Probing Pathways of Innate Immunity Through the Study of Familial Mediterranean Fever

by

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FIRE & ICE

Dedication

To all the members of my family, by blood and by choice.

Your love gives me strength every day.

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List of Abbreviations

AIDS	Aquired Immune Deficiency Syndrome
ASC	Apoptosis-associated Speck-like protein containing a CARD
BAR	Bin-Amphiphysin-Rsv
BHI	Brain and Heart Infusion
CAPS	Cryopyrin Associated Periodic Syndromes
CARD	CAspase Recruitment Domain
CC	Coiled-Coil
CD2BP1	CD2 Binding Protein 1
CINCA	Chronic Infantile Neurologic Cutaneous and Articular syndrome
CRP	C-Reative Protein
CV3B	CoxsackieVirus B3
DAPI	4,6-DiAmidino-2-PhenylIndole
DD	Death Domain
DDF	Death Domain Fold
DDHR	Death Domain Homology Region
DED	Death Effector Domain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	DiMethyl SulfOxide
DNA	DeoxyriboNucleic Acid

EFC	Extended FCH domain
FCH	Fes/CIP4 Homology
FCU	Familial Cold Urticaria
FIIND	Function to FIND
FMF	Familal Mediterranean Fever
HIDS	HyperImmunoglobulinemia D Syndrome
HIV	Human Immunodeficiency Virus
HPFS	Hereditary Periodic Fever Syndromes
ICE	Interleukin Converting Enzyme
IκB	Inhibitor of κB
IL-18	InterLeukin-18
IL-1β	InterLeukin-1β
IP	ImmunoPrecipitation
IRB	Institutional Review Board
LPS	LipoPolySaccharide
LRR	Leucine Rich Repeats
MDP	Muramyl DiPeptide
MEFV	*Mediterranean FeVer* locus
mRNA	Messenger RiboNucleic Acid
MWS	Muckle-Wells Syndrome
NACHT	Domain present in NAIP, CIITA, HET-E and TP1
NBD	Nucleotide Binding Domain
NF-κB	Nuclear Factor κB

NIH	National Institutes of Health
NLR	NOD-Like Receptor
NMR	Nuclear Magnetic Resonance
NOMID	Neonatal-Onset Multisystem Inflammatory Disease
OMIM	Online Mendelian Inheritance in Man
PAMPs	Pathogen-Associated Molecular Patterns
PAPA	Pyogenic sterile Arthritis, Pyoderma gangrenosum and Acne
PBL	Peripheral Blood Leukocytes
PBS	Phosphate Buffered Saline
PFA	ParaFormAldehyde
PMN	PolyMorphoNuclear leukocytes
PSTPIP1	Proline, Serine, Threonine-rich Phosphatase Interacting Protein 1
PyD	Pyrin Domain
RFP	Ret Finger Protein
SAA	Serum Amyloid A
SAP	Serum Amyloid P
SH3	Src Homology domain
siRNA	Small Interfering RiboNucleic Acids
SPRY	SP1A and RYanodine receptor
TNFα	Tumor Necrosis Factor α
TNFR1	Tumor Necrosis Factor Receptor 1
TPR	TetratricoPeptide Repeat
TRAPS	Tumor Necrosis Factor Receptor-Associated Periodic Syndrome

TRIM	TRIpartite Motif

List of Cell Lines

293T	Human embryonic kidney cells
3T3	Mouse embryonic fibroblasts
A549	Human carcinomic alveolar basal epithelial cells
COS-7	African green monkey kidney fibroblast cells
HCT116	Human colon carcinoma cells
HEK293	Human embryonic kidney cells
HeLa	Human cervical epithelial carcinoma cells
HL-60	Human promyelocytic leukemia cells
J774	Mouse macrophages
KG-1	Human acute myeloid leukemia cells
MAIL8	293T-derived that stably express C12N2 and ASC
MCF-7	Human breast adenocarcinoma cells
MNK45	Human gastric cancer cells
Rat-1	Rat embryonic fibroblasts
RAW	Mouse leukemic monocyte/macrophage cells
SW-13	Human adrenal carcinoma cells
SW-480	Human colon adenocarcinoma cells
THP-1	Human acute monocytic leukemia cells

Abstract

Probing Pathways of Innate Immunity Through the Study of Familial Mediterranean Fever

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Familial Mediterranean fever (FMF) is an autoinflammatory disease characterized by self-limiting attacks of fever, pain, and neutrophil influx in the joints, abdomen, chest or skin. FMF is caused by mutations in the *Mediterranean Fever* locus, which encodes pyrin, a protein expressed primarily in neutrophils and monocytes. Clues as to pyrin's function have come from our identification of several pyrin-interacting proteins; the further exploration of these interactions have revealed roles for pyrin in the modulation of inflammation through cytoskeletal and apoptotic signaling. First, we establish that pyrin is recruited to regions of actin polymerization by actin, VASP and Arp3, and show that the central region of the pyrin protein is responsible for these interactions. Second, we identify the pro-apoptotic protein Siva as a pyrin binding partner.

This interaction involves the C-terminal domain of pyrin and modulates Siva-induced apoptosis. Third, we examine the biology of PSTPIP1, a pyrin binding protein that links PEST-type phosphatases to their substrates. We show that PSTIPIP1 forms membrane-associated tubular filaments that radiate from the center of the cell and establish that an extended Fes-Cip4 homology (EFC) domain of PSTPIP1 is required for this function. Co-expression of pyrin with PSTPIP1 causes a marked change in tubule distribution, an activity that requires the B-box and coiled-coil region of pyrin. Finally, we examine ASC (apoptotic speck-like protein with CARD domain). The pyrin:ASC interaction involves a modified death domain located at the N-terminus of both proteins. ASC forms large cellular aggregates called specks that can act as a platform for inflammation (inflammasome) and/or cell death (pyroptosome). We show that specks prognosticate cell death, and that pyrin modulates speck formation. Treating cells with microtubule toxins dramatically reduces ASC speck aggregation, a finding that could, at least in part, account for the ability of colchicine to ameliorate FMF attacks. In addition, we demonstrate that ASC:pyrin aggregates are remarkably stable in extracellular space, and speculate that they could act to nucleate amyloid, an often fatal complication of FMF. Together, these studies lend new insight into the pathoetiology underlying FMF, and reveal new information about several other inflammatory conditions involving the innate immune system.

Chapter 1

Introduction

Autoinflammatory diseases are a set of inherited human disorders that are characterized by sporadic bouts of generalized fever with localized pain and inflammation. These disorders differ from autoimmune diseases in that they lack autoantibodies and do not involve antigen-specific T-cells, but are instead characterized by a massive mobilization of myeloid cells. It has therefore been proposed that autoimmune diseases represent defects in adaptive immunity while autoinflammatory disorders are diseases of innate immunity. The first reference to autoinflammatory diseases in medical literature dates back to the early 19th century, when Heberden described a periodic disease of pain in the abdomen, chest, and occasionally the extremities (Heberden, 1806). Since that time, several distinct autoinflammatory disorders have been identified, and have been divided into categories based on clinical presentation. These classifications include hereditary periodic fever syndromes, idiopathic febrile syndromes, granulomatous disorders, pyogenic disorders, hemophagocytic disorders, complement disorders, vasculitic syndromes, metabolic disorders, storage diseases, and fibrosing disorders (reviewed in Kastner, 2005). The distinct pathophysiology of each of these diseases provides a unique look into the complexity of the innate immune system, and the interconnectedness of the

various pathways involved.

The hereditary periodic fever syndromes (HPFS) are the most common of the historically defined autoinflammatory diseases. HPFS diseases include familial Mediterranean fever (FMF; OMIM 249100), hyperimmunoglobulinemia D syndrome (HIDS; OMIM 260920), Blau syndrome (OMIM 186580), TNF receptor-associated periodic syndrome (TRAPS; OMIM 142680), and the allelic disorders familial cold urticaria (FCU; OMIM 120100), Muckle-Wells syndrome (MWS; OMIM 191100), and neonatal-onset multisystem inflammatory disease (NOMID, also known as CINCA, or chronic infantile neurologic cutaneous and articular syndrome; OMIM 607115). The causative gene for each of these diseases was identified between 1997 and 2002, and some of these disorders display simple Medelian inheritance patterns while others are more complicated. Each HPFS is clinically distinct, but all have several features in common. Patients experience recurring attacks characterized by very high fever and localized inflammation. An attack trigger for some of these disorders is known, such as exposure to cold in FCU or vaccination in HIDS, but for most diseases, the specific trigger remains unknown. Originally, the inflammation associated with these attacks was described to affect synovia, serosa, and skin, but is now known to affect a wider range of tissues (Kastner, 2005). Attacks are self-limiting and the symptoms last between 12 hours (as seen in FCU) to a few weeks (as seen in TRAPS patients). Attack frequency can range from every few days to every few years. The attacks are self-resolving, and (with the notable exception of NOMID) patients do not

experience symptoms between attacks, although biochemical indicators of inflammation, including acute phase proteins, remain elevated (Kastner, 2005; Lachmann et al., 2006).

