitin ligases [51, 52]. In the case of human CASP8, tyrosine Y^{380} can be phosphorylated by the Src tyrosine kinase [46]. This modification represses the auto-processing and protease activity of CASP8, leading to the inhibition of apoptosis. A corresponding tyrosine residue is conserved throughout mammals but is absent from the mouse and rat proteins (Fig. 3C). This difference between myomorph rodents and other mammals is likely to have important functional consequences. As Src is activated at the onset of mitosis [53], phosphorylation of human CASP8 by Src results in cells being insensitive to extrinsic apoptotic stimuli during cellular proliferation. Consistent with this, mitotic human cells have been shown to be resistant to Fas-mediated apoptosis [54]. However, in the mouse, because Casp8 cannot be phosphorylated by Src, mitotic cells remain sensitive to extrinsic apoptotic stimuli. Although whether or not the protein is subject to modification by Src potentially explains the distinct phenotypic consequences of *CASP8*-deficiency between man and mouse, in fish the situation is rather different. As in myomorph rodents, the CASP8 proteins of many fish lack a tyrosine residue corresponding to Y^{380} in human CASP8. On the basis of the mouse data one might predict that *casp8*-deficiency would result in embryonic lethality in fish. However, this is not the case, as, using the Targeting Induced Local Lesions IN Genomes (TILLING) method, we have generated *casp8*-deficient medaka fish (*Oryzias latipes*) and shown that these lines are fully viable (Sakamaki et al., unpublished data).

In a previous study, cell death could not be prevented in the heart of *casp8*-deficient mouse embryos by co-expression of the pan-caspase inhibitor protein, baculovirus p35, which is able to block apoptosis [55], in the same tissue [56]. This evidence suggests that non-apoptotic cell death can occur in the absence of Casp8. The causes of such cell death phenomena have recently become more clear, a receptor-interacting serine/threonine kinase 3 (RIPK3) being involved, ultimately resulting in necroptotic cell death [57]. Necroptosis is a form of regulated or programmed necrosis and its signaling pathway as currently defined is as follows [58-62]. When Casp8 is inactive, RIPK1 interacts with and phosphorylates RIPK3 [63-65]. Activated RIPK3 then phosphorylates a pseudo-kinase, mixed lineage kinase domain-like (MLKL), inducing its oligomerization and translocation into the plasma membrane [66-68], resulting in the initiation of necroptosis. The available data thus imply

that one of the major developmental roles of Casp8 and Fadd in mouse embryos is to antagonize the induction of necroptotic cell death by repressing the RIPK1/RIPK3 activation [57, 69, 70].

The difference between mouse and medaka with respect to *casp8* deficiency may be explained in terms of functional redundancy in the latter organism but not in the former, as fish and man have retained *casp10* whereas mouse and rat have lost it (Fig. 2). The CASP10 protein is a CASP8 paralog, and these two proteins have a high level of similarity not only in structure but also in function. It has been shown that CASP10 is recruited to the complex on death receptor engagement and transmits apoptotic signals [71, 72], and thus could potentially substitute for CASP8 in the event of *casp8* deficiency. It has been demonstrated that although CASP10 is recruited to the DISC, it is not able to functionally substitute for CASP8 directly [73]. However, some recent studies suggest that CASP10 may act in an atypical Fas-induced cell death pathway [74], thus a degree of functional redundancy amongst CASP8 paralogs remains likely. Therefore, in non-myomorph vertebrates, CASP10 proteins may be able to compensate for CASP8-deficiency acting in a manner independent of death receptor-mediated signaling, although the molecular mechanisms involved require further investigation.

Evidence from cnidarians implies deep origins for the intrinsic and extrinsic apoptotic pathways

As described above, the manipulability of the model invertebrates *Caenorhabditis* and *Drosophila* facilitated understanding of what is now known as the intrinsic apoptotic pathway, but until recently the absence of an equivalent of the vertebrate extrinsic apoptotic pathway in these organisms was interpreted to mean that this system evolved in the vertebrate lineage. However, recent data from cnidarians, an early diverging lineage of animals that includes the sea anemones and corals as well as the textbook representative *Hydra*, implies that both extrinsic and intrinsic apoptotic pathways predate the cnidarian – bilaterian divergence, which occurred at least 550 million years ago (MYA). Thus, the absence of an extrinsic pathway in the fly and worm is likely to be another example of the higher rates of loss and divergence of genes that characterize these animals [75].

