Fire and ICE: The role of pyrin domain-containing proteins in inflammation and apoptosis

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ABSTRACT

The genetic bases for several human autoinflammatory syndromes have recently been identified, and the mutated proteins responsible for these diseases are rapidly being characterized. Here, we examine two of these newly identified proteins, pyrin (also called marenostrin, product of the familial Mediterranean fever locus, MEFV) and cryopyrin (product of the CAIS1 locus, and mutated in familial cold urticaria, Muckle Wells syndrome and chronic infantile neurological cutaneous and articular syndrome). Both pyrin and cryopyrin contain an N-terminal domain that encodes a death domain-related structure, now known as the pyrin domain, or PyD. We trace the molecular interactions mediated by these PyDs, examine the evolution of the family of molecules containing this domain, and discuss the function of PyD-containing proteins and their homologues. Synthesis of the available data indicates that both pyrin and cryopyrin interact via their PyDs with a common adaptor protein, ASC. ASC itself participates in at least three important cellular processes: apoptosis, recruitment and activation of pro-caspase-1 (with associated processing and secretion of IL-1), and activation of NF- B (a transcription factor involved in both initiation and resolution of the inflammatory response). Through PyD:PyD interactions, pyrin and cryopyrin, as well as several related, but still uncharacterized PyD containing proteins, appear to modulate the activity of all three of these processes, each of which plays a crucial role in the inflammatory pathways that characterize the innate immune system.

The term "autoinflammatory" was recently proposed to describe a set of heritable human diseases characterized by inflammation, without evidence of high titer antibodies or apparent involvement of antigen-specific T cells (1). The hereditary periodic fever syndromes constitute a subset of these autoinflammatory diseases, and include: familial Mediterranean fever (FMF); tumor necrosis factor receptor-1-associated periodic syndrome (TRAPS); hyper IgD periodic fever syndrome (HIDS); and the allelic disorders, Muckle-Wells syndrome (MWS) and familial cold urticaria (FCU). A few additional, less well-characterized fever syndromes, such as PFAPA syndrome (periodic fever aphthous stomatitis and adenitis) and chronic infantile neurological cutaneous and articular syndrome or CINCA (also known as neonatal onset multisystem inflammatory disease, or NOMID) might also belong in this category. Though each of these syndromes has its own genetic and phenotypic peculiarities, there are also several similarities in their presentation. For example, many of these syndromes involve fever, urticaria, arthritis and serositis as common clinical elements and systemic amyloidosis as a potential complication. In many cases too, inflammatory attacks are accompanied by a robust acute phase response, and a prominent neutrophilia characterizes the inflammatory site. These features suggest that the hereditary fever syndromes are disorders of the innate immune system,an ancient arm of the immune system responsible for quick response to a variety of pathogenic insults to the organism. Clearly, insights into the molecular underpinnings of innate immunity, potentially available through the etiologic investigation of these dis-

orders, could have enormous clinical significance.

Among the presently known periodic fever syndromes, FMF (2, 3), MWS/-FCU (4) and CINCA/NOMID (5) are caused by mutations in proteins that contain a newly identified protein:protein interaction domain, variously called a pyrin domain (PyD) or PAAD or DAPIN (6-11). Growing interest in this 90 amino acid domain, hereafter called the PyD, stems from the fact that its six alpha helices form a death domain (DD)-related structure (6-11). The death domain is one of three related structures seen in molecules involved in apoptosis (the other two being the death effector domain or DED, and the caspase recruitment domain or CARD). DDs, DEDs and CARDs are known to mediate protein:protein interactions (12, 13). Though probably evolved members of a single family, the individual members interact only with their most similar relatives. That is, DDs interact with other DDs, DEDs contact DEDs and CARDs touch other CARDs (12, 13). The PyD forms a fourth group, and proteins with PyDs also seem to interact with other PyDs. Interestingly, though pyrin (also called marenostrin) represents the founding protein for the PyD, it was not recognized as a death domain-containing molecule by either of the two consortia that initially cloned it. This is because: a) at that time, no other protein with a PyD had been characterized, though several could be found in the EST databases and b) the sequence identity between a PyD and the related DDs, DEDs and CARDs was too low to be recognized by simple homology searches. However, molecular modeling strategies, combined with sophisticated fold recognition algorithms, carried out simultaneously in several laboratories, were able to make the structural diagnosis (6-11).

The fact that the PyD *looks* like a protein: protein interaction domain found on other molecules known to be involved in apoptosis and inflammation was intriguing, but when pyrin was found to actually *interact* with a pro-apoptotic protein (11), interest rose exponentially, especially since that interaction required pyrin's PyD. A yeast two hybrid screen identified ASC (apoptosis associated speck-like protein containing a CARD) as a pyrin-interacting protein (11). ASC was, at the time of this finding, itself newly identified (14), as a protein that forms large, hollow cytoplasmic aggregations called specks in apoptotic cells (Fig. 1). Specks are harbingers of apoptosis, as 100% of cells that form specks go on to complete the apoptotic program (11, 14). Indeed, up-regulation or over-expression of ASC in a variety of cells causes speck formation and apoptosis. Exon 1 of pyrin is both necessary and sufficient for interaction of pyrin with ASC and co-localization of pyrin with ASC in specks (11). Interestingly, ASC also has an N-terminal PyD, and its C-terminal end encodes a CARD. Thus, ASC functions as an adaptor, linking PyD containing proteins to CARD-containing ones.

It is still unclear what significance the speck structure itself might have. However, it is possible that specks are a macroscopic reflection of the tendency for ASC and pyrin (perhaps in cooperation with additional proteins) to form intermolecular aggregates. Indeed, it is likely that micrscopic aggregates form at lower concentrations of these proteins. Intermolecular aggregation is a hallmark of apoptotic proteins. In fact, the activities of the effector molecules of apoptosis, the caspases, are largely regulated by "induced proximity". That is, the localized concentration, in the same molecular aggregate, of a number of these molecules results in the localized expression of an otherwise weak effector activity (15). This enhanced effector activity sets in motion a cascade of events that shortly results in the death of the cell. The molecular aggregate itself has been called an apoptosome, and the presence of these multi-protein aggregates has been physically demonstrated, as has their central role in apoptosis (16, 17).

