REVIEW

Convergent evolution of defensin sequence, structure and function

Thomas M. A. Shafee[1] · **Fung T. Lay[1]** · **Thanh Kha Phan[1]** ·
Marilyn A. Anderson[1] · **Mark D. Hulett[1]**

Abstract Defensins are a well-characterised group of small, disulphide-rich, cationic peptides that are produced by essentially all eukaryotes and are highly diverse in their sequences and structures. Most display broad range antimicrobial activity at low micromolar concentrations, whereas others have other diverse roles, including cell signalling (e.g. immune cell recruitment, self/non-self-recognition), ion channel perturbation, toxic functions, and enzyme inhibition. The defensins consist of two superfamilies, each derived from an independent evolutionary origin, which have subsequently undergone extensive divergent evolution in their sequence, structure and function. Referred to as the *cis-* and *trans-*defensin superfamilies, they are classified based on their secondary structure orientation, cysteine motifs and disulphide bond connectivities, tertiary structure similarities and precursor gene sequence. The utility of displaying loops on a stable, compact, disulphide-rich core has been exploited by evolution on multiple occasions. The defensin superfamilies represent a case where the ensuing convergent evolution of sequence, structure and function has been particularly extreme. Here, we discuss the extent, causes and significance of these convergent features, drawing examples from across the eukaryotes.

Keywords Disulphide-rich protein · Protein superfamily · Evolutionary constraint · Divergent evolution · Evolvability · Antimicrobial peptide

Thomas M. A. Shafee and Fung T. Lay contributed equally to the work.

✉ Marilyn A. Anderson
    m.anderson@latrobe.edu.au

✉ Mark D. Hulett
    m.hulett@latrobe.edu.au

[1] Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, VIC 3086, Australia

Introduction

Defensins are one of the best-described groups of antimicrobial peptides, and are expressed by a wide array of plants, animals and fungi for host defence. These proteins are small (less than 10 kDa), cysteine-rich (forming three to six disulphide bonds) and are typically cationic (net charge inter-quartile range of +1 to +5). The defensins are best known for their antimicrobial activity at low micromolar concentrations against Gram-positive and Gram-negative bacteria, fungi, viruses, and parasitic protozoa [1–3]. Additionally, the defensin fold has proved highly evolvable, with defensin-like protein (DLP) families having divergently evolved to perform alternative functions to antimicrobial activity. Diverse cell signalling roles via interaction with cell-surface receptors have been described, such as involvement in immune cell recruitment in vertebrates [4] and self/non-self-recognition during fertilisation in plants [5–7]. The venoms from scorpions, spiders, platypus, snakes and lizards all contain protein families with defensin-like structures that disrupt ion channels [8–10]. Plants and sessile animals have also adapted them for enzyme inhibition functions to deter grazers and predators [11, 12].

The defensins from across the animal, plant and fungal kingdoms have recently been classified into two

superfamilies, the *cis*- and *trans*-defensins, each of which has an independent evolutionary origin [13]. The separation was established by analysis of 2714 defensin and defensin-like sequences and structures, covering 27 distinct disulphide connectivities (discussed in more detail in later sections) [13], in addition to the recently described sea anemone DLP, which constitutes a fifth *trans*-defensin fold [12]. Because their sequences are so divergent, sequence similarity and cysteine motifs are insufficient to resolve the more ancient evolutionary relationships; however, structural information has proved more useful in resolving these questions. Networks of structural similarity and topology separate the defensins into two groups, within each of which homology is statistically supported, but between which similarities are below the threshold of chance. This split results from incompatible differences in secondary structure and disulphide order and orientation between the two superfamilies [13].

The larger superfamily is named the *cis*-defensins, derived from the two parallel disulphides that bond the final β-strand to an α-helix. This superfamily, which is dominated by plant defensins, contains 11 of the structurally characterised defensin motifs and 11 motifs with currently unsolved structures. Conversely, members of the *trans*-defensin superfamily (accounting for the five remaining disulphide motifs) have two analogous disulphides that point in opposite directions from the final β-strand and thus bond to different secondary structure elements (Fig. 1a, b) [13].

This evolutionary analysis has addressed the historical difficulties in classifying defensins by coupling primary sequence information, with secondary structure orientation, disulphide bond connectivities and tertiary structure similarities. This extends the classifications in the existing defensin-specific databases (e.g. the Defensins Knowledgebase [14] and iDPF-PseRAAAC [15]). It is also relevant for resolving relationships within the ever-expanding small cysteine-rich protein and peptide databases such as the manually curated APD (and its subsequent updates) [16–18] and machine-learning databases and prediction servers, such as CAMP [19], iAMP-2L [20], LAMP [21], PhytAMP [22], YADAMP [23], and ATDB [24].

The extant defensin structural classes, therefore, represent the divergent evolution of two ancestral folds to a variety of elaborated structures that specialise the defensins to their diverse functions. Within these structural classes, the inter-cysteine regions have undergone further extensive divergent evolution, to the extent that defensins of the same fold often display only chance sequence identity. Given their independent evolutionary origins and subsequent divergent evolution, the *cis*- and *trans*-defensin superfamilies display remarkable convergent evolution of a diverse array of traits. This review explores the known distribution of defensins in the two superfamilies, and how members have undergone convergences at the levels of gene and precursor protein organisation, protein sequence and structure, and how this has translated to functional and mechanistic convergences. Furthermore, the evolutionary pressures, constraints and solutions that have caused this convergence and divergence are discussed.

Phylogenetic distribution

With a few notable exceptions, *cis*- and *trans*-defensins are produced by different phyla [13] (Fig. 1c). Most *trans*-defensins occur in vertebrates (fish, reptiles, birds and mammals), with big defensins produced in some molluscs, arthropods and basal chordates (lancelets), and anemone DLPs produced in cnidarians. The greatest exception to this distribution is the presence of transcripts encoding *cis* β-defensins in two spiny lobster species of arthropods [25]. Conversely, *cis*-defensins occur in a wider array of animals: hydra, annelids, nematodes, arthropods, molluscs, and lancelets. They are also common in fungi, and spermatophyte plants [26, 27].

Genes from both defensin superfamilies are present in lancelets, and some arthropods and molluscs [27–30]. Within the multicellular eukaryotes, defensins have yet to be described in the non-spermatophyte plants (e.g. bryophytes, monilophytes), non-chordate deuterostomes (e.g. echinoderms), and the non-arthropod/nematode ecdysozoans (e.g. tardigrades). This broad and patchy distribution of defensins may be the result of repeated gene loss in multiple lineages [31] or extensive horizontal gene transfer between phyla, as has been documented for other host defence genes [32, 33]. Although "defensin-like" sequences of prokaryotic origin have been reported, these have only four cysteines and lack any other sequence similarity [34]. Therefore, in the absence of structural information, it is not yet possible to assert their relatedness [13].