The archetypical HPFS is familial Mediterranean fever (FMF), so named because of its high incidence in populations originating from the Mediterranean basin including non-Ashkenazi Jews, Turks, Armenians, and Arabs. In fact, in some populations, carrier frequencies as high as 29% have been reported (Kogan et al., 2001). This high incidence exceeds Hardy-Weinberg expectations and cannot fully be accounted for by founder effects. Thus, a selective pressure for maintenance of this allele frequency has been proposed (Aksentijevich et al., 1999; Lachmann et al., 2006; Schaner et al., 2001). Though the underlying mechanism for this selective advantage has not been precisely identified, it has been shown that heterozygotes display increased levels of several pro-inflammatory indicators, including serum amyloid A (SAA) and C-reactive protein (CRP) (Lachmann et al., 2006). On this basis, it has been proposed that the innate immune system of heterozygotes is “ramped up” and better able to clear potential deadly pathogens (Aksentijevich et al., 1999; Lachmann et al., 2006). Interestingly, this heterozygote phenotype would predict that FMF is actually a dominant disease with reduced penetrance, rather than a recessive disease as first categorized. This could at least partially explain the finding that up to 30% of patients diagnosed with FMF seem only to have mutations on one allele (Aksentijevich et al., 1999; Touitou et al., 2007).

FMF patients typically begin experiencing attacks in childhood, with an increase in attack frequency appearing at puberty. Attacks generally last from one to three days and are characterized by a massive influx of neutrophils into the affected area. The tissues predominantly affected are the joints, serosa, or skin, but also may include the pericardium, pleura of the lungs, skeletal muscle and vascular tissue. During joint attacks, leukocyte counts in the synovial fluid can reach as high as $1x10^6/mm^3$ (Heller et al., 1966).

One distinguishing feature of FMF compared to other autoinflammatory disorders is the strikingly high incidence of multiorgan AA-type amyloidosis. Serum amyloid A (SAA) is a protein that is secreted in response to cytokines during the acute phase response, and can be increased from 100 to 1000-fold during fever (Simon and van der Meer, 2007). When misfolded, SAA is deposited in organs as plaques and can lead to organ failure. Amyloid deposition in FMF is particularly prominent in the kidney, and often results in death due to renal failure. Prior to the 1970s, amyloidosis affected as many as 70% of FMF patients over 40 (Gafni et al., 1968). In 1974, it was observed that an FMF patient receiving colchicine therapy for gout experienced markedly fewer FMF attacks during treatment (Goldstein and Schwabe, 1974). This led Goldstein to suggest daily prophylaxis with colchicine as a standard treatment for FMF (Goldstein and Schwabe, 1974), a recommendation that is followed to this day. Colchicine reduces both the frequency and severity of attacks, and also

prevents amyloidosis. In some cases, colchicine administration has even been shown to reverse the nephrotic syndrome caused by amyloidosis in FMF patients (Simsek et al., 2000), although the mechanism behind its efficacy is unknown.

The Pyrin Protein

The FMF gene, *MEFV* (for Mediterranean Fever locus), was identified independently by two consortia in 1997 (The International FMF Consortium, The French FMF Consortium). *MEFV* is located on the short arm of chromosome 16, and its 3.7kb transcript encodes a 781-amino acid protein named pyrin by the International Consortium (1997b) or marenostrin by the French Consortium (1997a). The name "pyrin" has become more widely accepted in the field. Pyrin is comprised of 10 exons encoding several identifiable domains, including an N-terminal death-domain related structure that is now known as a pyrin domain (PyD), a central B-box and coiled-coil region, and a C-terminal B30.2/SPRY/RFP domain (see Figure 1.1). These domains are discussed in more detail below. Through these domains, pyrin interacts with a variety of proteins that link it to several cellular pathways. To date, more than 70 disease-causing mutations in pyrin have been identified (INFEVERS website - http://fmf.igh.cnrs.fr/infevers), most of which are missense mutations clustered in exon 10 (with a smaller cluster in exon 2). The mechanism by which these mutations cause the robust inflammatory response seen in FMF is as yet unclear, but an altered binding

affinity for partner proteins or ligands seems plausible, at least for exon 10 mutations, since this region of the protein (encoding the B30.2/SPRY/RFP domain) is thought to be an interaction domain (Goulielmos et al., 2006; Grutter et al., 2006; Woo et al., 2006).

Examination of the first exon of pyrin revealed that its sequence weakly resembles that of a death domain fold (DDF). This type of domain is characterized by a bundle of six α-helices that mediate protein:protein interactions and are thought to be involved in apoptosis and inflammatory signaling pathways (Lahm et al., 2003). It soon became obvious that the variant of the DDF embodied by pyrin was also shared by many other proteins; this domain came to be known as the pyrin domain or PyD.

Besides the PyD, other members of the DDF family include the death domain (DD), death effector domain (DED), and caspase recruitment domain (CARD). Notably, interactions between DDF-containing proteins are usually (though not always) homotypic; that is, DDs typically bind DDs and CARDs normally bind other CARDs (Lahm et al., 2003). Such interactions among DDF proteins are often used for the purpose of self-assembly into larger multi-subunit complexes. Several such complexes have been identified, including inflammasomes and recently pyroptosomes. These complexes are described in detail later in the text, but as their names imply, they are involved in inflammatory and cell death pathways. Consistent with this function, all hereditary periodic

fever syndromes (with the exception of HIDS, which is caused by mutations in the gene encoding mevalonate kinase, a critical enzyme in the isoprenoid pathway), result from a mutation in a DDF-containing protein (pyrin in the case of FMF, NOD2 for Blau syndrome, TNFR1 for TRAPS, and cryopyrin for FCU, MWS, and NOMID/CINCA).

The C-terminal half of the pyrin molecule contains a tripartite motif (TRIM) consisting of B-box zinc finger, Coiled-coil and RFP domain (the RFP is also called a SPRY or B30.2 domain). The TRIM motif, like the PyD, is found on a wide variety of proteins that function in different contexts, including transcriptional regulation, differentiation, and growth control (Nisole et al., 2005). Many TRIM proteins have been shown to form higher-order multiprotein complexes (Reymond et al., 2001), a feature shared with pyrin (see below). Several TRIMs are reported to have anti-retroviral activity through a variety of mechanisms, leading to the hypothesis that TRIMs represent an anti-viral arm of the innate immune system (Nisole et al., 2005). Of particular interest is the protein TRIM5α, which is known to be a major block to HIV infection in primates, although the mechanism behind this action is only beginning to be understood (Stremlau et al., 2004). Mutations in the RFP domain of TRIM5α have been shown to interfere with the protein's viral restriction ability (Song et al., 2005). It has been proposed that the RFP domain of TRIM5α, as well as that of pyrin, forms antibody-like loops that bind specific proteins, and that mutations in these loops result in altered binding affinity (James et al., 2007).

The B-box and Coiled-coil regions of TRIM proteins are thought to encode protein:protein interaction rather than protein:DNA interaction motifs. It has been speculated that the location of the B-box in close proximity to the Coiled-coil serves to orient the Coiled-coil and facilitate protein:protein interactions, or to discourage non-specific binding (Borden, 1998). The *Xenopus* protein Xnf7 requires the Coiled-coil and RFP domains for microtubule binding and bundling activity (Maresca et al., 2005). Through its Coiled-coil domain, the ret finger protein (confusingly, this protein itself is called RFP) binds to SMC3 (structural maintenance of chromosome 3 protein) and may regulate its distribution within the cell (Patel and Ghiselli, 2005). Additionally, the RFP protein binds to EPC1 (enhancer of polycomb 1) (Shimono et al., 2000; Tezel et al., 2002) and to Mi-2β (Shimono et al., 2003) through its Coiled-coil domain, and these interactions play a role in transcriptional repression. During the course of my thesis work, we found that the B-box and Coiled-coil region of pyrin mediates its binding to PSTPIP1 and to proteins associated with polymerizing actin; this is described in more detail below.

The very C-terminal region of pyrin contains a domain that has been referred to as an RFP (Ret Finger Protein) domain, or a SPRY (SP1A and RYanodine receptor) domain or a B30.2 domain (named for the B30.2 exon in the Human Class I histocompatibility complex). Recently the structure of this domain was predicted by NMR (Masters et al., 2006) and by crystallography (Grutter et al., 2006; Woo et al., 2006). Both methods predict a β-sandwich

structure formed by anti-parallel β-sheets with protruding loops that appear to represent binding surfaces. The residues in these loops are extremely variable among RFP domain-containing proteins, which may reflect binding specificity to individual proteins (Woo et al., 2006). Many FMF-causing mutations in pyrin, notably M680I and M694V, are found within this loop structure (Grutter et al., 2006; Masters et al., 2006; Woo et al., 2006), suggesting that the FMF mutations may alter the binding affinity of the pyrin protein for partner proteins. At least one study supports this hypothesis, showing that Caspase-1 binds with less affinity to mutant pyrin than to wildtype (Chae et al., 2006). Notably, several of the exon 10 FMF mutations, including the common V726A mutation, are not found within the loop region; however, V726A has been correlated with a milder phenotype and may therefore represent another mechanism by which pyrin mutations lead to disease (Chae et al., 2006).