Roles for apoptosis in the normal development and metamorphosis of cnidarians have been established [15, 76, 77] and more recently apoptosis has been implicated in the

"bleaching" (loss of photosynthetic symbionts) process and mortality in corals and sea anemones [78, 79]. The availability of whole genome sequences for three cnidarians – the sea anemone *Nematostella vectensis* [80], *Hydra magnipapillata* [81] and the coral *Acropora digitifera* [82] – has enabled the identification of homologs of many of the proteins associated with mammalian apoptotic pathways. Surveys based on these genome data [83-85] not only highlight the highly derived nature of the intrinsic apoptotic systems of *Drosophila* and *Caenorhabditis*, but also imply the presence of an extrinsic apoptotic pathway.

Although functionally equivalent to those of mammals, the intrinsic apoptotic pathways of *Drosophila* and *Caenorhabditis* are simple by comparison, and differ in some important respects (see Introduction). Whereas there are more than a dozen multi-domain Bcl-2 family members in vertebrates, only two Bcl-2 family members, Debcl and Buffy are present in *Drosophila* and Ced-9 is the sole representative of this family in *Caenorhabditis*. However, *Drosophila* Debcl and Buffy do not seem to play central roles in apoptosis regulation [28]. Under the canonical model of intrinsic apoptosis, Bcl-2 proteins function primarily at the level of controlling the release of cytochrome c (Cyt-c) from mitochondria, which then triggers apoptosome formation and activation. Whereas in mammals, Cyt-c interacts with APAF-1 in apoptosome assembly, there is no evidence for an equivalent role for Cyt-c in either fly or worm. Rather than controlling Cyt-c release, the *Caenorhabditis* Bcl-2 protein Ced-9 prevents apoptosome activity by interacting with the APAF-1 homolog Ced-4. *Drosophila* Buffy is thought to act in a similar manner, its inhibitory activity being released by dimerization with Debcl. Whereas in both the fly and vertebrates an initiator caspase (CASP9 in vertebrates, Dronc in *Drosophila*) in the apoptosome activates effector caspases (CASP3/7 in vertebrates, Drice in *Drosophila*), in *Caenorhabditis* the apoptosome associated Ced-3 acts as both as an initiator and an effector caspase.

In contrast to the relative simplicity of *Drosophila* and *Caenorhabditis*, the Bcl-2 repertoires of cnidarians are complex and vertebrate-like. Nine Bcl-2 family proteins have been identified in *Hydra* [31, 86], and eleven in *Nematostella* [85], the latter including members of the vertebrate Bax, Bak and Bok pro-apoptotic subfamilies. When *Hydra* Bcl-2 proteins were expressed in mammalian cells, the Bax orthologs induced apoptosis, whereas some other Bcl-2 proteins had protective properties and all associated with mitochondria [86], suggesting vertebrate-like modes of action. Single homologs of APAF-1 are present in

each of the cnidarians so far examined, although the *Acropora* protein is atypical in containing two N-terminal CARD domains ([87], see Supplementary Fig. S3A). The caspase repertoires of cnidarians are complex, but include Casp9-like and Casp3/7-like proteins. Thus, most of the intrinsic apoptotic machinery appears to have been in place before the cnidarian - bilaterian divergence.

The key components of the extrinsic apoptotic pathway are death receptors, the adaptor molecule Fadd and Casp8, and each of these is present in *Acropora* and most likely other cnidarians (**Fig. 4**). In mammals, CASP8 indirectly interacts with canonical death receptors – the DED in CASP8 undergoes homotypic interactions with the FADD adaptor molecule, which in turn interacts homotypically with the intracellular DD of a death receptor. BLAST and HMM searches identified candidate Casp8 proteins in *Acropora* and *Nematostella* and, although these are atypical in terms of specific amino acid residues in the catalytic pocket previously considered diagnostic, the *Acropora* Casp8 protein displayed the same substrate specificity as its mammalian counterparts [29]. A clear homolog of FADD has also been identified in *Acropora*, and co-immunoprecipitation experiments demonstrate that the *Acropora* Casp8 and Fadd proteins are capable of interacting [29].

The *Acropora* genome encodes many members of both the TNF and TNFR protein superfamilies. Exposure of coral cells to human TNFα not only induced classical symptoms of apoptosis including blebbing and proteolysis, but also increased the rate of symbiont loss, suggesting that TNF ligands might be involved in the bleaching response [32]. Crude preparations of one of the *Acropora* TNF ligands (AdTNF1) caused apoptosis and bleaching in the coral, and purified AdTNF1 caused apoptosis in human T-lymphocytes, implying that the coral ligand can interact with a human death receptor [32].