Most of the disulphide connectivities are unique to a phylum; however, two *cis*-defensin disulphide connectivities are broadly distributed across multiple eukaryotic kingdoms. They are termed the C6 and C8 defensins in reference to their number of cysteines (Fig. 1c). The C8 defensins are found in plants, molluscs and insects and are mostly antimicrobial (Table 1). C6 defensins are distributed even more broadly and contain members with antimicrobial activity in invertebrates, plants and fungi, as well as members with signalling roles in plants and toxic roles in chelicerates.
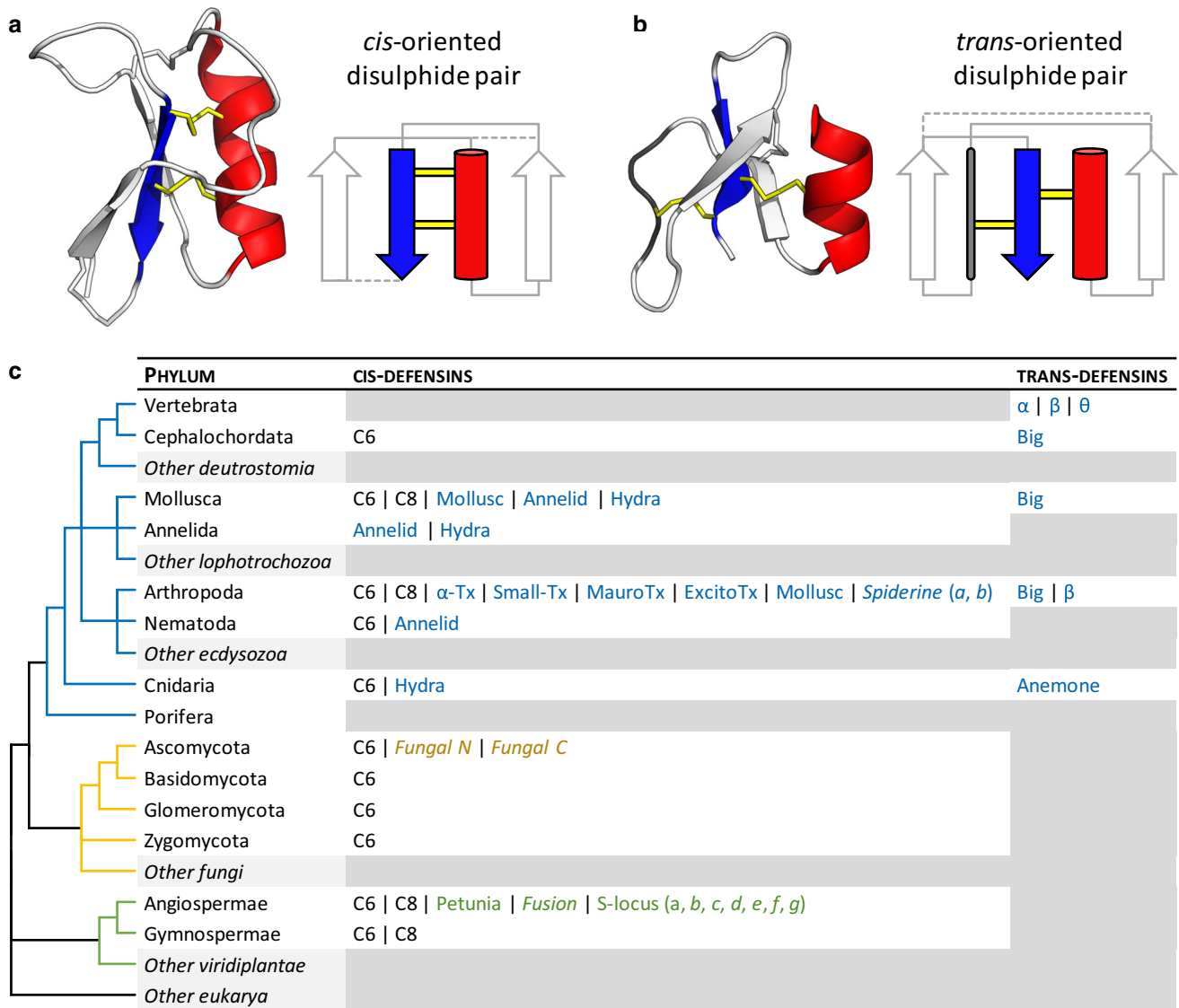
Fig. 1 Architecture and taxonomic distribution of *cis*- and *trans*-defensins. **a** The plant defensin NaD1 (PDB:1MR4) is a typical *cis*-defensin in which both of the most conserved disulphides (*yellow*) from the final β-strand (*blue*) point in the same direction and bond to the same α-helix (*red*). **b** The human β-defensin HBD-1 (PDB:1IJV) is a typical *trans*-defensin in which the disulphides from the final β-strand point in opposite directions, therefore, bonding to different secondary structure elements. Non-conserved disulphides are represented as *dashed lines* in the secondary structure diagrams. Adapted from [13]. **c** A simplified phylogeny of eukaryotic phyla, annotated with the occurrence of different structural classes and cysteine motifs (in *italics*) from each defensin superfamily. Classes specific to a kingdom are coloured as in the phylogeny. Classes are described in more detail in Figs. 4 and 5. Phyla with no known defensins from each of the superfamilies are filled in *grey*

Gene and precursor protein convergence

Gene copy number

Defensins from both superfamilies can be present in multiple copies in the genome of an organism, having evolved by tandem gene duplication with subsequent sequence diversification [46–49]. For instance, over 300 defensin and defensin-like sequences have been identified in *Arabidopsis* and *Medicago* [48, 50, 51]. Orthologues frequently derive new functions (i.e. neofunctionalise) due to positive selection, a common feature of host immune proteins co-evolving against pathogens or parasites as the host competes in an arms race [52, 53].

The β-defensin gene clusters on chromosome 8p23.1 form one of the most copy number variable regions in the human genome [54] and gene copy number variation correlates with a range of disease susceptibilities. The *DEFB4* gene, encoding human β-defensin-2 (HBD-2) has been particularly well studied in this regard where increased

Table 1 Distribution and functions of examples from the shared C6 and C8 *cis*-defensin scaffolds

	Taxon	Function	Example	Species	Accession	References
C6	Plant	Unknown	Nodule defensin	*Astragalus sinicus*	77994681	[ds]
		Fertilisation	LURE1	*Torenia fournieri*	225320707	[7]
	Fungus	Antibacterial	Micasin	*Arthroderma otae*	2LR5	[35]
	Cnidarian	Antifungal	Galiomicin	*Helicoverpa zea*	528880428	[ds]
	Cephalochordate	Unknown	BfD1	*Branchiostoma floridae*	260803302	[29]
	Nematode	Unknown	CreD1	*Caenorhabditis remanei*	308463700	[ds]
	Mollusc	Antibacterial	MGD-1	*Mytilus galloprovincialis*	1FJN	[36]
	Insect	Antibacterial	Nasonin-1	*Nasonia vitripennis*	2KOZ	[37]
	Ixodid	Antibacterial	Varisin A1	*Dermacentor variabilis*	37999545	[38]
	Arachnid	Antibacterial, antifungal	oh-Defensin	*Ornithoctonus hainana*	none	[39]
	Chelicerate	Antibacterial	LqD1	*Leiurus quinquestriatus*	1169262	[40]
		Ion channel toxin	Bmtx2	*Mesobuthus martensii*	2BMT	[41]
C8	Plant	Antifungal	NaD1	*Nicotiana alata*	1MR4	[42]
		Serine protease inhibitor	ATT	*Arabidopsis thaliana*	1JXC	[11]
		Sweet taste	Brazzein	*Pentadiplandra brazzeana*	1BRZ	[43]
	Mollusc	Induced by bacteria	Hs-defn	*Hyriopsis schlegelii*	339646140	[44]
	Insect	Antifungal	Drosomycin	*Drosophila melanogaster*	1MYN	[45]

ds direct submission to NCBI database

DEFB4 gene copy number was associated with psoriasis [55, 56]. For Crohn's disease, the findings have been mixed with separate studies reporting correlations with low [57] or high [58] *DEFB4* gene copy number. In addition, β-defensin gene copy number may also contribute to susceptibility to other conditions and diseases such as HIV infection [59], cervical cancer [60] and ankylosing spondylitis [54].

Protein biosynthesis, processing and trafficking

All known *cis*- and *trans*-defensins are processed from precursor proteins during maturation and trafficking. Given the presence of cysteine residues that participate in disulphide bonds, defensins are produced with N-terminal endoplasmic reticulum (ER) signal sequences (Fig. 2a). The mature defensin is secreted in the absence of any other signalling information.

Defensins that are targeted to intracellular locations (e.g. vacuole or phagolysosome) have additional prodomains (also referred to as propeptides, prosequences, prosegments or propieces) [61–66]. This targeting prodomain can either precede or follow the mature defensin domain (Fig. 2b, c). For instance, plant class II defensins (mainly represented in the Solanaceae family) have long anionic prodomains on the C-terminal side of their C8 *cis*-defensin domains, in contrast to the more common plant class I defensins, which lack a prodomain and are secreted [42, 62]. Analogous negative prodomains are located at the N-terminus of mammalian α-defensins from the *trans*-defensin
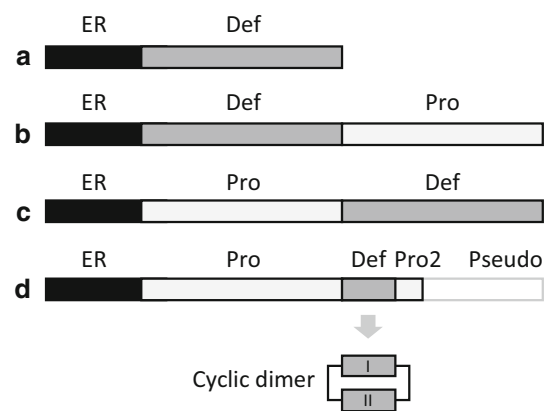

Fig. 2 Organisation of defensin precursor proteins. All defensins are produced with N-terminal endoplasmic reticulum (ER) signal sequences (to direct them to the ER for disulphide bond formation) in addition to the mature defensin domain (Def). Examples of defensins that adopt this structure include *a* scorpion C6 and plant C8 class I defensins. Other defensins are produced with additional prodomains (Pro) that can be positioned *b* C-terminally (e.g. mussel, and plant C8 class II defensins) or *c* N-terminally (e.g. insect C6 and vertebrate α- and β-defensins) of the mature domain. *d* θ-defensin precursors are truncated α-defensin prologues with a premature stop codon after the first 12 residues, from which a 9-mer fragment is excised, dimerised, and ligated to create the backbone-cyclised θ-defensin. The sequence after the stop codon is still highly similar to the α-defensin (Pseudo). Domain lengths not to scale

superfamily [67]. The fungal N-terminal and C-terminal defensin classes only occur as a two-domain fusion, and are proteolytically processed into two mature defensins [68] (Fig. S1). The plant 'fusion' class is similarly only found

fused to a C8 defensin in a two-domain gene and it is currently unknown whether or not it is proteolytically processed.