Do pyrin and ASC form an apoptosome-like structure ? It is clear that specks are large, well ordered molecular aggregates (11, 14, see Figure 1). Though smaller aggregates formed by these proteins are predicted to exist, the nature of such aggregates has not been probed. Moreover, evidence that ASC is pro-apoptotic has been presented (11, 14); and data indicating that pyrin can modulate the apoptotic process set in motion by ASC are also available (11), though there are some incongruities to deal with. First, in a variety of cell types, the co-expression of pyrin with ASC results in a dramatic increase in the number of cells that display specks (ASC, but not pyrin, causes speck formation when expressed alone). This finding would suggest that pyrin is pro-apoptotic, that it enhances the apoptotic signal elicited by ASC. However, paradoxically, the speck-positive cells seen after expression of ASC alone die much faster than the speck-positive cells seen after co-expression of pyrin with ASC. This indicates that pyrin enhances cell survival in the face of the apoptotic signal educed by ASC. At the time of this writing, it is still unclear whether we should place a "pro-apototic" or "anti-apoptotic" label on the pyrin protein. In fact, the problem seems further muddled (but in a very interesting way) by recent findings in our laboratory that the pyrin/ASC

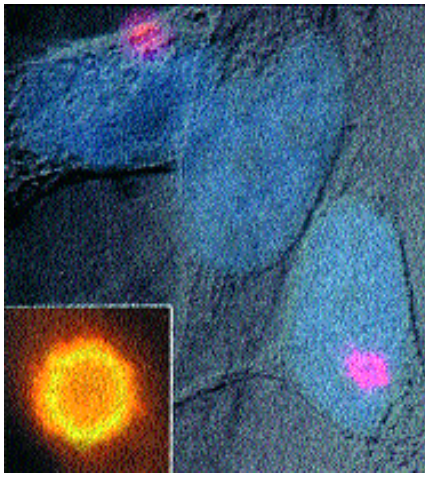

Fig. 1. *ASC specks are well-ordered cytoplasmic structures.* Flag-tagged ASC and myc-tagged pyrin constructs were transfected into HeLa cells and the proteins were recognized by double label immunofluorescence. Two cells in this image contain red specks (here visualized by rhodamine staining for the myc-pyrin protein). The inset shows a higher magnification of a speck; the yellow color arises from the co-localization of ASC (green) and pyrin (red) in the speck. Note the hollow character of the speck.

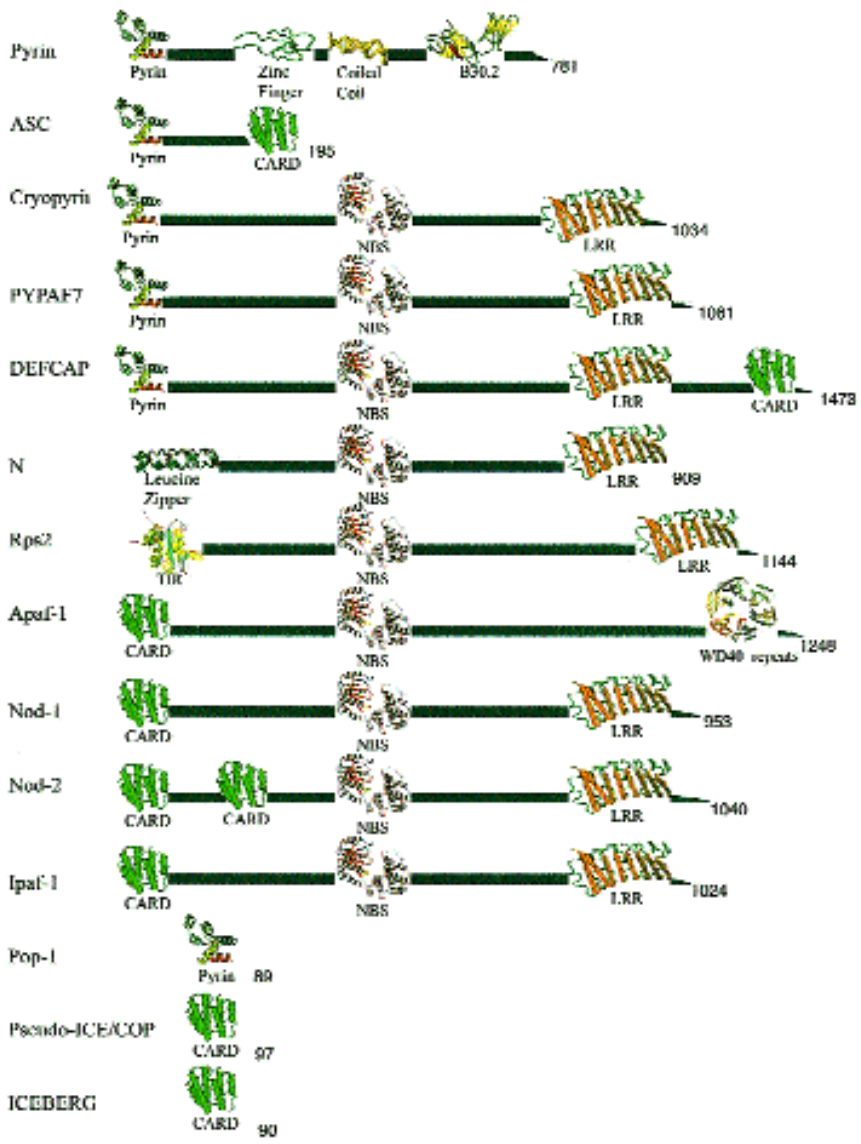

Fig. 2. *Structural comparison of pyrin domain-containing proteins.* As much as possible, and where known, motifs are shown as protein structure ribbon diagrams. These proteins contain many common motifs, including the pyrin domain (10), nucleotide binding site (NBS)(62), Leucine Rich Repeat (LRR) (63), and caspase recruitment domain (CARD) (64). Some of these proteins have domains unique to them as well, such as the WD40 repeats of Apaf-l (65), which have been implicated in apoptosome formation (16,17),the Toll/Interleukin 1 receptor (TIR) domain (66) of RPS2 from arabadopsis,and the leucine zipper motif (67) of the tobacco N protein. The protein most unlike the others in this structural family is pyrin itself, which, in addition to a pyrin domain, also contains a B-box type zinc finger (68), a coiled coil (69), and a B30.2 domain (70).

interaction may be different in different cells, and both labels could potentially fit (data to be presented).