Taken together, these experiments imply that the apoptotic pathways of bilaterians have unexpectedly deep origins, and that much of what was assumed to be mammal-specific elaboration of the intrinsic and extrinsic pathways may actually reflect ancestral states. However, whilst all or most of the molecular machinery was undoubtedly in place very early in animal evolution, much of the detail of cnidarian apoptotic pathways remains to be established – for example, the identity of the coral receptor for the AdTNF ligand. The coral data suggest that, as in mammals, release of Cyt-c from mitochondria, initiated either by Casp8 activation or in response to stress-induced damage, may be a key apoptotic trigger. Although the coral is not a genetically tractable organism, functional

analyses are now possible in both *Hydra* and the sea anemone [88, 89]. These cnidarians promise to provide novel insights and unique perspectives into the origins and diversification of apoptotic pathways during animal evolution.

Earlier origins of the metazoan apoptotic signaling systems?

Whilst a considerable body of data (summarized above) suggests the presence of complex and vertebrate-like apoptotic signaling systems in cnidarians, what about those animal phyla that diverged even earlier in animal evolution? In a previous paper, we have reported identification of *casp8* genes in both freshwater and sea sponges (*Ephydatia fluviatilis* and *Amphimedon queenslandica*) [29]. In general, DED1 of CASP8 proteins functions in binding to the DED of other molecules, such as an adaptor FADD, whereas DED2 is involved in oligomerization of CASP8 itself [90, 91]. The structural similarity between the sponge and cnidarian/bilaterian CASP8 proteins suggested Fadd-like proteins might also be present in sponges, and this was confirmed by searching the *A. queenslandica* genome database. The presence of a Fadd-like molecule containing both DED and DD motifs (**Fig. 5** and Supplementary Fig. S3B) suggests that the platform to recruit and activate Casp8 was already in place before the Porifera diverged from the eumetazoan stem. To date, there is no convincing evidence for the presence of a canonical *casp8* gene in either placozoans or ctenophores, although caspases are present in representatives of both phyla [29]. Broader phylogenomic surveys, including not only other "lower" animals, but also non-metazoan holozoans, will be informative, but it is already clear that the apoptotic systems of mammals have much deeper origins in animal evolution than was previously assumed.

Conclusions and outlook

In unraveling the molecular bases of apoptosis, mouse and the model invertebrates, *D. melanogaster* and *C. elegans* have been particularly important. However, in terms of understanding the evolution of apoptotic networks, it is now clear that these model invertebrates have misled us - the apoptotic cell death pathway of *C. elegans* is likely to be a highly derived process that reflects neither the general situation in invertebrates nor ancestral states. The absence of an equivalent of the vertebrate extrinsic apoptotic pathway in fly and worm is another example of the loss and modification of genes and pathways that is characteristic of these animals. By contrast, recent studies indicate that cnidarians

resemble mammals in being able to induce apoptosis through both extrinsic and intrinsic pathways, and a vertebrate-like apoptotic toolkit was most likely already present in the cnidarian-bilaterian ancestor. These counter-intuitive findings bring new light to the cell death field, and require a major change in thinking about the evolution of apoptotic systems.

Neither apoptosis nor true caspases have been identified in protists, fungi or plants [2, 3, 92]. No caspases were identified in the unicellular choanoflagellate (*Monosiga brevicollis*) by searching the JGI database [29]. These lines of evidence suggest that apoptosis is a process unique to, but ubiquitous across, multicellular animals. The high degree of conservation observed implies that apoptosis provided a strong selective advantage and may have contributed substantially to the spectacular evolutionary success of the Metazoa.