Reconstruction of ancestral α-defensin sequences indicates their acidic prodomains have co-evolved to compensate for the basic amino acids in the mature defensins [67]. Therefore, in addition to its role as a targeting signal, the prodomain either has a chaperone function to assist folding or protects against autocytotoxicity by shielding the extreme positive charge of the mature defensin from deleterious interactions with lipids or other cellular proteins [63, 69–73]. Similar targeting sequences are located between the N-terminal ER signal and mature defensin domains of other non-secreted defensins [28, 64, 74].

The complete activation of preprodefensins often involves a two-step process: cleavage of the ER signal peptide producing an inactive prodefensin, followed by removal of the prodomain [62, 63, 65]. The mature defensins can be stored as fully processed active proteins such as in HNP-1–4, which reside primarily in the intracellular compartment of the phagolysosome [64, 75, 76]. This is akin to the mature class II plant defensins that are stored in the plant vacuole [42, 62, 73]. In contrast, other defensins (e.g. human Paneth cell α-defensins HD-5 and HD-6) are stored as inactive prodefensins in secretory granules that are destined for extracellular activities in the intestinal lumen [77–79]. These defensins are activated proteolytically by a Paneth cell-derived trypsin after they are secreted [75]. In mice, the Paneth cell α-defensins (known as cryptdins) [80, 81] are activated by removal of the prodomain by matrix metalloproteinase-7 (matrilysin, MMP-7) [82] before secretion [69]. The importance of proteolytic removal of the prodomain for defensin activation is highlighted by the observation that mice deficient in the MMP-7 protease do not produce mature cryptdins and are more susceptible to oral challenges with *Salmonella typhimurium* bacteria [82].

The precursor proteins of θ-defensins are especially unusual. The prodomains are homologous to full-length α-defensins and undergo unique processing, in which two nine amino acid segments from two prodefensins are cyclised head-to-tail by transpeptidation to form a single 18-amino acid mature cyclic protein [83] (Fig. 2d). The cyclic product can consist of a homodimer produced by ligation of two identical precursors, or heterodimers from ligations of different precursors [84, 85]. Heterodimers are strongly favoured, although the mechanisms controlling their ligation are not yet known [86, 87]. Human θ-defensin pseudogenes are not expressed due to a premature stop codon in their precursor, which may contribute to the human susceptibility to HIV as compared to the resistance in old world monkeys [83].

Individual defensins from the *cis-* and *trans-* superfamilies are expressed under specific circumstances or at specific sites. For instance, they often have distinct, organ-specific expression patterns, particularly in tissues that are vulnerable to microbial attack, such as nutrient-rich reproductive tissues, root nodules and seeds in plants or epithelial tissues and neutrophils in animals. They can also be expressed constitutively or induced by infection and inflammatory factors [42, 48, 51, 88]. Mice cryptdins, for instance, constitute ∼70 % of the bactericidal activity that is secreted by the Paneth cells, with the concentration of cryptdins at the point of secretion in the intestinal mucosa reaching levels that are at least 1000 times greater than the antibacterial minimal inhibitory concentration (MIC) [89]. In humans, α-defensin HD-5 is stored at approximately 90–450 μg/cm^2 of the surface of the intestinal mucosa, sufficient to generate microbicidal concentrations in the lumen [75].

Structural convergence

Primary structure

The *cis-* and *trans*-defensin superfamilies have convergent features across their primary, secondary and tertiary structures. Both superfamilies are extremely sequence diverse. The inter-cysteine loops of homologues from the same phylogenetic order often share less than 20 % amino acid sequence identity and have multiple insertions and deletions. Despite this, there are several convergent sequence features between the 1820 *cis*-defensins and 894 *trans*-defensins [90]. Foremost, their sequence composition is highly biased towards cysteines, positively charged amino acids (arginine and lysine) and glycine, at the expense of the aliphatic hydrophobic residues (valine, leucine, isoleucine and methionine) which form the hydrophobic cores of globular proteins [91] (Fig. 3a). This amino acid bias parallels the overall hydrophilic and net positive charge distributions of proteins from both superfamilies (Fig. 3b–g).

The only non-cysteine residue that is broadly conserved within each superfamily is a glycine in a GxC motif. This motif occurs in 91 % of *cis*-defensins (excluding the S-locus proteins which have an additional disulphide at the homologous location) and 92 % of the α-helix-containing *trans*-defensins, with alanine being the most common alternative (Fig. 3h). This motif is a consequence of the disulphides, which constrain the β-strand such that the glycine's hydrogen side chain points back towards the α-helix (Fig. 3i, j). The R-groups of other residues cannot be accommodated in such a confined space and thus the potential steric clash causes them to be selected against. In
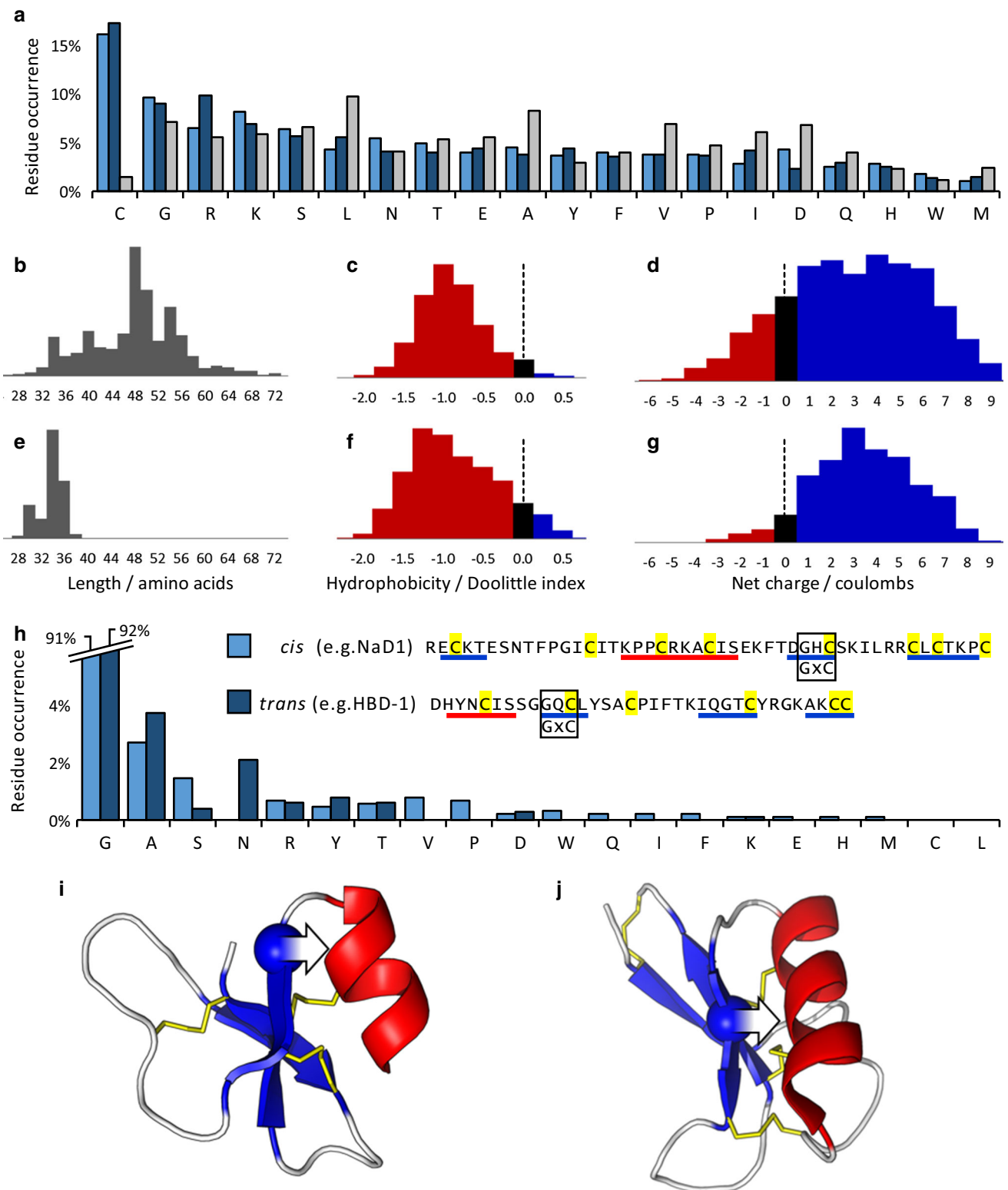
a

b, c, d, e, f, g

Length / amino acids   Hydrophobicity / Doolittle index   Net charge / coulombs

h

cis (e.g.NaD1) RECKTESNTFPGICITKPPCRKACISEKFTDGHCSKILRRCLCTKPC
GXC

trans (e.g.HBD-1) DHYNCISSGGQCLYSACPIFTKIQGTCYRGKAKCC
GXC

i

j

Fig. 3 Amino acid sequence properties of *cis-* and *trans*-defensins. **a** Average amino acid residue occurrence for the *cis*-defensins (*light blue*), *trans*-defensins (*dark blue*) and whole Uniprot database (*grey*). Distributions of length, hydrophobicity and charge for **b–d** 1820 *cis*-defensins and **e–g** 894 *trans*-defensins. The common GxC motif occurs in both *cis*-defensins (e.g. NaD1) and *trans*-defensins (e.g. HBD-1). **h** Residue bias in the first position of the GxC motif in the *cis*-defensins (excluding S-locus and spiderines, which have an additional disulphide at this location) and the *trans*-defensins (excluding α- and θ-defensins, which lack an α-helix and so are unconstrained at this location). In both **i** *cis*-defensins (PDB:1MR4) and **j** *trans*-defensins (PDB:1IJV), the glycine (*sphere*) is oriented such that a non-hydrogen R-group on any other amino acid in this position (*arrow*) would clash with the α-helix. β-Strands in *blue*, α-helices in *red*, disulphide bonds in *yellow*

this way, the constraints intrinsic to building similar cationic, disulphide-rich proteins cause convergence of both an overall residue bias, and the independent appearance of a defined GxC arrangement.