Pyrin Expression

Consistent with the FMF phenotype and its likely function in innate immunity, pyrin is expressed primarily in monocytes, neutrophils, eosinophils, dendritic cells (Centola et al., 2000; Diaz et al., 2004). It is also found in fibroblast-like stromal cells of the skin, synovial membranes, and peritoneum (Centola et al., 2000; Diaz et al., 2004), all common sites of inflammatory infiltrations in FMF. Subcellular localization of endogenous pyrin varies by cell

type: pyrin is nuclear in neutrophils, dendritic cells, and synovial fibroblasts while it is cytoplasmic in monocytes (Diaz et al., 2004). Certain pyrin binding partners contribute to the control of this subcellular localization (Jeru et al., 2005), as do alternative splicing events (Diaz et al., 2004). The differing subcellular locations of pyrin suggest that the function of pyrin may be cell type-dependent. Therefore, it is important to note that, in transfection studies performed in non-pyrin expressing cells such as HeLa, COS-7 and 293T, pyrin is exclusively cytoplasmic, and co-localizes with filamentous actin and microtubules (Mansfield et al., 2001).

Pyrin Splice Isoforms

Several pyrin isoforms in addition to the full-length protein have been identified and result from alternative splicing events in exons 2, 4, and 8 (Diaz et al., 2004). Combinations of these splicing events lead to a total of 9 possible alternative isoforms of pyrin, although several of these are very rare (summarized in (Diaz et al., 2004)). Two isoforms are worth considering further since their structures suggest possible important consequences for pyrin function. The first alternative isoform to be identified was found in human peripheral blood leukocytes (PBL). This transcript lacks exon 2, and so was designated as exon 2Δ (Papin et al., 2000). While exon 2Δ is seen in monocytes and is inducible by lipopolysaccharide (LPS), this isoform was undetectable in synovial fibroblasts

without LPS stimulation (Diaz et al., 2004). This isoform is interesting because exon 2 contains a binding site that is specific for certain members of the 14.3.3 protein family (Papin et al., 2000). The 14.3.3 proteins can act as cytosolic tethers; indeed, it has been demonstrated that loss of exon 2 promotes nuclear (rather than cytosolic) localization of pyrin (Papin et al., 2000). However, this is not the entire story, since pyrin in neutrophils and synoviocytes is nuclear, but the majority of pyrin mRNA transcripts in those cells contains exon 2 (Diaz et al., 2004).

Another pyrin isoform is generated from the use of an alternative splice donor site 3' to the normal exon 8 donor site; the resulting transcript contains extra bases downstream of the normal end of exon 8 and splicing to the normal exon 9 acceptor creates a frameshift. Thus, a truncated protein results that contains an extended exon 8, but is missing exons 9 and 10 (Diaz et al., 2004). Exon 9 is miniscule, but exon 10 encodes the entire RFP domain. This isoform is denoted exon 8ext. While PBL express both the consensus and exon 8ext messages for pyrin much more robustly than synovial fibroblasts, the exon 8ext transcript accounts for a much larger proportion of the total pyrin message in synovial cells than in PBL (27% of the pyrin transcript in synovial fibroblasts vs 10% for PBL). The exon 8ext isoform is of particular interest given that the mouse and rat orthologues of pyrin completely lack an RFP domain (Chae et al., 2000), and instead highly resemble exon 8ext. This could indicate that the wildtype pyrin protein in rodents has a function similar to that encoded by the

pyrin exon 8ext isoform of humans. Interestingly, one FMF mutation is a truncation mutant at amino acid position Met^{694}, located in the middle of exon 10. This finding predicts that loss of part (or potentially all) of exon 10 creates a pyrin molecule that promotes inflammation. By extrapolation then, the exon 8ext. form, as well as the rodent form, might also have a pro-inflammatory function in cells. However, this remains to be demonstrated.

Pyrin Binding Proteins

The pyrin protein contains a series of protein-protein interaction domains through which it networks with several binding partners and is connected to multiple cellular processes. To date, pyrin has been reported to interact with five proteins: ASC, Caspase-1, 14.3.3, PSTPIP1, and Siva. Here, we examine these partner proteins and their function in linking pyrin to cytoskeletal signaling, cytokine secretion, inflammation, and apoptosis.

ASC

Pyrin binds via its PyD encoded by exon 1 to ASC (apoptosis-associated speck-like protein containing a CARD). ASC is a critical mediator of apoptotic and inflammatory signaling (Taniguchi and Sagara, 2007) and pyrin appears to

modulate these ASC functions (Richards et al., 2001; Yu et al., 2007). ASC was originally identified as a 22kD protein in the Triton-X-insoluble fraction of HL-60 cells (Masumoto et al., 1999). Subsequent cloning of the ASC gene and analysis of the protein sequence revealed a 195-amino acid protein that contains an N-terminal PyD and a C-terminal Caspase recruitment domain (CARD). Thus, ASC is an interesting adaptor protein that can link PyD-containing proteins to CARD-containing ones. ASC expression is widespread, but it is found at particularly high levels in peripheral blood leukocytes (cells that also express pyrin) and in epithelial cells, both of which are cell types directly involved in innate immunity (Masumoto et al., 2001). ASC has also been implicated as a target of methylation-induced silencing in breast, prostate, lung, skin, gastric, and colon cancers (Collard et al., 2006; Conway et al., 2000; Guan et al., 2003; Liu et al., 2006; Moriai et al., 2002; Virmani et al., 2003; Zhang et al., 2006), indicating a possible role for ASC as a tumor suppressor.

Localization studies using an antibody against ASC have revealed that ASC can adopt two very distinct distributions within the cell. One distribution is a diffuse localization in both the cytoplasm and nucleus. The other is as a distinct speck structure that appears spontaneously and is associated with cell death (Masumoto et al., 1999). The specks are usually located near (although not within) the nucleus, and range in size from 0.5-3μm in diameter. Specks have a distinct structure with an apparently hollow center and filamentous projections that extend both toward the outside and into the center of the speck (Richards et

al., 2001). ASC is capable of binding to several proteins, most of which are involved in inflammation and cell death pathways (see Table 1.1). Distributed ASC may exist primarily as a dimer (Fernandes-Alnemri et al., 2007). The rapidity of speck formation and the fact that the speck contains nearly all of the cytoplasmic compliment of ASC implies that speck formation may occur in response to a whole-cell trigger such as an ion flux. Indeed, recent work by Alnemri et al. has shown that speck formation is immediately preceded by a potassium efflux, and that speck formation can be blocked by raising levels of extracellular potassium to prevent this efflux (Fernandes-Alnemri et al., 2007). ASC specks recruit several cellular proteins that contain either a PyD or a CARD (see Table 1.1). This rapid, triggered scaffolding function allows ASC to quickly assemble large signaling complexes (discussed further below).

The PyD and CARD-containing proteins that are known to bind to ASC are listed in Table 1.1 (reviewed in (McConnell and Vertino, 2004)). All of these proteins play a role in mediating apoptotic and inflammatory signaling, particularly Capsases 1, 8 and 10. Caspases 8 and 10 are the initiator Caspases in the receptor-mediated (extrinsic) apoptosis pathway, while Caspase-1 is responsible for the activation of interleukin-1β (IL-1β) and IL-18, critical inflammatory cytokines. All three of these Caspases, and several others, share a common activation pathway. The enzymes are produced as proenzymes that must have their pro-domain cleaved in order to be activated. This occurs when several of the proenzyme molecules, all of which have constitutive low-level

activity, are physically brought together. The concentration of several such molecules causes “proximity-induced” cleavage and subsequent activation (Bao and Shi, 2007). Indeed, ASC is an activating adaptor for Caspase-1 via a CARD-CARD interaction (Srinivasula et al., 2002; Stehlik et al., 2003). Co-expression of procaspase-1 and ASC leads to increased activation of procaspase-1 and secretion of mature IL-1β. Both domains of ASC are required for Caspase-1 activation (Srinivasula et al., 2002) as well as for speck formation (Richards et al., 2001). This establishes a role for ASC speck formation in inflammatory signaling through Caspase-1-dependent processing of IL-1β.