Thus far, we have established connections between clinical inflammation and the protein product of the FMF locus (pyrin), between pyrin and ASC, and between the ASC/pyrin interaction and apoptosis. At this point, we can postulate that at least part of the mechanism of the inflammatory response involves cytokine-induced activation of pyrin (18), followed by pyrin-induced modulation of ASC function. Exactly how the pyrin mutations affect this pathway is a major detail that remains to be filled in, but evidence that pyrin is a central component of the body's inflammatory response continues to build. There is now evidence that mutations in pyrin might also predispose an individual to other inflammatory conditions, including Bechet's disease (19, 20), inflammatory bowel disease (21), and other still unclassified diseases marked by fever and inflammation (22). There is also evidence that, both in human populations, and during primate evolution, pyrin has been subject to positive Darwinian selection (23-27). Interestingly, it appears that certain of the FMF-causing mutations have been selected for, indicating that under some environmental circumstances, a mutant pyrin allele actually confers a benefit to the host. One proposed scenario is that a heightened state of inflammatory alert (e.g., increased levels of acute phase response proteins such as CRP and SAA), which is apparently bestowed even on heterozygotes carrying a mutant pyrin gene, results in more certain clearance of dangerous pathogens (26, 27).

Cryopyrin, recently shown by Hoffman *et al.* to be the protein product of the causative gene (*CAIS1*) for MWS and FCU (4), also appears to be centrally linked to a myriad of inflammatory syndromes. Cryopyrin is so named for its N-terminal structural relationship with pyrin (both proteins contain a PyD) and for its role in cold-stimulated urticaria. Just as we were scratching our heads as to how mutations in a single protein can cause cold-induced rashes in some individuals (FCU) and sensorineural hearing loss in others (MWS), Feldmann *et al*. linked mutations in cryopyrin to another inflammatory syndrome, NOMID/CINCA (5). This syndrome has its own peculiarities, including migratory skin rashes, chronic meningitis and abnormalities in cartilage growth.

So what does cryopyrin do ? The first hints came from analysis of its structure. Figure 2 summarizes the structure of the proteins that are germane to this discussion, and Table I serves as a quick-reference guide for their functional significance and confusing nomenclature. At the same time that cryopyrin was first identified as the MWS/-FCU protein, Manji *et al.* reported the characterization of PYPAF1, or pyrin-containing Apaf1-like protein (28). The PYPAF family designation refers to Apaf-1-like proteins that contain a PyD at the N-terminus; PYPAF1 is cryopyrin. The cryopyrin structure was inter-

Table I. The players: PyD-containing proteins and proteins structurally related to PyD proteins.

Protein	Other names	Function	Reference
Pyrin	Marenostrin	Product of *MEFV* locus; modulates ASC-mediated apoptosis	2, 3, 11
ASC	TMS 1/PYCARD	Adaptor that binds pyrin, cryopyrin, DEFCAP, PYPAF7; activates NF- B at high concentrations; activates pro-caspase-1; forms"specks" in cells; pro-apoptotic	7, 11, 14, 49, 50
Cryopyrin	PYPAF1/NALP3	Responsible for FCU, MWS, NOMID/CINCA. Binds ASC, activating NF- B and caspase-1	4, 5, 28, 50
PYPAF7		Binds ASC, activating NF- B and caspase-1	50
DEFCAP	CARD7/NAC/ NALP1	Enhances Apaf-1-mediated caspase 9 activation; pro-apoptotic; binds ASC	7, 56, 57
N (tobacco)		Intracellular plant R protein	60
Rps2 (Arab)		Intracellular plant R protein	61
Apaf- 1		CED4-related; binds cytochrome c and ATP, activates caspase 9 by induced proximity	34, 35
Nodl	CARD4	Intracellular LPS receptor; activates caspase 9; activates NF- B; mediates JNK and NF- B activation by *S. flexneri*	31, 32, 37
Nod2		Intracellular LPS receptor; activates caspase 9; activates NF- B; mutations associated with Crohn's disease and Blau syndrome	38, 46-48
POP1	ASC2	Uncharacterized; predicted inhibitor of ASC function (see Figure 3)	50
ICEBERG		Binds pro-caspase-1, inhibits activation and caspase-1-dependent IL-1 secretion	53, 54
Pseudo-ICE	COP	Binds pro-caspase-1, inhibits activation and casp1-dependent IL-1 secretion; interacts with RICK and activates NF- B.	54, 55

Note that other, less well-characterized PyD-containing proteins are not listed here. These include the NOD-related proteins PYPAF2 (NBS1/NALP2), PYPAF3,PYPAF4/NALP4,PYPAFS, PYPAF6,PYPAF8/MATER, NAIP and CIITA discussed by others (29,50). In addition, proteins not in the NOD/Apaf family, but containing a pyrin domain (though more divergent) include:AIM2 (absent in melanoma), several interferon inducible genes such as IFI16 and MDNA,and the viral proteins M013L from myxoma virus and gp013L from rabbit fibroma virus (9). The latter two are PyD-only proteins; it remains to be seen whether these viral proteins can inhibit ASC-mediated caspase-1 activation.

esting because of its similarity to Apaf-1 (discussed further below), but more compelling was its similarity to the NOD proteins (members of the same Apaf-1-like structural family, but containing a CARD rather than a PyD at the N-terminus). Two structural domains are shared by cryopyrin and the NOD proteins (Fig. 2). First, there is a domain that binds ATP and mediates homo-oligomerization. We will refer to it as the NBS or nucleotide binding site, but it has also been called a NOD, nucleotide-binding oligomerization domain (29), or a NACHT (30). The second shared domain is composed of a series of leucine-rich repeats (LRRs); LRRs function as protein:protein interaction motifs. As shown in Figure 2, the NBS and LRR motifs are also found in other proteins besides cryopyrin and the NODs. Before getting back to the function of cryopyrin, a tangential diversion to provide a brief overview of some of these other proteins will be instructive from both a structural and a functional point of view.