Secondary and tertiary structure and disulphide connectivity

Both defensin superfamilies convergently use a double- or triple-stranded β-sheet (typically with an α-helix), cross-linked by a disulphide network into a compact core (Fig. 1a, b). Residues characterised as functionally important typically have highly solvent-exposed cationic side chains that bind to anionic ligands on the target. They may be located in the core (as in charybdotoxin) or on the displayed loops (as in NaD1) [26, 92–94]. Each superfamily has a conserved disulphide connectivity, which has been elaborated by divergent evolution to produce 22 *cis*-defensin and five *trans*-defensin classes with distinct, additional disulphides (Figs. 4, S2).

The disulphide bonding imparts another common feature to the defensins: their high stability to temperature, pH and proteolysis [42, 95–97]. The presence of disulphides limits the conformation entropy of the unfolded state and sterically occludes proteases. This may also account for the evolvability of the defensins, as disulphides make the structure robust to mutations in the loop regions, allowing extreme sequence diversification of the superfamilies [98].

The diversity of disulphide connectivities in the *cis*-defensins (Fig. 4) is generally derived from elaboration of the common C6 motif found in all eukaryotic kingdoms (Fig. 5a). Although such ancient evolutionary relationships are unresolved, it is tempting to speculate that the C6 class represents the ancestral fold of the *cis*-defensin superfamily. The C8 defensins, for example, have an additional disulphide compared to the C6 class, which constrains their longer N- and C-termini. The C10 petunia *cis*-defensins further elaborate on their C8 counterparts with a fifth disulphide which does not change the orientation of

secondary structure elements, but substitutes for the network of non-covalent interactions that are present in the C8 defensins [42, 99]. Conversely, fewer *trans*-defensin structural classes have been identified, but each is far more distinct from other classes of the superfamily (Fig. 5b).

Within each superfamily, insertion of secondary structure elements has generated different elaborations on the same core structure. Some such exemplifiers include the annelid and hydra 'macin' defensins and the big defensins (Fig. 5). These structures can be twice the size of the smaller members and have multiple insertions within their loops relative to smaller antimicrobial defensins, although they retain a similar charge density and hydrophobicity [13]. Even within each scaffold, families have divergent inter-cysteine loop lengths and composition, which adapts them to alternative functions. For example, scorpion toxins that use the C6 defensin fold have a shorter and more hydrophilic first loop for binding to their target ion channels [92]. Conversely, several cysteine pattern classes can be involved in the same function; for example, signalling by the seven S-locus 11 disulphide variant subclasses a–g (Figs. 5a, S2).

Quaternary dimerisation and oligomerisation

Several *cis-* and *trans*-defensins form homodimers or higher order oligomers [100–104]. The increased local charge density on the multimers is proposed to contribute to their high potency, broad-spectrum antimicrobial activity (elaborated further in the next section) [102, 105–107]. For such defensins in homogeneous solutions, there is an equilibrium between dimers and higher oligomers but dimers are the most prominent form adopted by the plant *cis*-defensins NaD1 (Fig. 6a) and TPP3 [100, 101], and the human *trans*-defensins including β-defensin HBD-2 (Fig. 6b) [102], and α-defensins HNP-3, HNP-4, HD-5 and HD-6 [103, 104]. The solved structures of specific human α-, β- and plant defensins are dimers with a six-stranded antiparallel β-sheet across the dimer interfaces. These dimeric structures have been proposed to provide a platform for lipid bilayer attachment and permeabilisation for innate defence against pathogens [2, 100]. It remains to be ascertained whether oligomerisation is a common feature for other *cis-* and *trans*-defensins.

NaD1 and TPP3 homodimers display a grip-shaped, cationic binding pocket, termed the "cationic grip" [94, 101]. The inner face of the cationic grip for NaD1 binds the anionic head group of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) via a network of ionic and hydrogen bond interactions (Fig. 6a). The grip region is comprised mainly of loop residues 36–40 (KILRR) in NaD1 and residues 37–41 (KLQRK) in TPP3. These loops are critical for lipid binding as well as for the
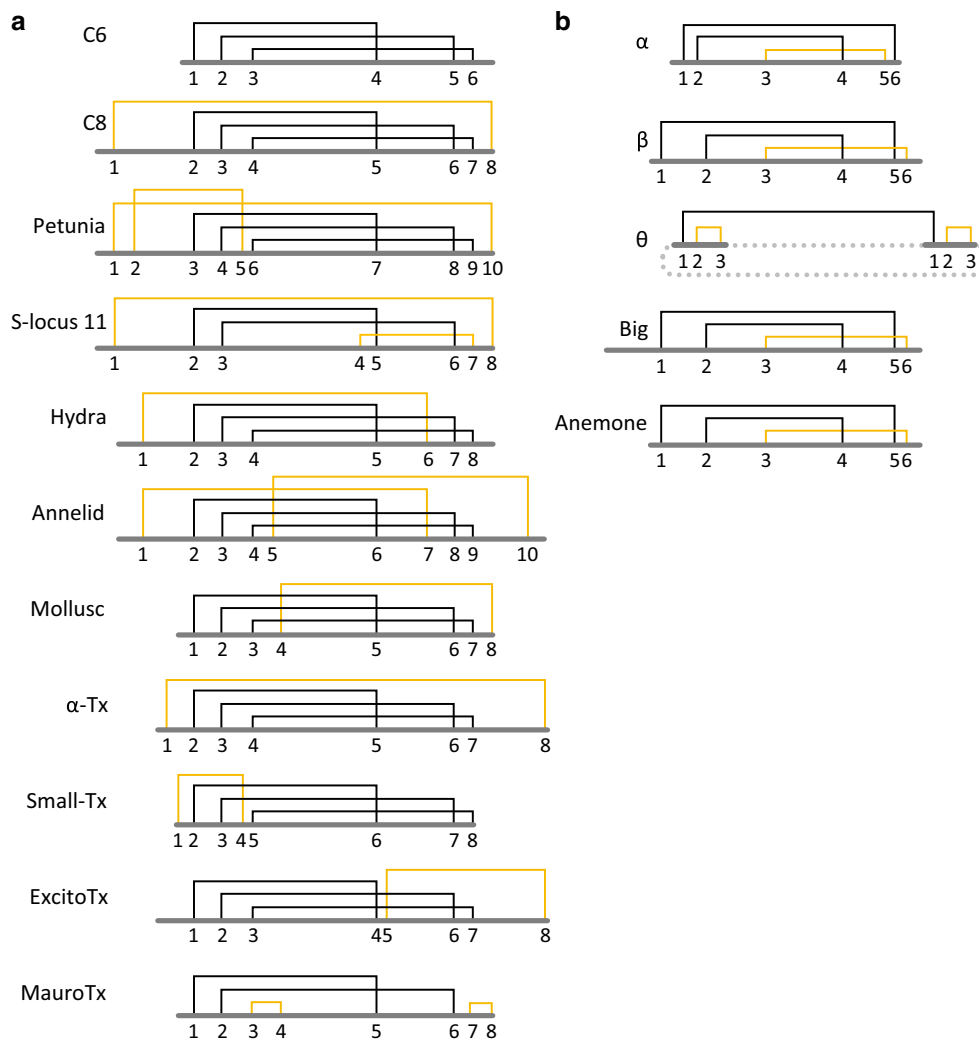
Fig. 4 Defensin disulphide connectivities. Disulphide connectivities for the **a** *cis*-defensins and **b** *trans*-defensins. The most highly conserved disulphides are indicated in *black* and disulphides that are unique to each class are indicated in *yellow*. The *dashed line* indicates cyclisation of the θ-defensin

antifungal and anticancer activities of these defensins [94, 100, 101]. The homologous loop of the *Medicago truncatula* defensin (MtDef4) consists of RGFRRR, which has been proposed to mediate antifungal activity by binding to phosphatidic acid (PA) as well as promoting entry into the fungal cell [108]. Whether dimerisation and oligomerisation is important for this PA interaction and antifungal activity has yet to be determined.