ASC speck formation is strongly associated with cell death, but the mechanism may be different in different cell types. In human monocytes and in the myeloid cell line HL-60, it has been shown that cell death is almost immediate following formation of the speck; death occurs through a Caspase-1-mediated process known as pyroptosis (Fernandes-Alnemri et al., 2007). Pyroptosis is a newly described cell death pathway that is most often seen in macrophages and other innate immune cells in response to microbial infection. Apoptosis ensues with cytoplasmic and nuclear condensation but with an intact plasma membrane; since cellular contents do not “leak” into the surrounding tissue, this form of death does not generally involve inflammation. In contrast, pyroptosis involves cell lysis and the release of cellular contents, and is thus inherently proinflammatory (reviewed in (Fink and Cookson, 2005)). Interestingly, in cells of a non-myeloid lineage that express ASC but do not express Caspase-1, cell death eventually

occurs, but only after several hours following speck formation (Richards et al., 2001). Thus, ASC must be capable of mediating cell death through multiple pathways, potentially involving Caspases other than Caspase-1.

The two major Caspase-mediated apoptotic pathways are the Caspase-8/10 receptor-mediated (extrinsic) cell death pathway and the Caspase-9 mitochondrial (intrinsic) cell death pathway (Chowdhury et al., 2006). ASC is able to bind to both Caspase-8 and Caspase-10 and recruits these molecules to specks (Schaner and Gumucio, 2005). Interestingly, Caspase-9 does not bind to ASC specks (Schaner and Gumucio, 2005). ASC has also been shown to activate BID, a proapoptotic member of the Bcl-2 family of proteins that acts downstream of Caspase-8 (Hasegawa et al., 2007). Use of dominant-negative forms of Caspase-8 (Masumoto et al., 2003) and siRNA knockdown of endogenous Caspase-8 (Hasegawa et al., 2007) prevent ASC-induced apoptosis in several cell types, indicating a general requirement for Caspase-8.

Although it does not bind to ASC, a context-dependent role for Caspase-9 in ASC-induced cell death has also been documented (Ohtsuka et al., 2004). Dominant-negative Caspase-9, siRNA against Caspase-9, and Caspase-9-specific inhibitors result in an attenuation of ASC-induced apoptosis in HEK 293, SW-480, and A549 cells, but not in 293T, MAIL8, HCT116, or MKN45 cells (Hasegawa et al., 2007; Masumoto et al., 2003; McConnell and Vertino, 2004). ASC may be also a transcriptional target gene for p53, an important tumor

suppressor gene that functions through the Caspase-9-mediated cell death pathway (Ohtsuka et al., 2004). Additionally, ASC binds directly to BAX, a proapoptotic member of the BCl-2 family of tumor suppressor genes and an important mediator in Caspase-9-initiated apoptosis (Ohtsuka et al., 2004). ASC is not able to induce apoptosis in BAX-deficient cells, and ASC may be required for BAX's translocation to mitochondria prior to cytochrome c release and apoptosis. These data indicate a role for ASC in Caspase-9-mediated apoptosis, but in an indirect, BAX-dependent and cell-type specific, manner.

The functional relevance of the pyrin:ASC interaction may stem from pyrin's ability to modulate ASC-mediated activation of the transcription factor NF-κB (nuclear factor-κB). NF-κB is expressed in nearly all animal cells and is part of a group of transcription factors known as "first responders." These factors are maintained in cells in an inactive state and are activated in response to stimuli such as cytokines, stress, irradiation, and viral or bacterial antigens (Hayden and Ghosh, 2008). Other transcription factors in this category include c-Jun, STATs, and nuclear hormone receptors. Because new protein synthesis is not required upon cell stimulation, these factors can be activated quickly to produce gene changes and provide the first line of cellular defense to harmful stimuli.

NF-κB is maintained as an inactive dimer, sequestered in the cytoplasm by a group of inhibitors known as IκBs (inhibitor of kappa B). The IκB proteins contain a series of ankyrin repeats that mask the nuclear localization signal of

NF-κB and prevent its translocation to the nucleus (Jacobs and Harrison, 1998). Upon cell stimulation, the kinase IKK (IκB kinase) phosphorylates IκB and targets it for ubiquitination and subsequent degradation by the proteasome. NF-κB is then free to enter the nucleus, where it activates genes that are tied to inflammation, cell survival and proliferation, and the immune response. The NF-κB response is self-limiting, as IκB is a direct target gene of NF-κB (Nelson et al., 2004). The pathogenesis of several diseases, including cancer, AIDS, and bubonic plague can be tied to perturbations in the NF-κB pathway. This highlights the importance of NF-κB regulation in cellular homeostasis, and implicates the pathway as a potential therapeutic target for multiple conditions.

NF-κB is activated in response to several extracellular stimuli, including the bacterial membrane component lipopolysaccharide (LPS) and the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα). This signaling is modulated by several DDF-containing proteins, although the mechanisms behind this process are poorly understood. ASC can differentially regulate NF-κB activation in a context-specific manner that depends on both the stimulus and the availability of partner proteins. Overexpression of ASC or of pyrin in unstimulated cells produces no detectable NF-κB response. However, expression of ASC or pyrin in cells exposed to TNFα results in inhibition of IKK and downregulation of NF-κB signaling compared to untransfected cells (Stehlik et al., 2002). In fact, all PyD-containing proteins tested, including cryopyrin, PYPAF2, and CARD7, reduced NF-κB activation in response to TNFα (Stehlik et

al., 2002). Additionally, suppression of endogenous ASC expression in MCF-7 or THP-1 cells results in enhanced NF-κB signaling in response to TNFα and LPS stimulation (Stehlik et al., 2003). These data indicate a role for PyD-containing proteins, particularly ASC, in curbing the NF-κB response.

Interestingly, transient co-expression of pyrin and ASC in HEK293 cells results in a 20-70 fold induction NF-κB signaling in the absence of inflammatory stimulation (Stehlik et al., 2003). Similar results were seen with co-expression of ASC and other PyD-containing proteins, such as cryopyrin and PYPAF7. This could indicate that ASC functions as a suppressor of NF-κB-mediated pro-inflammatory signaling, and that pyrin (as well as other PyD-containing proteins) can relieve this inhibition to result in enhanced NF-κB signaling. In this way, pyrin's interaction with ASC may be proinflammatory. These results are somewhat controversial, as the induction of NF-κB signaling appears to be dependent upon both the level of ASC expression and cell type used (Dowds et al., 2004; Yu et al., 2006). However, PyD-containing proteins consistently emerge from these studies as potential regulators of the NF-κB response.

As an ASC-binding protein, pyrin sits at the crossroads of several inflammatory and apoptotic signaling pathways, and thus has the potential to be a powerful modulator of these processes. Co-transfection of pyrin with ASC increases the percentage of transfected cells that contain ASC specks, but decreases the rate at which these cells die (Richards et al., 2001). This suggests

some manner of functional or regulatory role for pyrin in the context of speck function, although the exact details remain unclear. While exon 1 of pyrin is sufficient for localization to specks, it is not sufficient for the acceleration in speck formation, signifying a role for other domains of the pyrin protein in this process. It is possible that pyrin acts as an adaptor to bring proteins critical for apoptotic or inflammatory signaling to the speck, where they interact with other proteins in a macromolecular complex. Additionally, it is conceivable that pyrin links the signaling function of the speck to other cellular processes via its interaction with additional proteins. For example, Chae et al. suggest that the co-localization of pyrin and Caspase-1 in the ASC speck could affect IL-1β processing (Chae et al., 2006). Recent research from the Alnemri laboratory indicates that pyrin can also link ASC to PSTPIP1 and this may regulate pyroptosis (Yu et al., 2007).

Caspase-1

Caspase-1 (also called interleukin-converting enzyme or ICE) mediates fever and inflammatory signaling through activation of IL-1β and IL-18, two important inflammatory cytokines. Caspase-1 activation is mediated in part by ASC, and at least some recent evidence suggests that pyrin may play a role in this process (Chae et al., 2006; Yu et al., 2007). ASC can bind simultaneously via its PyD to pyrin and via its CARD to Caspase-1. This might have different consequences in various cell types: transfection of pyrin decreases ASC-induced

output of mature IL-1β in RAW cells (Chae et al., 2003) but in 293T cells, pyrin enhances this pathway (Yu et al., 2006).

Investigation into the role that pyrin and pyrin mutations might play in the activation of Caspase-1 led to the discovery that in addition to the CARD-mediated interaction between ASC and Caspase-1, pyrin also binds to Caspase-1 independently of ASC (Chae et al., 2006). Co-expression of pyrin and ASC resulted in reduction of IL-1β processing and secretion, suggesting that pyrin binding to Caspase-1 may have an inhibitory effect on this process. Investigation into the domains required for the interaction between pyrin and Caspase-1 revealed that both the p20 and p10 catalytic subunits of Caspase-1 and the RFP domain of pyrin are required. This is of particular interest given that most FMF-causing mutations in pyrin are clustered in the RFP domain. FMF-causing mutations in pyrin reduce pyrin's affinity for Caspase-1, and virtually eliminate the inhibitory effect of pyrin on IL-1β secretion. Additionally, an FMF patient treated with the IL-1β receptor antagonist anakinra showed a reduction in acute phase reactants and control of SAA and CRP (Chae et al., 2006), suggesting that IL-1β is a central player in the FMF disease process. However, initial reports indicate that this may not be the case for all FMF patients (unpublished data, FMF meeting, Bethesda, MD, 2005).