Nod1 (which also goes by the name CARD4, ref. 31) was originally identified on the basis of its structural homology to Apaf-1 (31, 32). Apaf-1 (apoptotic protease-activating factor-1) is a central component of the apoptosis machine; it was originally discovered in the worm, *C. elegans*, and named Ced-4 (33). Apaf-1/Ced-4 is a transducer of the mitochondrial or intrinsic pathway of apoptosis, connecting cell death signals to the activation of the execution pathway (33-36). The C-terminal WD-40 motifs of Apaf-1 bind cytochrome c released from mitochondria. This causes a structural alteration in the Apaf-1 molecule that allows its homo-oligomerization *via* the region containing the NBS and the recruitment of caspase 9 *via* its N-terminal CARD domain. The oligomerization results in the concentration of several caspase 9 molecules in the molecular aggregate, resulting in proximity-induced activation of caspase 9 (36). The NOD proteins function similarly (32, 37, 38). That is, the C-terminal LRRs act as a ligand binding domain (the ligand itself is discussed below); ligand binding induces a probable conformational change that

allows homo-oligomerization through the NBS region and recruitment of another protein *via* the CARD domain. In the case of Nodl and Nod2, this recruited protein is RICK (RIP2/CARDIAK), a CARD-containing kinase that itself binds to and activates IKK / NEMO, the regulatory subunit of the I- B (IKK) complex (39). Homo-oligomerization of Nodl or Nod2 results in the activation of RICK, and thence the IKK complex by induced proximity; the result is NF- B activation.

The activation of NF- B plays a central role in cellular responses to stress, inflammatory signals and pathogens (40, 41). Both Nodl and Nod2 also display pro-apoptotic tendencies, and activate the caspase 9 pathway, but the molecular details of this (e.g., do the Nods activate caspase 9 and NF- B in response to different triggers? Is there a temporal aspect to be considered?) have still to be worked out. In this context, it is interesting that the NF- B pathway seems to be critical both for the initiation of the inflammatory response, and for its resolution (42). While the initiation phase includes NF-KB-stimulated induction of pro-inflammatory cytokines and suppression of apoptosis, the NF- B-mediated resolution phase is characterized by the expression of anti-inflammatory molecules and the induction of apoptosis.

Another set of proteins with structural similarity to cryopyrin, NODs, and Apaf-1 is the intracellular group of plant cytosolic disease resistance (R) proteins. In the R proteins, the N terminal domain is not a CARD or PyD, but a different kind of protein recruitment domain (resembling either a leucine zipper or Toll/interleukin-1 receptor, TIR). The R proteins constitute a very large family of proteins that mediate resistance to a variety of pathogens, including bacteria, viruses and fungi (43, 44). The C-terminal LRR domain of each type of R protein is capable of recognizing and binding to a pathogen-specific molecule called an Avirulence factor (Avr), thereby constituting a "one R protein-one pathogen" recognition system. The consequence of this Avr:LRR interaction is the apoptotic death (the hypersensitive response) of the pathogen-containing plant cell.

Recognizing the remarkable structural similarities between the NOD proteins and the plant R proteins, Inohara *et al*. predicted that the LRRs of Nod1 and Nod2 would bind bacterial products such as LPS. Their predictions proved correct, as they were able to demonstrate the intracellular binding of LPS by Nodl and show that different bacterial molecules had differing abilities to activate these two NOD proteins (37). This finding suggests that NODs could function in a manner similar to the R proteins. That is, they may be involved in intracellular pathogen recognition, functioning as receptors and signal transducers for the innate immune system. In fact, Girardin *et al*. recently demonstrated that Nod1 mediates an intracellular host response to invasive *Shigella flexneri* (45), resulting in the activation of both NF- B and the stress-induced kinase, JNK (c-Jun N-terminal kinase). Dominant negative forms of Nod1 (versions lacking the CARD domain or consisting of only the LRR domain) inhibit both of these activities. These data, along with those of Inohara *et al*. reveal a novel intracellular response system in which Nod1 functions to sense bacterial LPS and initiate an inflammatory response.

If Nods are intracellular sensors that can regulate the innate immune response, then we might expect that mutations in these proteins might be involved in human inflammatory syndromes. This prediction too, has been borne out. Mutations in Nod2 (also called CARD15) are associated both with Crohn's disease, and with Blau Syndrome (46-48). It is interesting that the Crohn's disease mutations are thus far seen in the LRR domain, some causing a truncation of that domain (46, 47), while the Blau syndrome mutations lie in the NBS (48). Further work will be needed to determine how and why these different mutations in the same molecule encode different inflammatory phenotypes.

Coming back to cryopyrin, recall that its structure resembles a NOD protein, except that it has an N-terminal PyD rather than a CARD. However, as it happens, cryopyrin binds ASC, a protein that clearly functions as an adaptor. Like pyrin, cryopyrin/PYPAF1 interacts with ASC via PyD:PyD contact and is recruited to ASC specks (28). Whether cryopyrin also modulates ASC mediated apoptosis as pyrin does has not yet been tested. The interaction between cryopyrin and ASC requires the PyD of both proteins; thus the recruitment of ASC to cryopyrin essentially supplies cryopyrin with a CARD domain, which is then free to recruit still another effector protein to the complex. This recruited protein might be RICK or a functional homolog, since cryopyrin appears to enhance the otherwise weak ability of ASC to activate NF- B, and this activation proceeds through the IKK complex (28). Furthermore, deletion of the LRR domain from cryopyrin increases the ability of the truncated protein to activate NF- B. This suggests that for cryopyrin, as with Apaf-1 and the NODs, the C-terminal region might comprise a ligand binding domain that constitutes a molecular "switch". If so, identification of the ligand becomes an important goal with potential clinical significance.

There are a few more puzzle pieces that can be inserted (though these by no means complete the picture). First, recall that mutations in both pyrin and cryopyrin cause fever and inflammation, and note that both proteins interact with ASC. This turns out to be an important connection, since two different laboratories have now demonstrated that the CARD domain of ASC interacts directly with the CARD domain of pro-caspase 1 (49, 50). Caspase-1 (also called interleukin-1 converting enzyme or ICE) is the protease responsible for the processing and secretion of IL-1 and IL-18. It also processes the precursor of IL-6 and participates in the secretion of IL-4, IL- and TNF- (51, 52). Caspase-1 -/- mice exhibit a major defect in IL-1,3 processing, and diminished secretion of these other cytokines; in addition, they show minor deficits in some apoptotic pathways.