Human HBD-2 contains an analogous cationic loop that connects the first two β-strands. This loop, comprised of residues 22–25 (RRYK), forms a strikingly similar cationic grip structure in the HBD-2 dimer to that of the plant defensins NaD1 and TPP3 [102] (Fig. 6b). Despite a distinctly different dimer arrangement compared with HBD-2, HBD-6 also dimerises and forms a positively charged binding groove in the presence of glycosaminoglycan [109]. Therefore, the cationic binding pocket may be a common convergent feature in the structure–function relationship of defensin dimers.

For some defensins, high concentrations or the presence of ligand can promote the formation of higher order oligomers, illustrating an emerging role for defensin oligomers in innate host defence. HBD-2 oligomerises at high concentrations [102], NaD1 oligomerises in the presence of PIP_2 [94], and α-defensins HNP-1, HNP-2 and HD-6 oligomerise upon contact with artificial lipid membranes [110–112].

A high-resolution structure of an NaD1:PIP_2 complex was determined by X-ray crystallography and revealed an intriguing oligomeric arrangement. The oligomer comprises seven "cationic-grip" dimers of NaD1 in complex with the anionic head groups of 14 PIP_2 molecules. The seven NaD1 dimers assemble into an arch-shaped configuration with an extended cationic grove in which the
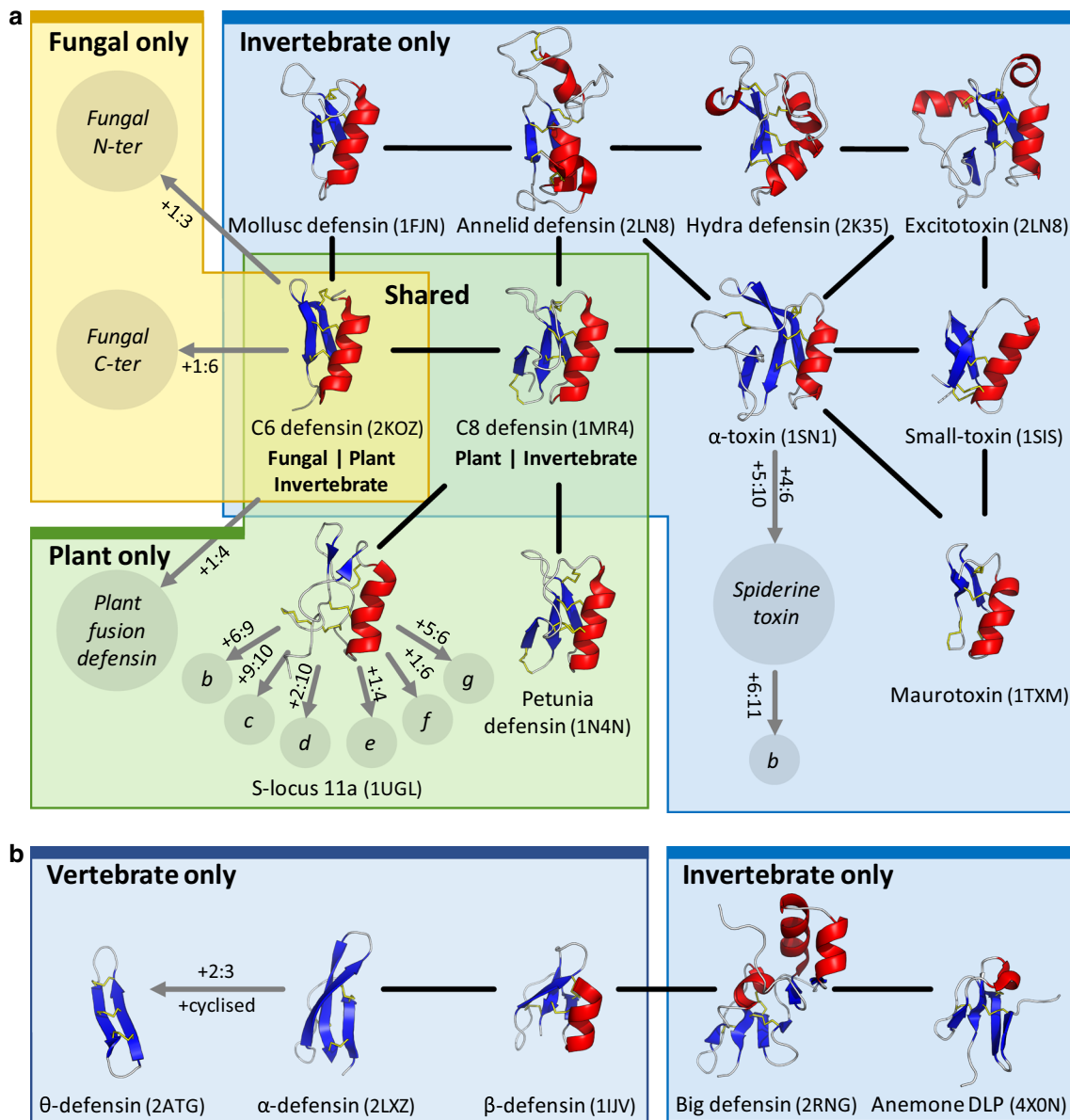
Fig. 5 Relatedness within the *cis-* and *trans-*defensins. Evidence for common origin in the **a** *cis-*defensins and **b** *trans-*defensins. Structures are shown for cysteine patterns with solved structures, classes with unresolved structures are represented by *italicised names in circles*. Putative disulphides unique to a class are denoted as *x:y* where *x* and *y* are the additional cysteines involved in the disulphide. Uncharacterised variants with additional disulphides are denoted by *single letters* (e.g. S-locus 11b, etc.). *Black lines* indicate homology evidence from structural similarity, *grey lines* indicate evidence from gene structure and organisation. The PDB codes for the proteins are given in *parentheses*. Structures are organised by kingdom, with a fungal representative as an example of the shared C6 defensins and a plant representative for the shared C8 defensins (*colours* as used in Fig. 1)

anionic lipid head groups are bound via a cooperative network of hydrogen bonds [94] (Fig. 6c). Interestingly, NaD1:PIP$_2$ complexes can assemble into long string-like fibrils in vitro, as revealed by transmission electron microscopy [94]. Whether such large oligomeric complexes can form in vivo and their functional importance remains to be determined.

A distinct oligomerisation event has been described for the α-defensin HD-6, which lacks direct antimicrobial activity, but self-assembles into ordered fibrils and nanonets to entrap bacteria [112]. The formation of multimeric transmembrane pores has also long been proposed for α-defensins, such as HNP-2 [111] and the C6 defensin, phormicin [113].
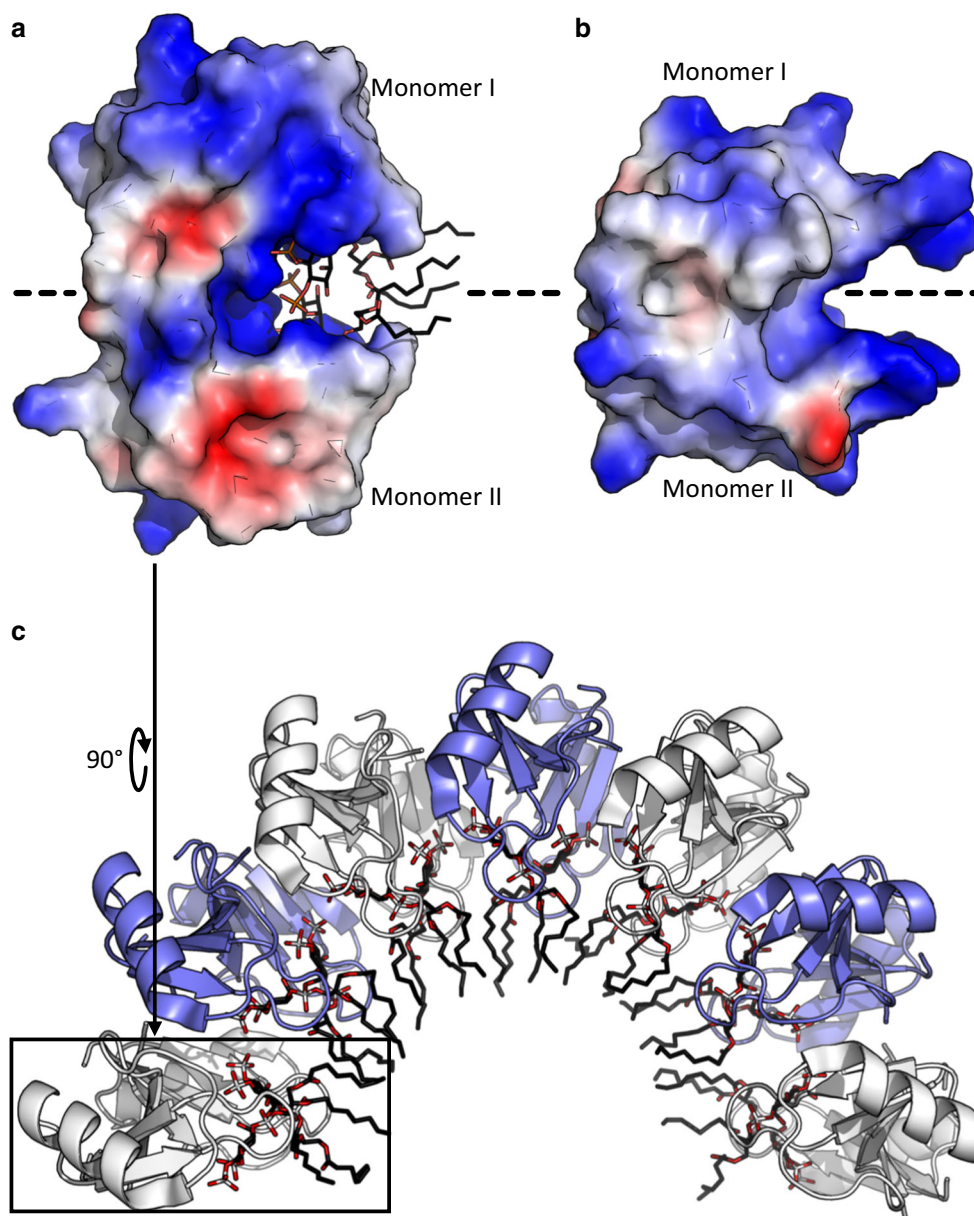
Fig. 6 Defensin dimerisation and lipid-mediated oligomerisation. **a** The plant C8 defensin NaD1 (PDB:4CQK) forms a homodimer that binds negatively charged phospholipid head groups via a cationic grip [94]. **b** The human β-defensin HBD-2 (PDB:1FD4) forms a structurally similar dimer [102]. Protein surface charge is indicated by *blue* (positive) and *red* (negative). Lipids are shown as *sticks* with phosphate in *white* and oxygen in *red*. **c** NaD1 dimers assemble into an arching oligomeric structure after interaction with the anionic head groups of PIP$_2$ within an extended cationic groove on the surface of the NaD1 oligomer (PDB:4CQK). Alternating dimers in *white* and *blue*