Thus, pyrin and Caspase-1 seem to interact via two distinct pathways. In the first pathway, both proteins interact with ASC, and the effect of pyrin on

Caspase-1 activation and subsequent IL-1β secretion remains controversial. In the second pathway, pyrin and Caspase-1 interact directly, independently of ASC. Wildtype pyrin binds to Caspase-1 and inhibits its ability to activate IL-1β; FMF mutations prevent the binding of Caspase-1, setting it free to activate IL-1β. Increased IL-1β secretion would lead to increased systemic fever and inflammation, accounting for the clinical presentation of FMF patients. A problem with this hypothesis is that the level of IL-1β activation in control and FMF patients is actually quite variable and the relationship with pyrin expression is not so clear (Chae et al., 2006). However, it is interesting that this hypothesis suggests that FMF is a gain of function rather than a loss of function disease as originally proposed. This idea actually fits well with the finding that pyrin is evolving under Darwinian selection as suggested by its history in primates (Schaner et al., 2001).

14.3.3

It has recently been discovered that pyrin binds to certain isoforms of 14.3.3 proteins. The 14.3.3 proteins are a family of highly conserved acidic proteins that are expressed in a wide variety of tissues. Each monomer in the functional dimer unit contains 9 antiparallel α-helices. The dimer itself creates a large channel with a highly negatively charged core. Several isoforms of 14.3.3 have been identified, and the variable residues between the isoforms appear to

be located on the outside of the channel as shown by X-ray crystallography (Benzinger et al., 2005; Wilker et al., 2005).

14.3.3 proteins are highly distinct, and do not appear to have significant homology to any other known proteins. The only somewhat recognizable domain in the protein is the tetratricopeptide repeat (TPR). The TPR domain is comprised of tandem repeats of a degenerate motif that is present in several proteins and acts as a scaffold for building multimeric signaling complexes. 14.3.3 proteins are known to be involved in a variety of cellular pathways through their binding partners, including cell cycle regulation, transcription, signaling, and cytoskeletal architecture (Aitken, 2006). Several proteins that interact with 14.3.3 proteins specifically bind to only one isoform, and control of this binding usually involves phosphorylation of the binding partner and occasionally the 14.3.3 protein itself, indicating that phosphorylation is key in the regulation of these proteins. Accordingly, 14.3.3 proteins interact with several kinases and phosphatases (Aitken, 2006).

14.3.3 proteins bind constitutively to several proteins that contain nuclear localization signals, masking these signals from the nuclear import machinery and sequestering the proteins in the cytoplasm (Yaffe, 2002). Among these proteins is the kinase c-Abl (Aitken, 2006). Upon phosphorylation by JNK kinases, 14.3.3 releases c-Abl and also the apoptotic protein BAD, allowing their transport to other cellular compartments. C-Abl is transported to the nucleus

where it activates apoptotic pathways in response to DNA damage (Yoshida and Miki, 2005; Yoshida et al., 2005). BAD is phosphorylated and translocates to the mitochondria where it interacts with the anti-apoptotic protein Bcl-2 (Yoshida and Miki, 2005; Yoshida et al., 2005). Thus, 14.3.3 proteins are linked to both intracellular trafficking and apoptosis. This ability of 14.3.3 proteins to regulate the trafficking of other proteins between various cellular compartments has since been repeatedly demonstrated with several of its binding partners (Brunet et al., 2002; Johnson et al., 2002; Merla et al., 2004; Muslin and Xing, 2000; Zhang et al., 1999). This unique mechanism of action indicates a critical role for 14.3.3 proteins in intracellular signaling pathways.

A yeast 2-hydbrid screen revealed that full-length pyrin binds to 3 isoforms of 14.3.3: τ, ε, and σ (Jeru et al., 2005). Since 14.3.3σ is expressed mainly in epithelial cells and not in cells of myeloid lineage (where pyrin is expressed), only isoforms τ and ε were studied further (both of these isoforms are expressed along with pyrin in the myoblast cell line KG-1 and in the lymphoblast cell line HL-60 (Jeru et al., 2005). The full-length pyrin protein is diffusely cytoplasmic in monocytes, although it is capable of localizing to the nucleus in other cell types (Diaz et al., 2004; Papin et al., 2000; Tidow et al., 2000). However, as stated previously, an alternative splice isoform that lacks exon 2 (exon 2Δ) is predominantly nuclear (Cazeneuve et al., 2004; Diaz et al., 2004; Papin et al., 2000). Both full-length pyrin and exon 2Δ contain two nuclear localization signals predicted by sequence analysis, but direct testing showed that neither of these

signals are required for transport of exon 2Δ to the nucleus (Papin et al., 2000). Given the known role that 14.3.3 proteins play in nuclear translocation, these proteins made an intriguing candidate for controlling the subcellular localization of pyrin.

The ability of pyrin (both full-length and exon 2Δ) to bind to 14.3.3 proteins was further investigated by both immunoprecipitation and the ASC speck assay. Both protein:protein interaction detection methods revealed that full-length pyrin binds efficiently to both 14.3.3τ and 14.3.3ε. However, exon 2Δ does not bind to either 14.3.3 isoform (Jeru et al., 2005). This immediately suggested that the binding sequence required for interaction with 14.3.3 proteins is contained in pyrin exon 2. Indeed, pyrin exon 2 alone is sufficient for binding to 14.3.3 proteins (Jeru et al., 2005). Furthermore, binding of full-length pyrin to 14.3.3 proteins requires phosphorylation of three critical serine residues in the domain encoded by exon 2 of pyrin (208, 209, and 242). This is in agreement with previous studies showing that most 14.3.3 binding proteins require phosphorylation in order for binding to occur (Aitken, 2006). When these critical residues were mutated to alanine, localization of full-length pyrin within the nucleus is dramatically increased (Jeru et al., 2005). This indicates that 14.3.3 proteins play a role in sequestering full-length pyrin in the cytoplasm. These data are of particular interest since one of the most common FMF mutations in pyrin is in exon 2 (E148Q), and this mutation is correlated with a less severe disease phenotype (Sarrauste de Menthiere et al., 2003). This may potentially be due to

the ability of this mutation to alter the subcellular localization and functional interactions of full-length pyrin, but not of exon 2Δ, though this explanation is really still hypothetical. Together, these data indicate a role for 14.3.3 in regulating pyrin localization, and thus its ability to interact with partner proteins to control cell function.

PSTPIP1

Pyrin interacts with PSTPIP1 (proline, serine, threonine-rich phosphatase interacting protein 1), a key protein associated with the cortical cytoskeleton, through its central B-box/Coiled-coil domain. Through this interaction, pyrin is linked to critical cytoskeletal signaling pathways and to cytokine secretion. PSTPIP1 is the mammalian orthologue of the yeast protein CDC15, a phosphorylated cytoskeletal-associated protein found in the cytokinetic cleavage furrow that is required for cytokinesis (Fankhauser et al., 1995). In mammals, PSTPIP1 is expressed in hematopoetic cells, in the lung and at high levels in synoviocytes (Arturo Diaz, unpublished data). PSTPIP1 functions as an adaptor protein, and is comprised of an N-terminal FCH domain, a central Coiled-coil (CC), and an SH3 domain. This series of protein-protein interaction domains facilitates the binding of PSTPIP1 to several other cellular proteins. Murine PSTPIP1 was initially isolated in a yeast-two hybrid screen as a protein that binds PEST-type phosphatases PTP-PEST and PTP-HSCF. The human protein

was subsequently identified as a binding partner of the cell surface protein CD2 (leading to the alternate name of CD2 binding protein 1, or CD2BP1 (Li et al., 1998)). In addition, PSTPIP1 binds other T cell immunophenotypic markers such as CD4, CD8, CD54, CD58, CD61, and CD62L, although no functional role for these interactions is known (Bai et al., 2001). In addition to binding the aforementioned PEST phosphatases, PSTPIP1 is also known to bind to several PEST phosphatase substrates, including the actin-binding tyrosine kinase c-Abl, and the cytoskeletal-associated protein WASP. Mutations in WASP cause Wiskott-Aldrich syndrome, an X-linked hematopoetic disorder that results in platelet defects and immunodeficiency. The importance of WASP as a key regulator of cytoskeletal signaling is becoming increasingly clear, and as a WASP-binding protein, PSTPIP1 sits at the intersection of several important signaling pathways. Through binding to PSTPIP1, pyrin therefore also sits in a critical position to modulate multiple cellular processes.