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FIGURE LEGENDS

Figure 1. The apoptotic signaling pathways defined in *C. elegans*, *D. melanogaster* and mammals. The core components of the cell death machinery in the nematode (*C. elegans*), fruit fly (*D. melanogaster*), and mammals are shown. These canonical apoptotic pathways are evolutionarily conserved and central to the regulation of apoptosis is the caspase family. In *C. elegans*, Egl-1 (a BH3-domain-only pro-apoptotic Bcl-2 family member), Ced-9 (an anti-apoptotic Bcl-2 family member), Ced-4 (an APAF-1-like adaptor) and Ced-3 (both as an initiator and an effector caspase) are involved in apoptotic signaling [14]. In *D. melanogaster,* Reaper (an inhibitor of apoptosis protein (IAP) antagonist), Diap1 (an IAP homolog), Dronc (an initiator caspase) and Drice (an effector caspase) [28]. Dark (an APAF-1-like molecule) is also involved in the signaling pathway. In the intrinsic pathway of mammals, Bim or other BH3-domain-only proteins allow the pro-apoptotic Bcl-2 family members Bax and Bak to bring about release of cytochrome-c from mitochondria, which then nucleates an apoptosome around APAF-1 (an adaptor) and activates the apoptotic initiator caspase-9. In the extrinsic pathway, death ligands, death receptors, FADD (an adaptor) and caspase-8 (an initiator caspase) are required for apoptotic signaling. The pathways converge when apical caspase activation in the DISC complex leads to the activation of downstream effector caspases such as caspase-3. The DISC complex [93] and mammalian and *Drosophila* apoptosomes [94] are indicated by single and double asterisks with a square bracket, respectively.

Figure 2. The complex evolutionary history of CASP8 and its paralogs in the vertebrate lineage. Comparative genomics suggests that a single ancestral *CASP8* gene gave rise to the four paralogs *CASP8*, *CASP10*, *CASP18*, and *CFLAR* early in vertebrate evolution. These four genes remain tightly linked in some vertebrates, but the ancestral cluster has been fragmented during teleost evolution, and a novel *card-casp8* gene that possibly arose from CASP18 by domain swapping is adjacent to the *casp8* gene in some fish. CASP18 genes have been lost in some vertebrate lineages; the *CASP18* gene is incomplete in amphibians and completely lost in eutherian mammals. Loss of the *CASP10* gene has also occurred in one major rodent lineage. The phylogenetic tree of the species shown at the left half was generated based on previous reports [95, 96].

Figure 3. Comparison of human and mouse caspase-8.

A: The morphological phenotype of Casp8-deficient mutant mice. Homozygous *Casp8*-defect mutant embryos (right), but not wild-type embryos (left), typically show hemorrhaging in the cardiac cavity and die during mid-gestation. **B:** Three-dimensional structures of the active forms of human and mouse CASP8 proteins superimposed to emphasize similarity. **C:** Amino acid alignment of the protease domains of human, dog, mouse and rat CASP8 and human CASP10 proteins. Amino acid residues highlighted in color and numbered (1) – (6) are targets for phosphorylation, and those numbered (7) and (8) for ubiquitinylation. Residues numbered (9) and (10) indicate positions in human CASP8 and CASP10 at which loss of function mutations have been identified. Information on, and citations to, these modifications and mutations is summarized in Table 1.

Figure 4. Apoptotic pathway components in the coral mapped onto the intrinsic and extrinsic pathways of mammals.

Many of the key network components have been identified in cnidarians, as indicated. Components outlined by red boxes have been definitively identified [29, 31, 32, 86, 87], whereas blue boxes indicate that candidates are present. Some important components of the mammalian apoptotic systems are unlikely to be present in cnidarians; CASP8 is the only *Acropora* caspase known that has N-terminal death effector domains (DEDs), thus orthologs of CASP10 and c-FLIP are undetectable.

Figure 5. Key components of the apoptotic machinery are present in non-bilaterian animals.

Cnidarian and sponge genomes encode clear homologs of key components of the apoptotic signal transduction pathways of bilaterians. In particular, the cnidarian apoptotic repertoire is surprisingly complex and vertebrate-like. In terms of morphology, placozoans are the simplest metazoans, but this is likely to reflect secondary loss of complexity that is also a characteristic of the genome. The situation in ctenophores is unclear, and their evolutionary position remains contentious. Above the table, the phylogenetic tree of the species was generated based on the previous report [97]. A question mark ("?") indicates that a homologous gene is unconfirmed. Accession data for the FADD and APAF-1 sequences are given in Supplemental Figure S3. CARD-containing CASP9-like sequences have been

identified in the sponge *A. queenslandica* (UniProt: I1GD28_AMPQE) and the coral *Acropora digitifera* (Superfamily: adi_v1.09611). Photo credits: A. Omori (Ctenophora, *Beroe forskalii*), N. Funayama (Porifera, *Ephydatia fluviatilis*), PLoS ONE (Placozoa, *Trichoplax adhaerens*), and T. Koide (Bilateria, mouse).

Table 1 Summary of modifications and mutations of human CASP8 and CASP10.