Functional and mechanistic convergence

Antimicrobial activity by targeting membrane lipids

Host defence by antimicrobial activity was the first described activity and is the most commonly reported function for members of both defensin superfamilies. It is also the likely ancestral role of each superfamily, with other functions having divergently evolved in various eukaryote taxa. Antimicrobial action is often achieved via complex, multi-step interaction mechanisms, which remain poorly characterised for the majority of defensins. These diverse mechanisms include interaction with cell wall carbohydrates, membrane transport machineries, cytoplasmic cell components, nucleic acids, or induction of reactive oxygen species, and typically cannot be generalised across even closely related defensins [114–121]. However, the most common antimicrobial mechanism of both superfamilies involves lipid binding that either directly disrupts membranes, inhibits lipid-dependent cell wall synthesis or

aggregates pathogens. Specific defensin-lipid interactions distinguish between host and pathogen by taking advantage of differences in cell wall and membrane composition. Vertebrate defensins additionally regulate the interaction between innate and adaptive immunity via signalling mechanisms, and are described in more detail in the next section [122, 123].

Lipid targeting is a property that extends throughout the antifungal C8 plant and insect *cis*-defensins. For example, glucosylceramide lipids located in the cell walls and plasma membranes of filamentous fungi are targeted by the plant defensins RsAFP2 [117], Psd1 [124] and MsDef1 [125] and the insect C6 defensin heliomicin [117]. Other plant defensins interact with structurally related membrane lipids such as mannosyl-diinositolphospho-ceramide (bound by DmAMP1) [126] and phosphatidic acid (bound by MtDef4) [108]. As mentioned, the *cis*-defensins NaD1 and TPP3 bind the phospholipid PIP$_2$ as does the *trans*-defensin HBD-3 [94, 101, 127, 128]. Indeed, the above-mentioned lipid binding loops of NaD1 and TPP3 are strikingly analogous to that of HBD-3 (residues 36-39, RGRK), suggesting a convergent 'phospholipid recognition code' between the defensin superfamilies [129].

Both superfamilies also contain members that bind the membrane-anchored lipid II peptidoglycan precursor, to block cell wall biosynthesis. Fungal C6 *cis*-defensins, including plectasin [130], oryzeasin [131] and eurocin [132] use this mechanism, and lipid II binding has also evolved in the mollusc *cis*-defensins MGD-1 and Cg-Def [36, 133]. Convergent use of lipid II binding is reported for the *trans*-defensins, human α-defensin 1 (HNP-1) [134] and human β-defensin 3 (HBD-3) [135].

Finally, binding to the cell surfaces of potential microbial pathogens by several *cis*- and *trans*-defensins may block pathogen adsorption and entry into host cells [136, 137], or cause aggregation of the microbes [112, 138]. Proposed aggregation mechanisms include the simultaneous binding of two microbial cells by a defensin with two hydrophobic interfaces, or by the formation of extended fibril networks [112, 138].

Achieving high affinity as well as specificity for a particular lipid in a pathogen's membrane requires high binding energy, whether for membrane disruption [128] or lipid extraction (of the order of 100 kJ mol^{-1} [139]). Hydrophobic interactions are typically neither energetic, nor specific enough to achieve this [140, 141] and consequently the proteins rely on multiple electrostatic contacts with the charged head groups (on the order of 10 kJ mol^{-1} energy each [142]. This contrasts with the non-specific plant lipid transfer proteins, which use an extensive binding tunnel to form hydrophobic interactions with fatty acyl lipid tails [143].

Signalling by receptor interaction

Both *cis*- and *trans*-defensin superfamilies have convergently evolved members with signalling activities. The two most common signalling functions are immune cell recruitment and self-recognition, mediated by interactions with cell-surface receptors. In the *trans*-defensin superfamily, multiple human α- and β-defensins selectively chemoattract leukocytes and stimulate cytokine release [144–147]. These immunomodulatory effects are mediated by interaction with a number of chemokine receptors. For human β-defensins, these receptors include CCR6 (immature DCs, neutrophil, T cells), CCR2 (monocytes), and Toll-like receptors (TLR) 1, 2 and 4 (monocytes, myeloid DCs and immature DCs) [147–151]. In contrast, human α-defensins HNP-1, 2 and 3 potently inhibit the phospholipid/Ca^{2+} protein kinase C-mediated signalling pathway [152]. They, therefore, link innate and adaptive immunity, and effectively enhance pathogen killing and clearance.

An example of signalling by an antimicrobial *cis*-defensin is the plant C8 defensin Psd1, which mediates its antifungal action against *Neurospora crassa*, not from direct membrane disruption but rather from protein internalisation and signalling via cyclin F, which interferes with nuclear division and disrupts the cell cycle [153]. This mechanism was supported by localisation of Psd1 to the nucleus and its interaction with cyclin F [153].

The largest group of signalling *cis*-defensins (the S-locus 11 class) functions in self/non-self-recognition during fertilisation in angiosperm plants and lack antimicrobial activity. S-locus protein 11 (SP11) variants, also referred to as S-locus Cysteine-Rich (SCR) proteins, are important signalling mediators of plant self/non-self-recognition in the sporophytic incompatibility system that prevents inbreeding [154]. SP11 variants or haplotypes are expressed by polymorphic genes that reside at the multi-allelic S-locus and serve as the pollen S-haplotype specificity determinants. They are paired with cognate stigmatic S-haplotype specificity determinants known as S-locus receptor kinases (SRKs), which are single-pass serine/threonine receptor kinases present in the plasma membrane of stigmatic papilla cells [6, 155]. In a self-pollination, binding of the pollen SP11 protein to the "self" SRK on the stigma leads to SRK autophosphorylation and results in pollen rejection. This is mediated by the initiation of a transduction pathway that results in the inhibition of pollen hydration and penetration of the pollen tube through the epidermal cell walls of the stigma. During cross-pollination, there is no interaction between SP11 and SRK and fertilisation proceeds unimpeded [6]. Binding to cognate SRKs is largely determined by the exposed loop regions between the third and fourth as well as the fifth and sixth cysteine residues in SP11, as identified by site-directed alanine mutagenesis and loop

swapping experiments [6]. This suggests that new allelic specificity has evolved readily in the SP11 folds. Indeed, over 100 haplotypes can exist in a given species, and consequently as many SP11 and SRK proteins [154, 156]. This is reflected in the extraordinarily high variation in protein sequence and seven different disulphide connectivities (Figs. 4a, S2) [13].

An additional plant fertilisation role is played by another group of defensins called LUREs (C6 defensin fold). LUREs are secreted by the two synergid cells on the side of the egg cell and act as diffusible, species-specific signals that chemoattract and guide the pollen tube to the ovule for fertilisation [7, 157].