The interaction between ASC and pro-caspase-1 potentiates pro-caspase-1 activity by induced proximity and thereby potently induces the processing and se-

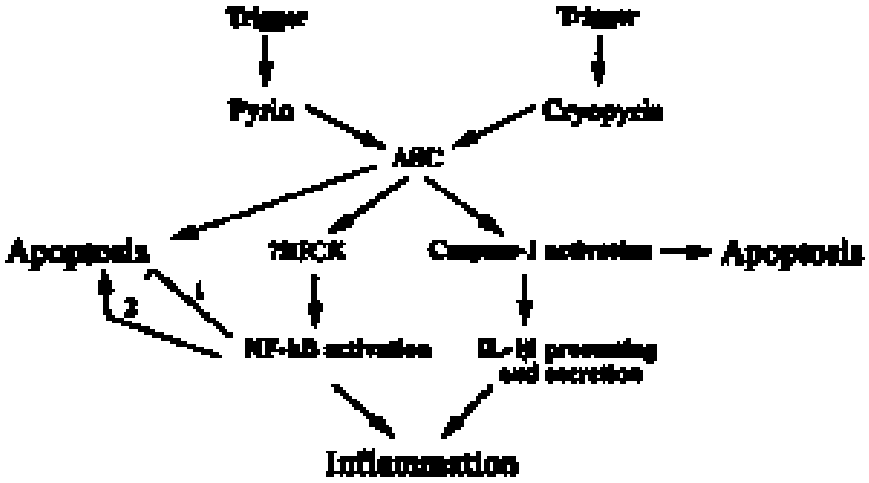

Fig. 3. Summary of molecular interactions and signaling pathways in which pyrin and cryopyrin funotion. Both pyrin and cryopyrin interact with ASC and stimulate one or more of three ASC-mediated activities: apoptosis, NF- B activation (possibly through RICK) and caspase-l-activation, leading to IL-1 processing and secretion. Caspase-1 activation can also trigger apoptosis, but it is not clear that this is the path followed in this case. ASC itself, however, induces apoptosis, and this pathway is modulated by pyrin. NF- B activation is believed to have two, temporally separate activities: early induction of the pro-inflammatory response and later resolution of the inflammatory response. These two activities rely on different effector genes,and have opposite effects on apoptosis. It is likely that other PYPAF proteins also figure in the upstream portion of these pathways. See text for details.

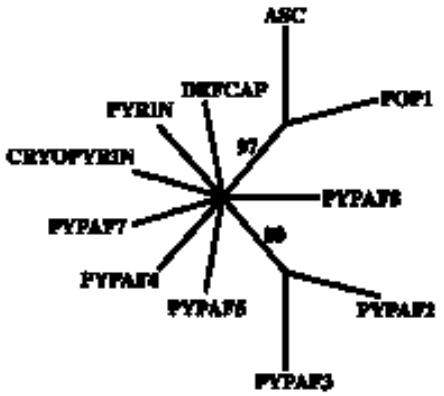

Fig. 4. Bootstrapped consensus cladogram of pyrin domains from pyrin, ASC, POPI and the seven other PYPAF proteins identified to date. The pyrin domains of ASC (accession number NM_013258), POP1 (accession number AF454669), PYPAF8 (accession number AY054986), PYPAF2 (accession number-AF310106), PYPAF3 (accession number AF464765), PYPAF5 (accession number XM_113700), PYPAF4 (accession number XM_085972), PYPAF7 (accession number AY095146), cryopyrin (accession number AF410477), DEFCAP (accession number NM_013258) and pyrin (accession number AF018080) were aligned using ClustalX. A maximum parsimony phylogenetic tree was constructed with PAUP version 4.0blO. A bootstrap analysis was performed with 1000 replicates, using a full heuristic search and including groups compatible with 50% majority-rule consensus. Significant support for related sub-groups within the family is only found for the PYPAF2/-PYPAF3 pair and for ASC/POP1. Some trees also supported a grouping for PYPAF1/crypopyrin and PYPAF7, but the bootstrap value for this grouping, 57, was too low to be considered significant.

cretion of IL-1 , a major pyrogenic and pro-inflammatory cytokine. Srinivasula *et al.* suggest that the interaction of ASC and caspase-1 creates a signaling complex,the inflammasome, in response to pro-inflammatory stimuli (49). PYPAF7 and its cousin, cryopyrin (PYPAF1), can each impart a synergistic activation of pro-caspase-1 and a resultant increase in IL-1 secretion (49). It remains to be tested whether other PyD containing proteins known to interact with ASC (pyrin, DEFCAP and the remaining PYPAFs) also regulate the activity of this putative inflammasome. If so, then a more complete (if still rather fuzzy) pathway begins to emerge: an inflammatory trigger signals activation of PyD-containing proteins, recruitment of ASC, activation of caspase-1 and processing and secretion of IL-1 . In addition, for some PyD proteins (e.g., cryopyrin), ASC mediated activation of NF- B is enhanced, adding to the pro-inflammatory signal, while for others (e.g., pyrin), ASC-mediated apoptosis is altered (Fig. 3).

As might be predicted, these processes of apoptosis and inflammation require tight control. Thus, it is perhaps not surprising that there are inhibitors of the NOD-like and PYPAF-like proteins. Experiments in several laboratories had shown that expression of just the interacting domains (e.g., the CARD or PyD) of these proteins has a dominant negative effect on effector function. Apparently, these experiments had already been done by the genome itself. Two proteins, pseudo-ICE (which has also been named COP, or CARD-only protein) and ICEBERG (53-55), have been identified that consist essentially of only a CARD domain that is highly homologous to the CARD domain of pro-caspase-1 (92% and 53%, respectively). Either of these proteins can prevent the caspase-1 dependent processing and secretion of IL-1 . However, the two are functionally different, since pseudo-ICE can activate NF- B and can enhance TNF- induced NF-kB activation, but ICEBERG cannot (54).