	Amino acid & position	Contributing enzyme	Effect	Reference
Phosphorylation				
(1)	T263	RSK2	Degradation	[48]
(2)	S305	Plk1	Inactivation	[49]
(3)	S347	p38 MAPK	Inactivation	[45]
(4)	Y380	Src	Inactivation	[46]
(5)	S387	Cdk1	No processing	[47]
(6)	S387	MAPK	Inactivation	[50]
Ubiqutinylation				
(7)	K229, K231	TRAF2	Degradation	[52]
(8)	K461	Cullin-3	Stability	[51]
Mutation				
(9)	R248W		Inactivation	[44]
(10)	*L285F		Inactivation	[98]
(10)	*V410I		Inactivation	[98]

Asterisks indicate the mutation of the *CASP10* gene.

Figure 1

Figure 2

Figure 3

Figure 4

	Ctenophora	Porifera	Placozoa	Cnidaria	Bilateria
Adaptor(s)		+	+	+	+
FADD		+	?	+	+
APAF-1		+	+	+	+
Caspase(s)	+	+	+	+	+
caspase-8		+	?	+	+
caspase-9(-like)		+	?	+	+

Figure 5

SUPPORTING INFORMATION

Database search

The following web sites were used to access nucleotide and protein sequence databases:
NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Ensembl
(http://www.ensembl.org/index.html), SUPERFAMILY of the HMM library and genome
assignments server
(http://supfam.cs.bris.ac.uk/SUPERFAMILY/cgi-bin/taxonomic_gen_list.cgi), Compagen,
a comparative genomics platform for early branching Metazoa
(http://compagen.zoologie.uni-kiel.de). Proteins were identified and characterized using
TBLASTN [1]. Domain searches were performed using either SMART (Simple Modular
Architecture Research Tool)
(http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) or UniProt
(http://www.uniprot.org/uniprot/). Multiple sequence alignment was performed using
Clustal W (http://www.genome.jp/tools/clustalw/).

**Construction of three-dimensional model of mouse Casp8 protein and comparison
with human CASP8**

Three-dimensional (3D) models of a CASc domain of mouse Casp8 protein were built
based on alignments with 4JJ8 [2] and 4JR1 [3] calculated by FORTE, a profile–profile
comparison method for protein structure prediction [4], using MODELLER9.8 [5].
Side-chain conformations of the model were optimized using SCWRL4.0 [6]. The model of
mouse Casp8, which was selected and validated using VERIFY3D [7], was superimposed
onto the structure of human CASP8.

Cytotoxic assays of chicken CASP18 protein in human culture cells

To express chicken CASP18 protein in cultured human cells, the plasmid construct
pCMV-GgCASP18 was generated by inserting the cDNA fragment from *G. gallus* into the
expression vector pCMV-SPORT6 (Invitrogen). Plasmids pCAG-p35 and pCAG-CrmA,
for the inhibition of protease activity of caspases and the specific inhibition of Casp8
activation, and plasmid pCAG-mCherry, for the detection of transfected cells, were
generated previously, respectively [8-10]. Human cervical carcinoma HeLa cells were

cultured in Dulbecco's Modified Eagle's medium with 10% fetal calf serum. Transfection of plasmid DNAs into cells was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. For the check of pro-apoptotic activity of CASP18 proteins, HeLa cells were transiently co-transfected with pCMV-GgCASP18 and pCAG-mCherry with or without pCAG-p35 or pCAG-CrmA. At 2 days after transfection, fragile and vulnerable cells were washed out with Phosphate Buffered Saline (PBS) and the remaining cells were fixed in PBS containing 3.7% formaldehyde. Phase-contrast and fluorescent images were acquired using a fluorescent microscope.

Assessment of pro-apoptotic activity of chicken CASP18 protein

Previous studies have demonstrated that overexpression of human and mouse CASP8 proteins induces cell death in mammalian culture cell lines without exogenous apoptotic stimulation [11-13]. Human HeLa cells are known to be sensitive to extrinsic apoptotic signals in association with CASP8 activation. Therefore, this killing assay is used to examine whether CASP18 protein could induce cell death when expressed in HeLa cells. After transfecting into HeLa cells, the killing activity of chicken CASP18 proteins was assessed by monitoring mCherry (a variant of red fluorescent protein)-positive transfected cells in the presence or absence of cytokine response modifier A (CrmA) or baculovirus P35 (Supplementary Fig. S2). CrmA is known as a viral product that specifically blocks the protease activity of CASP8 whereas P35 plays a role as a pan-caspase inhibitor [14, 15]. The number of mCherry-positive cells that co-expressed with chicken CASP18 decreased compared to the number of control cells, suggesting that many transfected cells died and disappeared. When either CrmA or P35 inhibitor was additively expressed, the number of mCherry-positive cells was recovered, indicating that CASP18-induced cell death is prevented by these inhibitors.