The diverse signalling interactions by defensins from both superfamilies are a product of the small and stable defensin folds, which allow for the display of highly divergent loop sequences and the selection of molecules that form specific receptor interactions.

Toxic function by ion channel perturbation

Within the two defensin superfamilies, several subfamilies of animal DLPs have been converted to neurotoxic functions. These DLPs retain a defensin-like scaffold, but inter-cysteine loop sequences have been selected that enable specific interactions with ion channels, and they have typically radiated into large, diverse multigene families [158].

The *cis*-defensins include several classes from scorpions and spiders that are uniquely used for toxic functions, such as the α-toxins (note that α-toxins are not related to α-defensins) [159, 160] (Fig. 7a). Spiderines are based on an α-toxin scaffold with additional cysteines (up to 12 total) and, in some cases, an additional and unique N-terminal domain [8].

In addition to the classes that uniquely perform toxic functions, distinct subfamilies specialised to toxic function are present in both C6 *cis*-defensins and β-*trans*-defensins (Fig. 7a, b). In line with their highly divergent function, their sequences are clearly specialised [92]. Toxins of the C6 class, such as charybdotoxin, contain the conserved KCφN motif for ion channel binding, not present in antimicrobial defensins (Fig. 7c). They also lack the segregated, amphiphilic, cationic surface charge distribution typical of antimicrobial defensins, and have altered loop lengths to allow for specific interactions with ion channels, rather than lipids. Similarly, the β-defensin fold has been adapted to a toxic function in the well-characterised snake crotamines, as well as the putative toxins helofensin from bearded lizard venom and ovDLP-A from platypus venom [161, 162].

The toxic members from both superfamilies act by binding cation channels (K^+, Na^+, Ca^{2+}) with the exception of chlorotoxin, which binds Cl^- channels [163]. The best characterised of these is the scorpion charybdotoxin, which binds to voltage-gated K^+ channels and inserts a lysine to block the channel's selectivity filter (Fig. 7d) [92, 93]. The snake toxin crotamine (*trans*-defensin fold) is also proposed to bind and block voltage-gated K^+ channels via analogous residues R31-Y32 [164]. Conversely, a different region of the *cis*-defensin scaffold has been repurposed for toxic function in the scorpion excitatory toxins, such as Bj-xtrIT. These toxins bind Na^+ channels using the opposite surface to charybdotoxin [165], and likely interact with the channel's regulatory regions, rather than the pore itself [163].

It is, therefore, likely that ancestral antimicrobial defensins from both superfamilies were convergently neofunctionalised to toxicity by extensive adaptation of the sequence and length of their inter-cysteine loops for specific interaction with new ion channel targets. This convergent recruitment is indicative of the versatility of defensin folds when subjected to suitable selection pressures. Their short length and secretion is suitable for the large-scale expression required for toxin production [166]. The stability afforded by the disulphide-rich structures is beneficial for storage in venom sacs and persistence in prey, as well as allowing the sequence diversification of the loop regions [167]. Indeed, similar evolution from innate immunity function to toxicity has also occurred in other defence gene families where their mechanisms for pathogen defence are repurposed for offence [166].

Enzyme inhibition

Some plant defensins exhibit proteinase [11, 168, 169] and α-amylase [170–172] inhibitory activities, probably evolved to fend against insect predation. The tight, stable disulphide-linked topology of the defensins appears to make them well suited to enzyme inhibition. For instance, the *A. thaliana* trypsin inhibitor ATT uses the *cis*-plant C8 defensin fold to competitively inhibit PA clan proteases, such as trypsin [11]. The putative reactive site P1–P1′ residues are contained in the first solvent-exposed loop [11] (Fig. 8a). *Trans*-defensins including α- and θ-defensins are also protease inhibitors, but by non-competitive mechanisms, whereby they bind to the active site at a location other than the substrate binding site [173, 174].

More recently, a big-defensin-like protein from the sea anemone *Stichodactyla helianthus*, helianthamide, was identified with highly potent ($K_i = 10$ pM) and selective inhibitory activity against human pancreatic α-amylase [12]. Helianthamide adopts a four-stranded *trans*-defensin fold highly similar to the big defensins and binds into and across the α-amylase active site and is thought to act as an antifeedant. Three aromatic residues (Y7, Y9, and H10) constitute all of the important polar contacts of helianthamide with the enzyme's catalytic machinery, along with I11 and V12, which create a nonpolar interface to complement the hydrophobic ridges bordering the active site of
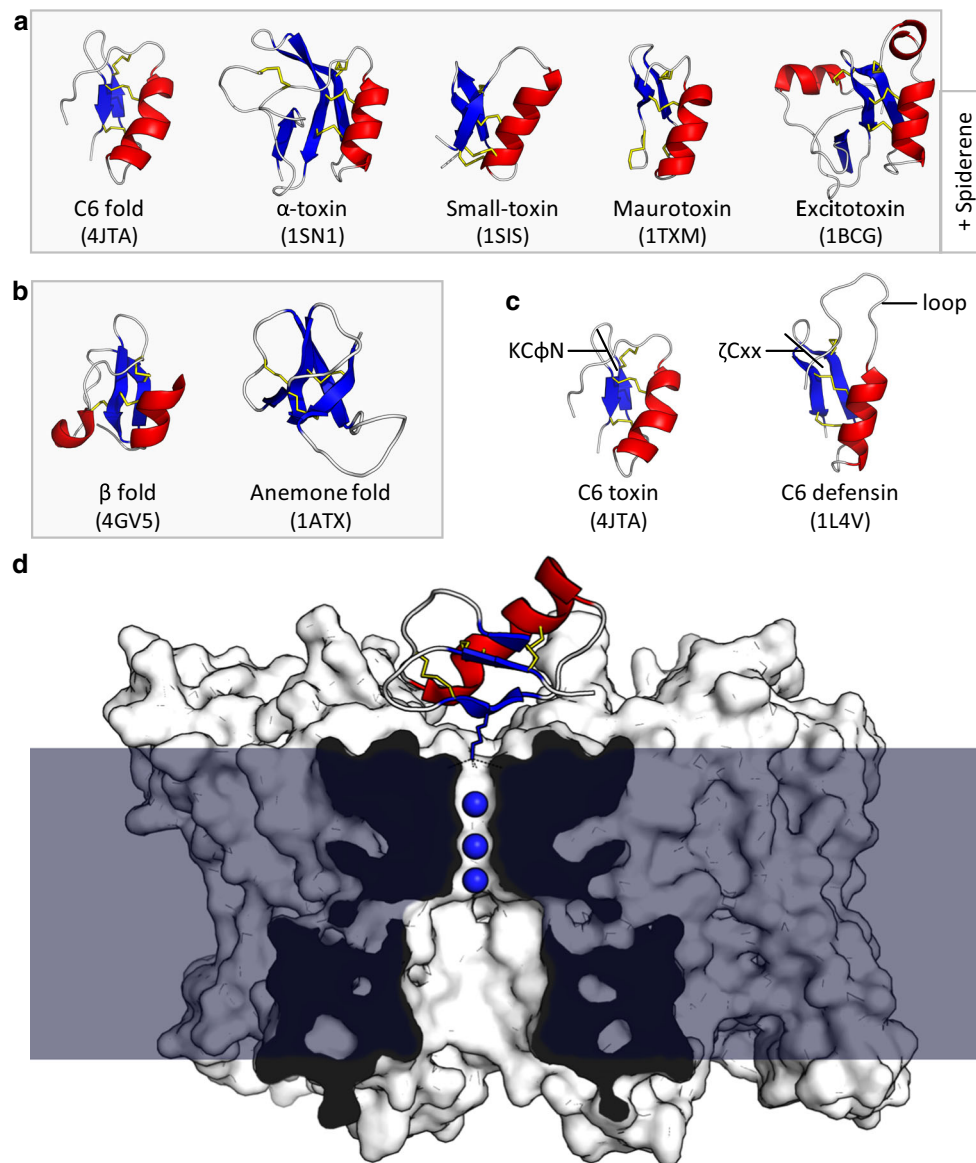
Fig. 7 Blocking of ion channels by defensin-like peptides. **a** The common *cis*-defensin C6 fold is adapted in some scorpion toxins, along with four toxin-specific structural classes with distinct additional disulphides. A structurally uncharacterised *cis*-defensin is also present in lynx spider venom (spiderine). **b** The *trans*-defensin fold has been recruited to toxic function such as crotamine in snakes, OvDLP from platypus and helofensin from bearded lizards. The anemone fold is also used in sea anemone neurotoxins. **c** Comparison of the C6 defensin fold with different functions. Toxins contain a conserved KCφN motif, whereas antimicrobial defensins contain the broader ζCxx motif at the same location, in addition to a large, flexible loop (φ = hydrophobe, ζ = hydrophile). **d** Charybdotoxin binds to the tetrameric K_v channel (*white surface*) and inserts a lysine residue into the first of the channel's four K^+ binding sites, blocking the transport of K^+ ions (*blue spheres*) through the cell membrane (*blue*) (PDB:4JTA) [93]

the enzyme [12]. It is interesting that both plants and sessile animals independently converted defensins to effective antifeedant activities to deter their respective grazers or predators.