One of the most well characterized functions of WASP is its role in activating Arp2/3-mediated actin polymerization. In this system, WASP acts as a platform for assembling the actin polymerizing machinery near the cell membrane in lamellipodia. Indeed, hematopoetic cells from Wiscott-Aldrich syndrome patients have abnormal cortical cytoskeletons and defects in actin polymerization (Andreu et al., 2007; Molina et al., 1992; Ochs et al., 1980).

Pyrin's relevance in connection with PSTPIP1 and WASP may be found in the modulation of a structure known as the immunological synapse, a term used to describe the antigen-induced interface of an antigen-presenting cell and a T-cell. The mechanism behind formation of the synapse is poorly understood, but it is associated with increased actin polymerization at the border between the two interacting cells; synaptic interaction is reduced by agents that perturb the actin cytoskeleton (Ryser et al., 1982; Wulfing et al., 2000). WASP localizes to the immunological synapse, and this is dependent upon the proline-rich regions of the protein (Badour et al., 2003). PSTPIP1 binds to proline-rich regions of WASP through its SH3 domain (Wu et al., 1998), and is able to simultaneously bind CD2 and CD2AP (an intracellular adaptor protein that links CD2 to the cytoskeleton) through its Coiled-coil domain (Badour et al., 2003). Since WASP, CD2, and CD2AP are all present in the immunological synapse, PSTPIP1 emerged a potential facilitator for the formation of this structure. This hypothesis led to a series of experiments that showed PSTPIP1 not only localizes to the immunological synapse, but is required for synapse formation and for the linking of CD2 engagement to actin polymerization at the cell interface (Badour et al., 2003). PSTPIP1 plays the additional role of connecting PTP-PEST to WASP at the immunological synapse. This results in dephosphorylation of WASP and a decrease in WASP-mediated actin polymerization, and thus the dissolution of the immunological synapse (Cote et al., 2002). In this way, PSTPIP1 may act to limit the longevity of the synapse and its subsequent signaling.

Another intriguing feature of PSTPIP1 is that mutations in the gene encoding this protein result in PAPA syndrome, a dominantly-inherited autoinflammatory disease characterized by arthritis, pyoderma gangrenosum, and acne (OMIM #604416 (Wise et al., 2002)). The disease is similar to FMF, although the clinical presentation is more severe. During PAPA attacks, the accumulation of sterile, neutrophil-rich material within the joints, skin, and muscle results in significant tissue damage. Attacks are not self-limiting, often requiring drainage of the infiltrate or use of intra-articular steroids. At the molecular level, a dramatic upregulation of IL-1β can be observed. The similarities between FMF and PAPA syndrome, and the fact that pyrin and PSTPIP1 bind to each other, imply that pyrin and PSTPIP1 might be linked in an as yet unknown inflammatory pathway connected to the innate immune system. When *PSTPIP1* was identified as the causative gene in PAPA syndrome, it was discovered that PAPA-causing mutations result in a nearly complete elimination of binding with PTP-PEST (Wise et al., 2002;Shoham et al., 2003). PSTPIP1 is both a binding partner and a substrate for the PEST phosphatases. Thus, reduced PEST phosphatase binding to mutant PSTPIP1 also means that the mutant PSTPIP1 is hyperphosphorylated, primarily by c-Abl. While these changes do not affect PSTPIP1's ability to bind to WASP or CD2 by co-IP, binding affinity for pyrin is markedly increased (Wise et al., 2002).(Shoham et al., 2003). Conversely, mutation of a critical tryptophan at residue 232 of PSTPIP1 dramatically reduces pyrin binding.

Experiments have suggested a normal state in which PSTPIP1 is maintained as a homotrimer that is bound to WASP, and thus is connected to the actin cytoskeleton (Wu et al., 1998; Yu et al., 2007). Phosphorylation releases PSTPIP1 from WASP, freeing it to move to other cell compartments. Phosphorylation of PSTPIP1 must be tightly controlled in a spatiotemporal manner if it is to be bound to the appropriate partners and thus linked to appropriate pathways.

Recent experiments have shown the importance of the pyrin-PSTPIP1 interaction in the regulation of IL-1β signaling through ASC. Evidence has been presented that pyrin is maintained as an autoinhibited homotrimer, in which the PyD encoded by exon 1 of pyrin is bound to its own B-box, encoded by exon 3. Such an interaction is predicted to mask the PyD and prevent its interaction with ASC (Yu et al., 2007). PSTPIP1 could terminate this autoinhibition by binding to the B-box and Coiled-coil domain of pyrin, thereby releasing the PyD and allowing pyrin's interaction with an ASC inflammasome, resulting in downstream IL-1β processing and secretion. Since mutant forms of PSTPIP1 bind with higher affinity to pyrin (Shoham et al., 2003), they may be more efficient at activating the pyrin molecule, ultimately resulting in increased IL-1β production (Yu et al., 2007). This model proposes an intriguing mechanism for PAPA syndrome and provides a potential functional relevance for the PSTPIP1-pyrin interaction. Importantly, however, it predicts that, in the absence of PSTPIP1, pyrin will be autoinhibited and will not interact with ASC, a prediction that does not seem to be

borne out by the available evidence (Cazeneuve et al., 2004; Richards et al., 2001).

Siva

Siva, named for the Hindu god of death, is the most recent pyrin binding partner to be identified. It appears that, to a large extent, the pyrin:Siva interaction is mediated by the RFP domain, encoded by exon 10 of pyrin (Balci-Peynircioglu et al., 2008). This is the region that harbors most of the known FMF-causing mutations. This interaction places pyrin in position to modulate a second cell death pathway that is independent of ASC. Siva was originally identified as a binding partner of the cytoplasmic tail of CD27, a tumor necrosis factor receptor family member that can induce apoptosis (Prasad et al., 1997). Siva was further found to bind to the structural protein VP2 of Coxsackievirus B3 (CVB3) and to contribute to the CD27-mediated apoptosis seen in CVB3-induced cardiomyopathy (Henke et al., 2000; Henke et al., 2001). The *Siva* gene contains 4 exons, and encodes a protein comprised of an N-terminal region with homology to a death domain homology region (DDHR) and a cysteine-rich carboxy terminus that contains a B-box-like ring finger followed by a second zinc finger-like domain. Two alternative splice isoforms have been identified: full-length Siva-1 and a shorter form, Siva-2 that lacks the 65 amino acids encoded by exon 2. Siva-1 is almost exclusively nuclear while Siva-2 is both cytoplasmic

and nuclear (Py et al., 2004), although both proteins can move between cellular compartments (Balci-Peynircioglu et al., 2008). The sequence missing from Siva-2 includes most of the DDHR, and all of a 13-amino acid sequence that is 100% conserved between mouse and human (residues 82-94 in murine Siva-1 (Yoon et al., 1999)). However, both isoforms retain the N-terminal region that is important for nuclear localization (Py et al., 2004) and the cysteine-rich carboxy tail required for CD27 binding. While overexpression of Siva-1 induces apoptosis as detected by DNA fragmentation, overexpression of Siva-2 does not. It has therefore been proposed that Siva-2 acts to regulate the activity of Siva-1, perhaps by preventing the association of Siva-1 with CD27, and therefore restricting CD27-mediated apoptosis (Yoon et al., 1999).

Siva's apoptotic activity may be mediated in part through its interaction with the Bcl-2 family member Bcl-X_L. Bcl-2 proteins are powerful mediators of an apoptosis pathway that acts through the mitochondria. The proapoptotic members of the Bcl-2 family include the tumor suppressor gene BAX, as well as BAK, BAD, and BID, while the antiapoptotic members include Bcl-2 itself and Bcl-X_L (Adams and Cory, 2001; Korsmeyer et al., 1999; Strasser et al., 2000). In this system, stimulation of the cell by TNFα results in the binding of procaspase-8 to the cytoplasmic tail of the type I TNFα receptor. Proximity-induced activation of Caspase-8 results, and the active enzyme cleaves and activates BID. BID then translocates to the mitochondria where it complexes with other members of the Bcl-2 family to modulate cell death through the release of cytochrome c and

SMAC/DIABLO. Siva-1, but not Siva-2, binds directly to the anti-apoptotic Bcl-X_L by immunoprecipitation and by GST pulldown (Xue et al., 2002). Cells that overexpress Bcl-X_L are highly resistant to UV-induced apoptosis (Srinivasan et al., 1998), and co-expression of Siva-1, but not Siva-2, abrogates this effect and results in cell death (Xue et al., 2002). This suggests that Siva-1 acts to suppress the protective effect of antiapoptotic Bcl-2 proteins.