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FIGURE LEGENDS

Figure S1. Evolutionary diversity and convergence of *CASP8* and its paralogous genes. **A:** The chromosomal location of the *casp8* gene and its paralogous genes in fish. Both *casp8* and *cflar-like* genes were identified in the lamprey genome. The question mark suggests it is unknown whether two genes are linked or separated. As the *cflar* gene was identified as the gene straddling between two Scaffolds, JH127063 and JH126818 in the coelacanth genomic database, it is probable that four genes localize in the same locus. The *cflar* gene is separated from other three genes in spotted gar. Furthermore, both *casp8* and *casp10* genes were segregated and a new gene, *card-casp8* appeared in bony fish such as stickleback. **B:** The conservation of the *CASP8* and other paralogous genes in animals from lobe-finned fish to marsupials. However, the *Xenopus CASP18* gene lacks the region corresponding to CASc. The *CASP18* gene was deleted from the original place in eutherians such as human and dog. **C:** The loss of the *Casp10* gene in mouse, but not in guinea pig and squirrel, of the rodent family. The *CASP10* gene was identified in the genome databases of animals belonging to Hystricomorpha and Sciuromorpha. Abbreviated genes: *abca1*, ATP-binding cassette subfamily A member 1; *ALS2CR12*, amyotrophic lateral sclerosis 2 chromosomal region candidate gene 12 protein; *ap1m1*, AP-1 complex subunitmu-1; *BZW1*, basic leucine zipper and W2 domain-containing protein 1; *catip*, ciliogenesis associated TTC17 interacting protein; *dytn*, dystrotelin; *NDUFB3*, NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3; *STRADB*, STE20-related kinase adaptor beta; *TRAK2*, trafficking protein, kinesin binding 2. The blue bold arrows indicate the coding region and orientation of the gene. Genes were searched from the databases,

Pmarinus_7.0 (lamprey), LatCha1 (coelacanth), LepOcu1 (spotted gar), Zv9 (zebrafish), JGI_4.2 (*Xenopus*), Galgal4 (chicken), BROADO5 (opossum), CanFam3.1 (dog), GRCh38 (human), cavPor3 (guinea pig), spetri2 (squirrel), GRVm38 (mouse) of Ensemble and AWM45574v1 (alligator) of NCBI.

Figure S2. Cytotoxicity assay of human HeLa cells expressing chicken CASP18. The pCMV-GgCASP18 plasmid encoding chicken CASP18 and the pCAG-mCherry plasmid were cotransfected into HeLa cells with or without pCAG-CrmA or pCAG-P35. CrmA is a CASP8-specific inhibitor and P35 is a pan-caspase-inhibitor. After 2 days of culture, cells were washed to remove floating cells, and then fixed; viability was determined by monitoring red fluorescence-positive cells. Both phase-contrast and fluorescence images were captured for each field under the microscope. Scale bars represent 100 μm.