Adaption to abiotic stresses

In addition to their induction by biotic stresses such as pathogen infection, the plant *cis*-defensins have been co-opted for response to abiotic stressors, including drought [175], salinity [176, 177], cold [178, 179] and metals [180]. For instance, the AhPDF1.1 defensin from *Arabidopsis halleri* (the only *Arabidopsis* species adapted to metal-contaminated soils and displaying high zinc and cadmium tolerance and hyper-accumulation capacities) has been functionally linked to conferring zinc tolerance in studies with yeast (*Saccharomyces cerevisiae*) and transgenic plants (*A. thaliana*) [180].
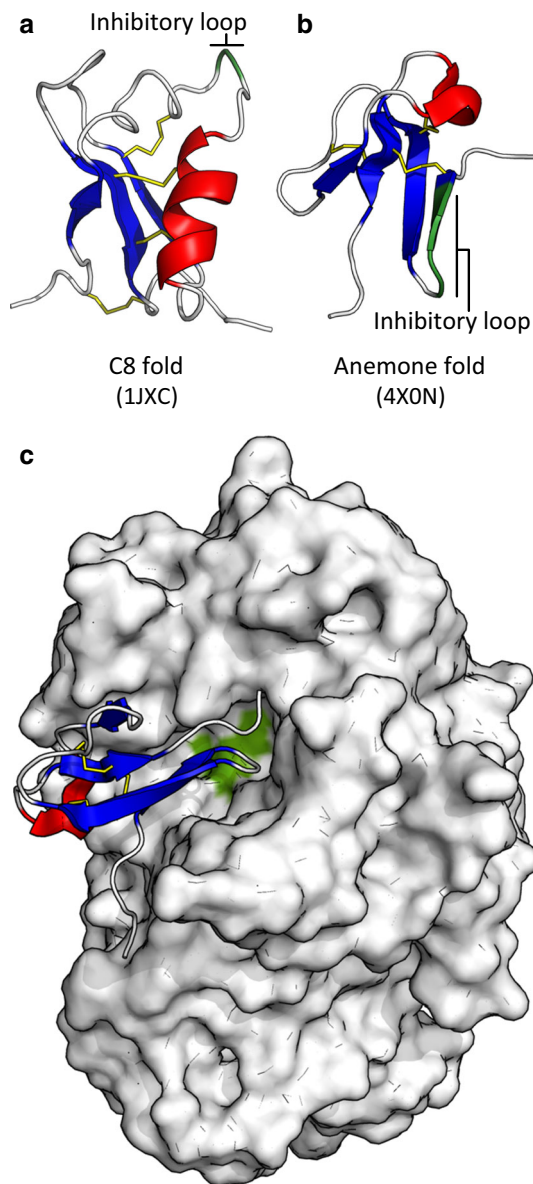
Fig. 8 Enzyme inhibition by defensin-like peptides. **a** The C8 *cis*-defensins fold has been adapted to enzyme inhibitory function in the *Arabidopsis thaliana* trypsin inhibitor (ATT) (PDB:1JXC) [11], and **b** the *trans*-defensins contain an α-amylase inhibitor, helianthamide, from sea anemones (PDB:4X0N) [12]. Inhibitory loop highlighted in green (putative for ATT) [11]. **c** The enzyme α-amylase (*white surface*) uses an aspartate-glutamate dyad in its active site for hydrolysis (*green*), which is competitively inhibited by the bound helianthamide (PDB:4X0N)

Anticancer activity

Several defensins from both superfamilies have specific cytotoxic anti-proliferative activities on cancer cell lines as well as solid and haematological tumours, and have minimal effects on healthy cells [181–183]. Examples include human α-defensins HNP-1 to HNP-3 [184, 185], their rabbit orthologues NP-1 to NP-3 [184], human β-defensin

HBD-1 and HBD-3 [127, 186], frog defensin brevinin-2 [187] and the plant *cis*-defensins NaD1 [94] and TPP3 [101]. They are cytotoxic to several cancer cell lines such as Raji and WIL-2 (lymphoma), L1210 and Jurkat (leukaemia), human HeLa (cervical carcinoma) and MCF-7 (breast carcinoma) and do so via direct membrane disruption and cell lysis [127, 184] or DNA damage [188]. Greater activity towards cancerous over healthy cells is likely to be due, in part, to an increase in affinity for the dysregulated tumour cell plasma membranes. The changes to the membrane include an increase in negatively charged phospholipids [189, 190] and glycoproteins [191, 192] in the outer leaflet, as well as increased surface area and fluidity [193, 194].

In contrast, murine β-defensins and human HBD-2 exert indirect anticancer activity via chemotactic and immunoadjuvant activities that promote adaptive immune responses [195–197]. Furthermore, HBD-3 and HNP-1, 2 and 3 additionally exert inhibitory effects on metastasis and angiogenesis [198–200]. These activities may be physiologically relevant in mammalian defensins, but are certainly a promiscuous side-activity in plant defensins, and reflect the propensity of antimicrobial defensins to interact with cell membrane targets.

Causes and significance of convergence

Convergent evolution occurs when similar selection pressures coincide with biophysical constraints that favour only a small number of accessible, adaptive solutions within a fitness landscape. In such cases, selection funnels evolutionary lineages towards similar solutions in that fitness landscape [201, 202]. Convergent evolution has been widely described for a range of biological phenomena: from physiology and behaviour to gene organisation and recruitment; however, examples at the protein level are rare [201–203].

Convergent evolution of specific sequences or structural folds is less common than functions, since equivalent functions can typically be achieved by different structural folds, and equivalent structures can be formed by many different sequences [204]. For example, the ability to cleave peptide bonds by a variety of chemical mechanisms has convergently evolved in the different classes of proteases. Indeed, even the same mechanism of covalent proteolysis using the same catalytic triad geometry has evolved independently at least 24 times in distinct superfamilies of serine and cysteine proteases [205]. Sequence convergence has also occurred in the transmembrane (TM) helix of the mitochondrial import receptor subunit TOM20. The plant and fungal analogues have evolved the same sequence motif, but in reverse order, as the TM helix passes though the membrane in opposite directions in the

different analogues [206]. Similarly, sequence convergence of linear motif peptides in pathogens is driven by selection to mimic their host's sequences and so bind host targets and disrupt cellular processes [207]. Finally, the convergent evolution of particularly favourable protein folds is thought to be extremely rare, and possible examples (such as the β-barrel fold) are highly contentious [206, 208, 209].

The extent of convergence between the *cis*- and *trans*-defensins is, therefore, particularly remarkable and derives from several intrinsic constraints of fold and function. First, the structures of small cysteine-rich proteins (CRPs) are freed from the requirements of a hydrophobic core, but have additional packing constraints when secondary structural elements are forced into close proximity. Second, the activities of the defensins dictate additional biophysical requirements that further constrain functional structures. Yet there are simultaneous evolutionary pressures that favour very high sequence divergence within these constraints, largely driven by specialisation to different targets and subsequent co-evolution with those targets [210].

The constraints of small CRPs impose a limited number of viable secondary structure orientations and disulphide topologies [211–213]. Consequently, similar CRP structures can be converged upon by evolution whereas larger globular proteins remain more diverse. This constraint is strong enough that additional unrelated proteins such as the MARCO receptor have also converged on similar folds [13].

The use of a compact, disulphide-stabilised core to display a set of cationic loops has, therefore, proven to be remarkably evolvable for a number of functions. Both the *cis*- and *trans*-defensin superfamilies independently evolved innate immune functions, using positively charged loops for membrane disruption. Analogous loops can interact with negatively charged lipid head groups in a 'cationic grip' formed by protein dimers. Each superfamily also contains members that have evolved to bind cell surface receptors to perform a variety of signalling roles, some immune related, some as divergent as fertilisation. Similarly, on multiple occasions, both folds have been recruited to a toxic function using exposed positive residues for ion channel blocking. The two defensin superfamilies, therefore, represent one of the most extensive occurrences of convergent evolution and demonstrate how evolution can favour extremely similar solutions to a selection pressure even when run independently.

Concluding remarks

In summary, this review provides insights into the unique features of the two defensin superfamilies, which arose by extensive convergent evolution from independent origins.

It highlights how the defensins represent a thorough case study in the evolvability of small CRP scaffolds, which is unlike that for more commonly studied globular proteins [214]. This recent work establishes the foundation for understanding evolutionary relatedness in defensins, and highlights how the elaboration of stable scaffolds has enabled both superfamilies to span an uncommonly wide array of biological roles [13].

With new sequences and structures being described at an ever-increasing rate, it is inevitable that additional defensin structural classes will be discovered. The integration of evolutionary, structural and functional data will inform design principles to enable the engineering of improved or novel variants for therapeutic and agricultural applications.

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