In some cell lines that have been tested, Siva-1 does not induce apoptosis despite relatively high expression levels of the protein (Yoon et al., 1999). This indicates that the apoptotic action of Siva is context dependent, and emphasizes the importance of uncovering the role of pyrin in this process. It has been shown that Siva-1 is phosphorylated by ARG on Tyr^{48}. ARG is a non-receptor tyrosine kinase that is known to induce apoptosis in response to oxidative stress (Cao et al., 2001). Eliminating phosphorylation of Siva-1, either by using ARG-deficient cells or by mutating the critical amino acid residue, prevents apoptosis and suggests a mechanism by which Siva-1-induced apoptosis may be regulated (Cao et al., 2001). Additionally, this demonstrates the involvement of Siva in apoptotic pathways independent of CD27.

The Inflammasome

As discussed above, through its interaction with binding partners, pyrin

sits at the intersection of several critical signaling pathways. Given that pyrin and several of its interacting proteins are made up of a series of protein:protein interaction domains, the potential for building large multi-protein signaling complexes within the cell is clear. In fact, two such complexes that contain ASC have been identified (see Figure 1.2). One is termed the pyroptosome, and serves as a platform for Caspase-1-dependent cell death through the ASC speck (see above). The other complex is known as the inflammasome, and is a critical mediator of inflammatory signaling through inflammatory caspases, particularly Caspase-1.

In 2002, it was shown that ASC acts as a platform for the coalescence of a large (>700kD) cytoplasmic inflammatory signaling complex that involves members of the NALP and Caspase protein families (Martinon et al., 2002). The assembly of this complex results in the proximity-induced activation of Caspase-1 and Caspase-5, and the subsequent processing of the inflammatory cytokines IL-1β and IL-18. This structure was termed the "inflammasome," and has since been shown to be a central hub in inflammatory signaling. Three sub-types of inflammasome have been identified, all of which contain ASC in association with one of the NALPs (Petrilli et al., 2005). The NALPs are members of the NOD-like receptor (NLR) family of proteins, and are thought to act as cytoplasmic detectors of pathogen-associated molecular patterns (PAMPS). To date, 14 NALPs have been identified in humans.

The natural stimuli that lead to formation of the inflammasome are beginning to be identified. One subtype of inflammasome (the cryopyrin inflammasome, see below) is specifically induced by muramyl dipeptide (MDP). Since MDP is a degradation product of peptidoglycan from bacterial cell walls, it has been proposed that inflammasomes form in response to PAMPs. Additionally, recent research has uncovered the fact that IL-1β processing in macrophages occurs in response to adenoviral DNA in the cytoplasm in a process involving the inflammasome (Muruve et al., 2008). These data have led to the hypothesis that inflammasomes are an integral arm of the innate immune system, working as an intracellular pathogen detection and response system.

The NALP1 inflammasome was the first to be identified, and is comprised of a central NALP1 molecule (also known as CARD7, NAC, or DEFCAP) bound via its N-terminal PyD to the PyD of ASC, and via its C-terminal CARD to the CARD of Caspase-5 (Martinon et al., 2002). In addition, Caspase-1 is bound to the CARD of ASC, making a complex comprised of 4 types of molecules (see Figure 1.2, panel A). NALP1, in addition to its PyD and CARD domains, contains central NACHT, NBD, LRR, and FIIND domains. While the FIIND domain is of as yet unknown function, the NACHT domain is considered critical for oligomerization of the complex, and the LRR may be important for recognizing PAMPs that may trigger inflammasome formation.

The NALP2 inflammasome is similar to the NALP1 inflammasome, but

contains a NALP2 molecule (also known as PAN1, NBS1, or PYPAF2) in place of NALP1 (see Figure 1.2, panel B; Agostini et al., 2004). NALP2 is smaller than NALP1, and lacks the C-terminal FIIND and CARD domains. Instead, the NALP2 inflammasome contains an additional small adaptor molecule known as Cardinal, a known inhibitor of NFκB signaling that contains an N-terminal FIIND domain and a C-terminal CARD (Agostini et al., 2004). Together, NALP2 and Cardinal contain all of the domains found in NALP1 (PyD, NACHT, NBD, LRR, FIIND, and CARD), indicating that each of these domains may play a critical role in inflammasome function. Additionally, the NALP2 inflammasome does not include Caspase-5, but recruits two Caspase-1 molecules for every subunit of the complex (Agostini et al., 2004).

The cryopyrin (also known as PYPAF1, CIAS1, or NALP3) inflammasome is nearly identical to the NALP2 inflammasome in its make-up, but is notable in the fact that it contains a molecule called cryopyrin in place of NALP2 (see Figure 1.2, panel B). Cryopyrin is comprised of the same type of domains as NALP2, and it therefore seems likely to have similar function. Like pyrin, cryopyrin contains a PyD, but the remainder of the two molecules is very different. Importantly, however, mutations in cryopyrin are responsible for three autoinflammatory diseases (FCU, MWS, and NOMID/CINCA). It has been proposed that these diseases, known collectively as the "cryopyrinopathies" or "CAPS," are caused by an increased ability of mutant cryopyrin to self-associate, which leads to increased signaling through the cryopyrin inflammasome and an

overactivation of IL-1β (Yu et al., 2006). Overproduction of IL-1β is the primary feature in these diseases, and all are successfully treated with the IL-1 receptor antagonist anakinra. These data suggest a central role for cryopyrin in inflammatory signaling, particularly through IL-1β.

It has been previously noted that pyrin can bind the inflammasome components ASC and Caspase-1. Dowds et al. showed using in vitro systems that through these interactions, pyrin could disrupt cryopyrin-ASC binding, thus preventing the formation of the cryopyrin inflammasome (Dowds et al., 2004). However, these results have been disputed (Yu et al., 2007). Additionally, pyrin has recently been shown to bind Caspase-5, NALP1, NALP2, and cryopyrin (Papin et al., 2007). Although this interaction depends on the RFP domain of pyrin, binding affinity was not altered when FMF-causing mutations in the RFP domain were introduced. Furthermore, pyrin can also bind to the immature pro-IL-1β molecule, although not to the mature, cleaved form (Papin et al., 2007). This binding results in inhibition of Caspase-1 activity, and ultimately a decrease in IL-1β secretion. These findings implicate pyrin as a cyropyrin inflammasome inhibitor. While in this context pyrin appears to have an anti-inflammatory function, other research has shown that pyrin and ASC can form an inflammasome-like complex known as the pyroptosome that results in increased Caspase-1 activation and IL-1β processing (see Figure 1.2, panel C (Yu et al., 2006)). In this way, pyrin may be proinflammatory. This may once again

emphasize that pyrin activity is context dependent and is therefore strictly regulated, perhaps by binding partner availability and cellular activation state.

Summary

Much remains to be discovered about the pyrin molecule and its function in modulating cellular pathways such as inflammation and apoptosis. My thesis work has involved the study of three pyrin-associated proteins: ASC, PSTPIP1 and Siva. In Chapter 2, I describe the co-localization of pyrin and ASC with regions of polymerizing actin, and identify the cellular proteins responsible for this recruitment. Chapter 3 details the interaction of pyrin with Siva and documents the effect this interaction has on Siva-induced apoptosis. Chapter 4 examines the nature of the PSTPIP1:pyrin interaction and identifies the cellular elements that determine in the distinct distribution of these proteins. In Chapter 5, I explore some previously unknown aspects of the pyrin:ASC interaction, and propose a novel hypothesis regarding the mechanism by which FMF leads to renal amyloidosis. Finally, Chapter 6 presents a summation of my work, placing my findings into context with a discussion of critical topics for future study in the field of pyrin biology.

Table 1.1 List of ASC Binding Proteins

ASC-Binding Protein	Alternate Name(s)	ASC-Binding Domain	Capable of ASC Speck Co-localization?	Associated Cellular Pathways
Pyrin	Marenostrin	PyD	Yes	Inflammation, apoptosis, cytoskeletal signaling
Caspase-1	Interleukin-converting enzyme (ICE)	CARD	Yes	IL-1β, IL-18 processing (inflammasome)
Caspase-5	-	CARD	Yes	Enhances inflammation
Caspase-8	-	DED	Yes	Initiates extrinsic apoptosis cascade
Capsae-10	-	CARD	Yes	Initiates extrinsic apoptosis cascade
PYPAF1	Cryopyrin, NALP3, CIAS1	PyD	Yes	Enhances inflammation and apoptosis
PYPAF2	NALP2, PAN1, NBS1	PyD	No	Inflammation (through the inflammasome)
PYPAF5	NALP6, PAN3	PyD	Yes	Inflammation (NF-κB)
PYPAF7	NALP12, PAN6	PyD	Yes	Inflammation
CARD7	NALP1, NAC, DEFCAP	PyD	Yes	Inflammation (through the inflammasome)
CARD12	Ipaf, Clan	CARD	Yes	Enhances inflammation and apoptosis
PYNOD	-	PyD	Yes	Inhibits inflammation and apoptosis
cPOP1	ASC2	PyD	Yes	Enhances inflammation
cPOP2	-	PyD	No	Inhibits inflammation

Abbreviations: PyD: pyrin domain; CARD: caspase recruitment domain; DED: death effector domain