Figure S3. Identification of adaptor proteins APAF-1 and FADD in simple invertebrates. **A:** Schematic representations of protein structure of human, coral (*A. millepora*), placozoan (*T. adhaerens*) and calcareous sponge (*S. ciliatum*) APAF-1 proteins. All proteins contain CARD, single NB-ARC (nucleotide-binding domain of APAF-1, R proteins and Ced-4), and several tandem repeated WD40 motifs. Abbreviation: TM, transmembrane domain. **B:** Multiple alignment of amino acid sequences of FADD proteins from human, *A. millepora*, sea anemone (*N. vectensis*) and sponge (*A. queenslandica*). Identical and similar amino acids are indicated by red and blue, respectively. The regions corresponding to DED and DD are indicated by color boxes, respectively. The α-helix structure elements are cited from the previous study [16]. Identification numbers are as follows: human (UniProt: O14727), *A. millepora* (GenBank accession number: KP170508), *T. adhaerens* (UniProt: B3SBJ1) and *S. ciliatum* (Compagen: scpid10642) APAF-1 proteins and human (UniProt: Q13158), *A. millepora* (GenBank accession number: KJ639817), *N. vectensis* (GenBank accession number: FC212566) and *A. queenslandica* (GenBank accession number: GW184847) FADD proteins.
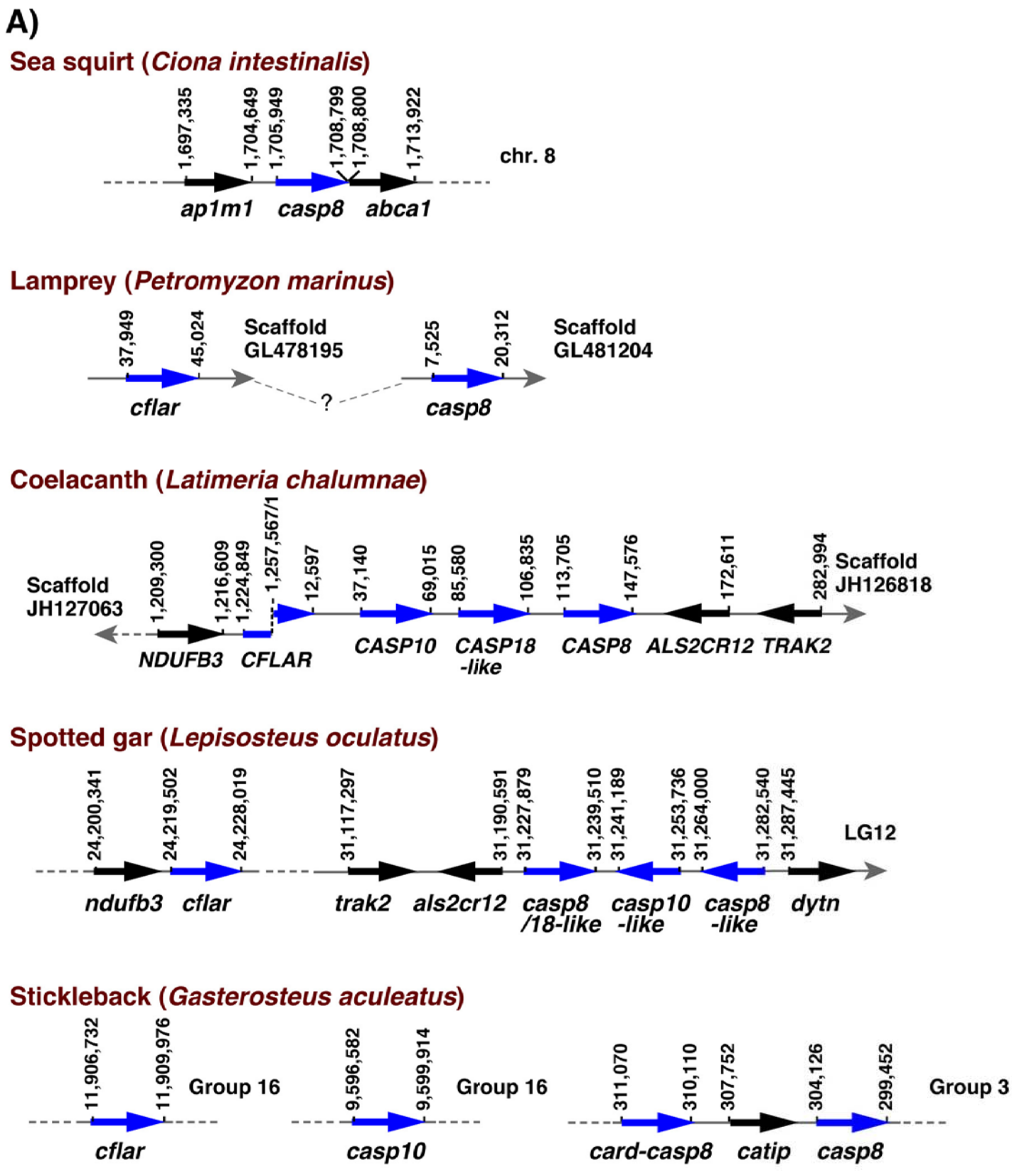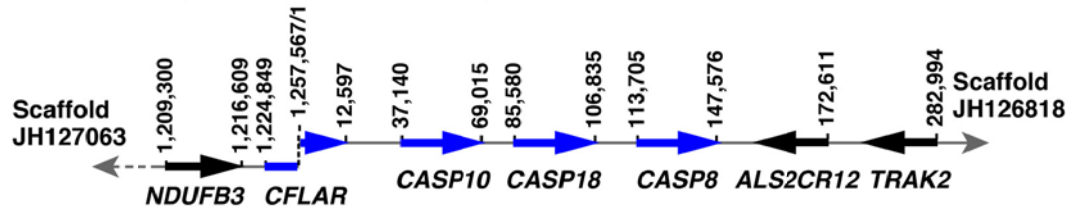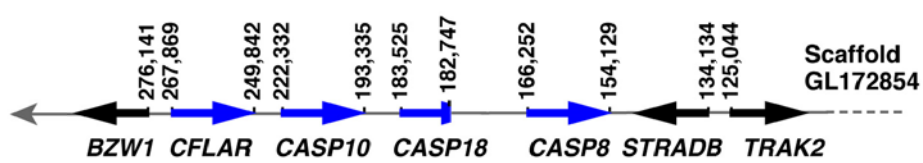
Figure S1