Interestingly, the database also contains a PyD-only protein (POP), noted previously by Wang *et al.* (50). Since POP has not yet been characterized, we examined its relatedness to the PyDs of pyrin, ASC and several of the other PyD-containing proteins of the PYPAF group. Figure 4 shows the consensus tree from a bootstrapped maximum parsimony analysis. The starburst distribution of branches suggests that ancient gene duplications exploded the number of these proteins, and that since that time, few additional gene duplications have taken place. However, two clusterings are well supported by the bootstrap analysis: PYPAF2 and PYPAF3 as well as ASC and POP. The latter pair invites some further functional speculation. Recall that ASC appears to act as an adaptor for several of the PyD-containing proteins, though not all have been tested for ASC binding. Since the analysis shows that of all the PyD proteins, POP is most closely related to AS C, and since for all of the death domain-related structures including the PyD, similarity breeds familiarity (i.e., interaction), POP might be ex-

pected to bind ASC readily, thus inhibiting ASC's interaction with other PyD motifs. If this prediction is borne out, POP's future value may lie in its therapeutic potential for the treatment of those inflammatory diseases in which ASC may play a role (FMF, FCU/MWS, CINCA/NOMID, and potentially others).

It is interesting that the interactions between PyD proteins in general seem to be weak, or perhaps unstable. For example, using GST pull-down assays, Srinivasula *et al*. found no evidence that ASC forms stable complexes with pyrin, cryopyrin, DEFCAP or PYPAF3 (49). However, other laboratories, using yeast two hybrid screens, immunoprecipitation, immunolocalization or the mammalian two-hybrid system, find evidence for interactions, albeit lower affinity ones (7, 11, 50). Perhaps these weak interactions are important. CARD-CARD domain interactions may be of high affinity because most are critical for the kind of velcro-like stable recruitment needed for induced proximity activation. On the other hand, PyD-PyD interactions may be primarily of a regulatory nature; thus, by necessity, easy to perturb to allow for a sensitive on/off switch. A propensity to act as a regulatory switch could also ultimately underlie the fact that mutations in these proteins cause diseases that present in a "periodic" fashion. A potential modulatory role has already been suggested for pyrin (in regulating ASC induced apoptosis, ref. 11), cryopyrin (in activating NF- B and caspase-1-dependent IL-1 processing ref. 28, 50), PYPAF7 (in regulating NF- B activation and caspase-1-dependent IL-1 processing, ref. 50), and DEFCAP (in modulating Apaf-1-dependent apoptosis, ref. 56, 57).

Though some shape is beginning to emerge from the connection of these molecular dots, there are still many outstanding questions. For example, what are the triggers for the periodic inflammatory attacks suffered by individuals with FMF, MWS or FCS ? Though cryopyrin seems to look and function like a NOD protein, and might be expected to be able to sense pathogens, it is clear that at least one inciting trigger is simply temperature, and it seems unlikely that the attack-producing ligand (if there is one) for cryopyrin, in FCU at least, is a bacterial or viral product. [NOMID/CINCA may differ in this regard.] Thus, there may be an intracellular, stress induced molecule to which the cryopyrin LRR can respond. Then there is the question of the mutations, all of which are thus far observed in and around NACHT/-NBS/NOD domain (the domain responsible for oligomerization). Interestingly, this is true for FCU and MWS as well as NOMID, despite the phenotypic differences in these three syndromes. Could it be that one type of mutant molecule in the oligomer group leads to failure to reach a critical mass for induced proximity activation, while another mutation causes homo-oligomerization to occur too readily ? Do some mutations alter the conformation of cryopyrin so as to change either the upstream sensing or downstream effector functions? More data is needed.

And what of pyrin? In the region outside of the PyD, it is clearly the most divergent of the PyD containing proteins. Most of its mutations fall within the C-terminal rfp or B30.2 domain (58), suggesting that this domain is critical for the function of the protein. However, the mouse and rat genomic sequences both contain a frameshift that results in the expression of a truncated form of pyrin that lacks the rfp domain entirely (59)! How is it that this domain, so seemingly critical to the function of pyrin, as judged by the effects of the mutations that it harbors, is missing altogether in rodents? Extrapolating from the function of other PyD-containing proteins, it is tempting to think that this apparent paradox could be reconciled if the C-terminal rfp domain acts as an intramolecular regulatory domain, functionally (but not structurally) equivalent to Apaf-1's WD40 motifs or the LRRs of the NOD-like proteins. Would this mean that the rfp domain binds a ligand that then controls the on/off switch for pyrin function? If so, what is the ligand? And is apoptosis the function (the only function?) that is controlled?

We have examined here only a subset of the PyD-containing proteins related to pyrin and cryopyrin. Several more lurk in the database. However, it is already possible to appreciate the amazing interconnectedness of the molecular interactions that encircle the critical processes: inflammation, apoptosis, NF- B activation, caspase-1 activation and cytokine secretion. These interactions describe a remarkable set of structural and functional parallels between pathogen recognition systems and components of the innate immune pathway inside of mammalian cells. The large number of remaining questions still to be answered, however, is sure to keep this field "hot" for some years to come.

References

1. GALON J, AKSENTIJEVICH I, McDERMOTT F, O'SHEA J, KASTNER DL: TNFRSF1A mutations and autoinflammatory syndromes. *Curr Opin Immunol* 2000; 12: 479-86.
2. INTERNATIONAL FMF CONSORTIUM: Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. *Cell* 1997; 90: 797-807.
3. FRENCH FMF CONSORTIUM: A candidate gene for familial Mediterranean fever. Nat Genet 1997; 17:25-31.
4. HOFFMAN HM, MUELLER JL, BROIDE DH, WANDERER AA, KOLODNER RD: Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet* 2001; 29: 301-5.
5. FELDMANN J, PRIEUR AM, QUARTIER P *et al.*: Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in *CMS1*, a gene highly expressed in polymorphonuclear cells and chondrocytes. *Am J Hum Genet* 2002; May 24 (epub ahead of print).
6. BERTIN J, DiSTEFANO PS: The PYRIN domain:A novel motif found in apoptosis and inflammation proteins. *Cell Death Differ* 2000; 7:1273-4.
7. MARTINON F, HOFMANN K, TSCHOPP J: The pyrin domain:A possible member of the death domain-fold family implicated in apoptosis and inflammation. *Curr Biol* 2001; 11: R118-20.
8. STAUB E, DAHL E, ROSENTHAL A: The DAPIN family:A novel domain links apoptotic and interferon response proteins. *Trends Biochem Sci* 2001; 26: 83-5.
9. PAWLOWSKI K, PIO F, CHU Z, REED JC, GODZIK A: PAAD - A new protein domain associated with apoptosis, cancer and autoimmune diseases. *Trends Biochem Sci* 2001; 26: 85-7.
10. FAIRBROTHER WJ, GORDON NC, HUMKE EW *et al.*: The PYRIN domain: a member of the death domain-fold superfamily. *Protein Sci* 2001; 10: 1911-8.
11. RICHARDS N, SCHANER P, DIAZ A *et al.*:

Interaction between pyrin and the apoptotic speck protein (ASC) modulates ASC-induced apoptosis. *J Biol Chem* 2001; 276: 39320-9.
12. ARAVIND L, DIXIT VM, KOONIN EV: The domains of death: Evolution of the apoptosis machinery. *Trends Biochem Sci* 1999; 24: 47-53.
13. HOFMANNK: The modular nature of apoptotic signaling proteins. *Cell Mol Life Sci* 1999; 55: 1113-28.
14. MASUMOTO J, TANIGUCHI S, AYUKAWA K *et al.*: ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. *J Biol Chem* 1999; 274: 33835-8.
15. SALVESEN GS, DIXIT VM: Caspase activation: The induced-proximity model. *Proc Natl Acad Sci USA*: 1999; 96: 10964-7.
16. SHI Y: Apoptosome: The cellular engine for the activation of caspase-9. *Structure (Camb)* 2002; 10: 285-8.
17. ACEHAN D, JIANG X, MORGAN DG, HEUSER JE, WANG X, AKEY CW: Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell* 2002; 9: 423-32.
18. CENTOLA M, WOOD G, FRUCHT DM *et al.*: The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. *Blood* 2000; 95:3223-31.
19. LIVNEH A, AKSENTIJEVICH I, LANGEVITZ P *et al.*: A single mutated MEFV allele in Israeli patients suffering from familial Mediterranean fever and Behcet's disease (FMF-BD). *Eur J Hum Genet* 2001; 9: 191-6.
20. TOUITOU I, MAGNE X, MOLINARI N *et al.*: MEFV mutations in Behçet's disease. *Hum Mutat* 2000; 16: 271-2.
21. CATTAN D, NOTARNICOLA C, MOLINARI N, TOUITOU I: Inflammatory bowel disease in non-Ashkenazi Jews with familial Mediterranean fever. *Lancet* 2000; 355: 378-9.
22. BOOTH DR, LACHMANN HJ, GILLMORE JD, BOOTH SE, HAWKINS PN: Prevalence and significance of the familial Mediterranean fever gene mutation encoding pyrin Q148. *QJM* 2001; 94: 527-31.
23. SCHANER P, RICHARDS N, WADHWA A, AKSENTIJEVICH I, KASTNER D, TUCKER P, GUMUCIO D: Episodic evolution of pyrin in primates: human mutations recapitulate ancestral amino acid states. *Nat Genet* 2001; 27: 318-21.
24. AKSENTIJEVICH I, TOROSYAN Y, SAMUELS J *et al.*: Mutation and haplotype studies of familial Mediterranean fever reveal new ancestral relationships and evidence for a high carrier frequency with reducedpenetrance in the Ashkenazi Jewish population. *Am J Hum Genet* 1999; 64: 949-62.
25. STOFFMAN N, MAGAL N, SHOHAT T *et al.*: Higher than expected carrier rates for familial Mediterranean fever in various Jewish ethnic groups. *Eur J Hum Genet* 2000; 8: 307-10.
26. BOOTH DR, GILLMORE JD, LACHMANN HJ *et al.*: The genetic basis of autosomal dominant familial Mediterranean fever. *QJM* 2000; 93: 217-21.
27. TUNCA M, KIRKALI G, SOYTURK M,AKAR S, PEPYS MB, HAWKINS PN: Acute phase response and evolution of familial Mediterranean fever. *Lancet* 1999; 353: 1415.
28. MANJI GA, WANG L, GEDDES BJ *et al.*: PYPAF1, a PYRIN-containing Apaf1-like protein that assembles with ASC and regulates activation of NF-kappa B. *J Biol Chem* 2002; 277: 11570-5.
29. INOHARA N, OGURA Y, NUNEZ G: Nods: A family of cytosolic proteins that regulate the host response to pathogens. *Curr Opin Microbiol* 2002; 5: 76-80.
30. KOONIN EV, ARAVIND L: The NACHT family - A new group of predicted NTPases implicated in apoptosis and MHC transcription activation. *Trends Biol Sci* 2000; 25:223-4.
31. BERTIN J, NIR WJ, FISCHER CM *et al.*: Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. *J Biol Chem* 1999; 274: 12955-8.
32. INOHARA N, KOSEKI T, DEL PESO L *et al.*: Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J Biol Chem* 1999; 274: 14560-7.
33. METZSTEIN MM, STANFIELD GM, HORVITZ HR: Genetics of programmed cell death in *C. elegans*: Past, present and future. *Trends Genet* 14: 410-16.
34. ZOU H, HENZEL WJ, LIU X, LUTSCHG A, WANG X: Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997; 90: 405-13.
35. LI P, NIJHAWAN D, BUDIHARDJO I, SRINIVASULA SM,AHMAD M,ALNEMRI ES, WANG X: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; 91: 479-89.
36. SRINIVASULA SM, AHMAD M, FERNANDES-ALNEMRI T, ALNEMRI ES: Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell* 1998; 1: 949-57.
37. INOHARA N, OGURA Y, CHEN FF, MUTO A, NUNEZ G: Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J Biol Chem* 2001; 276: 2551-4.
38. OGURA Y, INOHARA N, BENITO A, CHEN FF, YAMAOKA S, NUNEZ G: Nod2,a Nodl/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* 2001; 276: 4812-8.
39. INOHARA N, KOSEKI T, LIN J *et al.*: An induced proximity model for NF-kappa B activation in the Nodl/RICK and RIP signaling pathways. *J Biol Chem* 2000; 275: 27823-31.
40. TAK PP, FIRESTEIN GS: NF- B:A key role in inflammatory diseases. *J Clin Invest* 2001; 107: 7-11.
41. LI X, STARK GR: NF B-dependent signaling pathways. Exp Hematol 2002; 30: 285-96.
42. LAWRENCE T, GILROY DW, COLVILLE-NASH PR,WILLOUGHBY DA: Possible new role for NF B in the resolution of inflammation. *Nat Med* 2001; 7: 1291-7.
43. FEYS BJ, PARKER JE: Interplay of signaling pathways in plant disease resistance. *Trends Genet* 2000; 16: 449-55.
44. DIXON MS, GOLSTEIN C, THOMAS CM, VAN DER BIEZEN EA,JONES JD: Genetic complexity of pathogen perception by plants: The example of Rcr3, a tomato gene required specifically by Cf-2. *Proc Natl Acad Sci USA* 2000; 97: 8807-14.
45. GIRARDIN SE, TOURNEBIZE R,MAVRIS M *et al.*: CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive *Shigella flexneri*. *EMBO Rep* 2001; 2: 736-42.
46. OGURA Y, BONEN DK, INOHARA N *et al.*: A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001; 411: 603-6.
47. HAMPE J, CUTHBERT A, CROUCHER PJ *et al.*:Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet* 2001; 357: 1925-8.
48. MICELI-RICHARD C, LESAGE S, RYBOJAD M *et al.*: CARD15 mutations in Blau syndrome. *Nat Genet* 2001; 29:19-20.
49. SRINIVASULA SM, POYET J-L, RAZMARA M, DATTA, P, ZHANG Z, ALNEMRI ES: The PYRIN CARD protein ASC is an activating adaptor for caspase-1. *J Biol Chem* 2002; (epub ahead of print).
50. WANG L, MANJI G, GRENIER JM *et al.*: PYPAF7: A novel PYRIN-containing Apaf1-like protein that regulates activation of NF-kB and caspase- 1-dependent cytokine processing. *J Biol Chem* 2002; May 17 (epub ahead of print).
51. KUIDA K,ZHENG TS, NA S *et al.*:Altered cytokine export and apoptosis in mice deficient in interleukin-lb converting enzyme. *Science* 1995; 267: 2000-3.
52. LI P, ALLEN H, BANERJEE S, SESHADRI T: Characterization of mice deficient in interleukin-1 beta converting enzyme. *J Cell Biochem* 1997; 64: 27-32.
53. HUMKE EW, SHRIVER SK, STAROVASNIK MA, FAIRBROTHER WJ, DIXIT VM: ICEBERG: A novel inhibitor of interleukin-1beta generation. *Cell* 2000; 103: 99-111.
54. DRUILHE A,SRINIVASULA SM,RAZMARA M, AHMAD M, ALNEMRI ES: Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. *Cell Death Differ* 2001; 8: 649-57.
55. LEE SH, STEHLIK C,REEDJC: Cop,a caspase recruitment domain-containing protein and inhibitor of caspase-1 activation processing. *J Biol Chem* 2001; 276: 34495-500.
56. HLAING T, GUO RF, DILLEYKA *et al.*: Molecular cloning and characterization of DEFCAP-L and -S, two isoforms of a novel member of the mammalian Ced-4 family of apoptosis proteins. *J Biol Chem* 2001; 276: 9230-8.
57. CHU ZL,PIO F, XIE Z *et al.*:A novel enhancer of the Apafl apoptosome involved in cytochrome c-dependent caspase activation and apoptosis. *J Biol Chem* 2001; 276: 9239-45.
58. TOUITOU I: The spectrum of familial Mediterranean fever (FMF) mutations. *Eur J Hum Genet* 2001; 9: 473-83.
59. CHAE JJ, CENTOLA M, AKSENTIJEVICH I *et al.*: Isolation, genomic organization, and expression analysis of the mouse and rat homologs of MEFV, the gene for familial mediterranean fever. *Mamm Genome* 2000; 11: 428-35.
60. WHITHAM S, DINESH-KUMAR SP, CHOI D, HEHL R, CORR C, BAKER B: The product of

the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. *Cell* 1994; 78:1101-15.

61. MINDRINOS M, KATAGIRI F, YU GL, AUSUBEL FM: The *A. thaliana* disease resistance gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. *Cell* 1994; 78: 1089-99.
62. JAROSZEWSKI L, RYCHLEWSKI L, REED JC, GODZIK A: ATP-activated oligomerization as a mechanism for apoptosis regulation: fold and mechanism prediction for CED-4. *Proteins* 2000; 39: 197-203.
63. SCHUBERT W-D, GOBEL G, DIEPHOLZ M *et al*.: (Leucine rich repeat structure) MMDB Id: 17799; PDB Id: 1H6T.
64. SHI Y: (CARD structure) MMDB Id: 13221 PDB Id: 2YGS.
65. CARR S, PENFOLD CN, BAMFORD V, JAMES R, HEMMINGS AM: The structure of TolB, an essential component of the tol-dependent translocation system, and its protein-protein interaction with the translocation domain of colicin E9. *Structure Fold Des* 2000; 8: 57-66.
66. XU Y, TAO X, SHEN B, HORNG T *et al*..: Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. *Nature* 2000; 408: 111-5.
67. PHELAN P, GORFE AA, JELESAROV I, MARTI DN, WARWICKER J, BOSSHARD HR: Salt bridges destabilize a leucine zipper designed for maximized ion pairing between helices. *Biochemistry* 2002; 41: 2998-3008.
68. BORDEN KLB, FREEMONT PS: (Zinc finger structure) MMDB Id: 5201 PDB Id: 1FRE.
69. DAY CL, ALBER T: Crystal structure of the amino-terminal coiled-coil domain of the APC tumor suppressor. *J Mol Biol* 2000; 301: 147-56.
70. SETO MH, LIU HL, ZAJCHOWSKI DA, WHITLOW M: Protein fold analysis of the B30.2-