A)

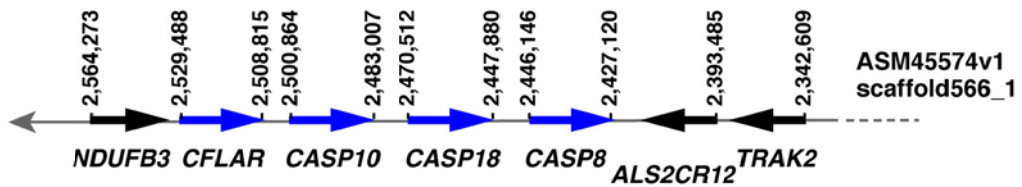### Sea squirt (*Ciona intestinalis*)

Lamprey (*Petromyzon marinus*)

Coelacanth (*Latimeria chalumnae*)

Spotted gar (*Lepisosteus oculatus*)

Stickleback (*Gasterosteus aculeatus*)
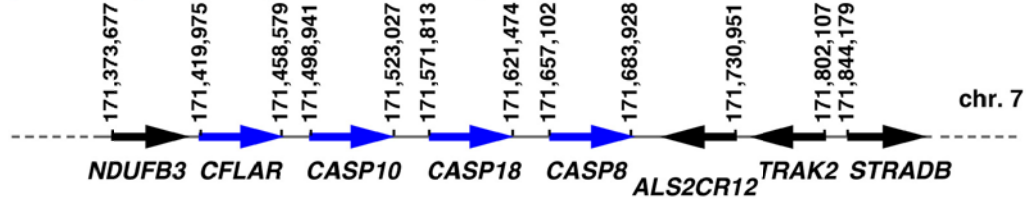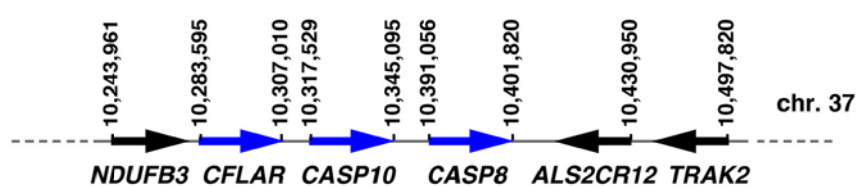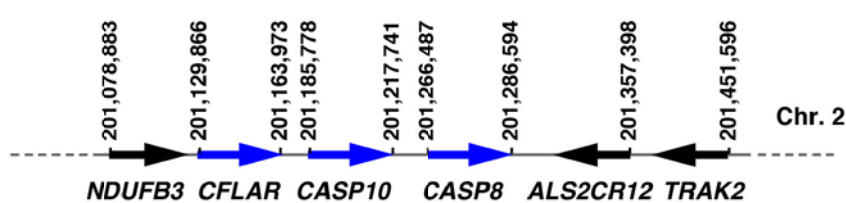
Figure S1

Figure S1 C)

Guinea pig (*Cavia porcellus*)

Scaffold 104

683,588 670,009 658,575 633,684 591,798 579,696

CFLAR   CASP10   CASP8

Squirrel (*Ictidomys tridecemlineatus*)

Scaffold JH393536

400,282 423,311 460,556 485,518 582,282 595,729

CFLAR   CASP10   CASP8

Mouse (*Mus musculus*)

Chr. 1

58,783,281 58,810,766 58,880,787 58,903,598

Cflar   Casp8

Figure S2

Empty vector

CASP18

CASP18 + P35

CASP18 + CrmA

Figure S3

A)

B)