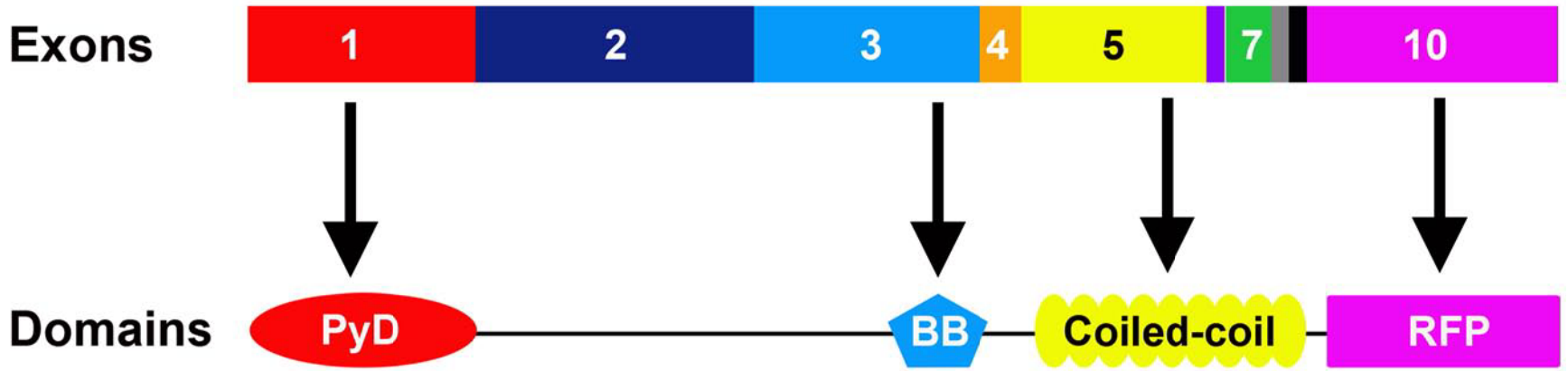

Figure 1.1: Schematic of Pyrin. The gene that encodes pyrin (*MEFV)* is comprised of 10 exons that encode several identifiable domains. Exon 1 codes for a death domain-related structure known as a pyrin domain (PyD). Exons 3-5 encode a Bbox/Coiled-coil domain, and exon 10 encodes a domain variably known as an RFP, SPRY, or B30.2 domain. Each of these domains is thought to function in protein:protein interactions.

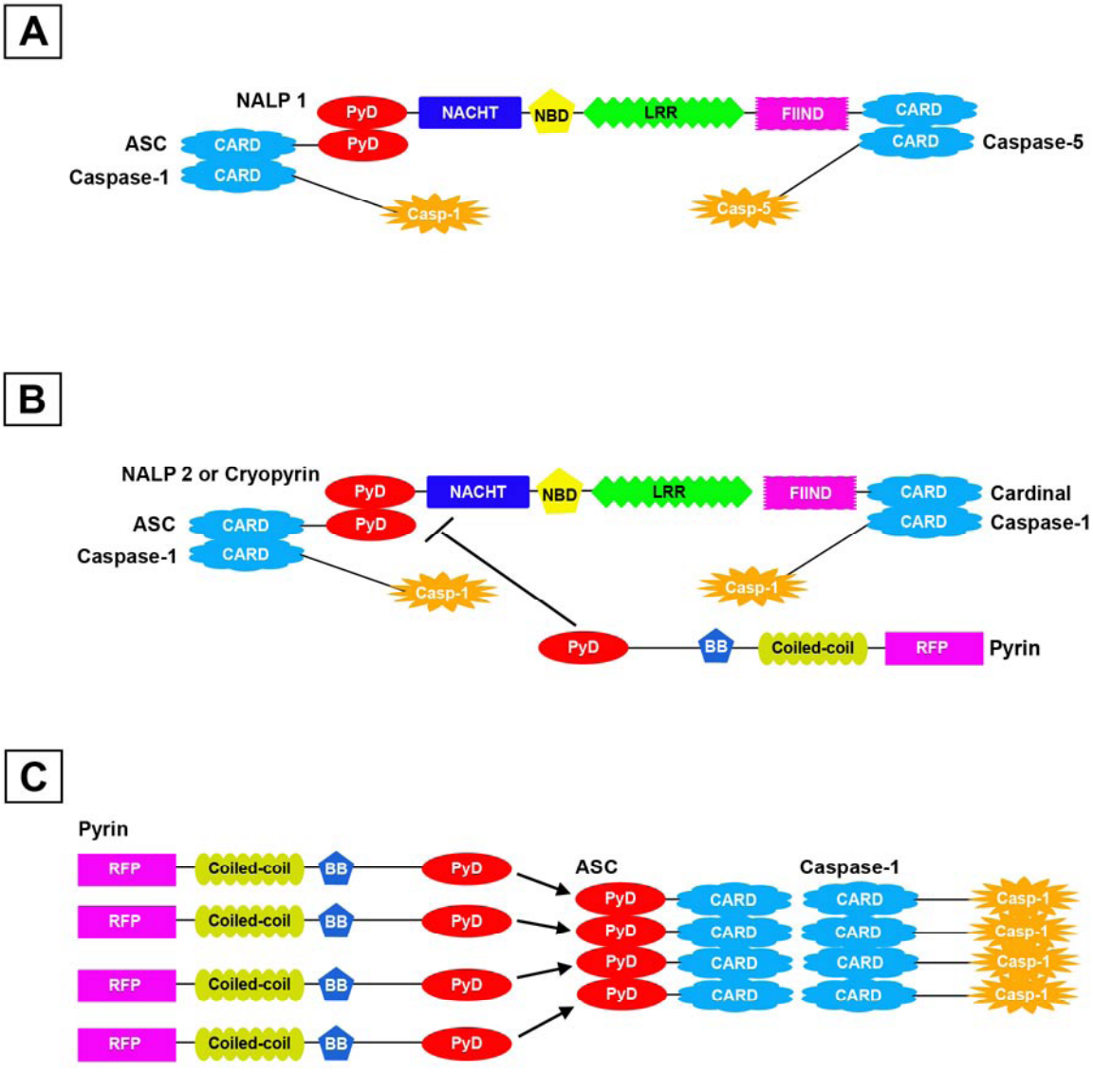

Figure 1.2: ASC Signaling Complexes. ASC serves as a scaffolding protein for several large cell signaling complexes. The NALP1 inflammasome (A) consists of ASC, NALP1, Caspase-1, and Caspase-5. Another form of inflammasome contains ASC, Cardinal, Caspase-1, and either NALP2 or cryopyrin (B). It has been shown that pyrin can interfere with the cryopyrin:ASC interaction, and prevent the formation of this inflammasome. ASC also forms a signaling complex known as the pyroptosome (C). Oligomerized ASC recruits several molecules of procaspase-1, resulting in proximity-induced activation and eventual cell death through an inflammatory process known as pyropptosis. Pyrin can bind to this complex through its PyD, although how this affects inflammatory signaling a is unclear.

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Chapter 2

Pyrin and ASC co-localize to cellular sites that are rich in polymerizing actin[1]

Abstract

Familial Mediterranean fever (FMF) is an autoinflammatory disorder characterized by sporadic attacks of fever with painful inflammation that is most often localized to synovial, pleural or peritoneal membranes. FMF is caused by mutations in the *MEFV* locus, which encodes pyrin, a protein expressed in myeloid cells and several fibroblastic cell types. The exact function of pyrin in these cells and the mechanism underlying the pathological effect of pyrin mutations have yet to be revealed. Here, we document that in migrating human monocytes, pyrin protein is dramatically polarized at the leading edge, where it co-localizes with polymerizing actin. ASC (Apoptosis-associated Speck protein with CARD domain), a known pyrin-interacting protein and a critical component of the inflammasome, is also located at the leading edge in migrating monocytes. Similarly, both pyrin and ASC concentrate in dynamically polymerizing actin-rich tails generated by *Listeria monocytogenes*. Pyrin's B-box and coiled-coil region is required for its association with *Listeria* tails. Pyrin also binds, with low affinity and via the same domains, to actin, VASP, and Arp3. Though disease-causing mutations in pyrin do not appear to alter its localization to the leading edge or to

Listeria rocket tails, they could potentially have important functional consequences in the context of processes such as cell migration and synapse formation. The co-localization of pyrin and ASC together at such sites may provide an important link between cytoskeletal signaling and inflammasome function.

Introduction

The term “autoinflammatory” is used to describe a set of heritable human diseases characterized by fever and inflammation without apparent T- or B-cell involvement (Galon et al., 2000). These diseases are believed to represent disorders of the innate immune system. Familial Mediterranean fever (FMF) is the most common of these autoinflammatory syndromes, and is particularly prevalent in the Mediterranean region. In fact, the frequency of several of the known *MEFV* mutant alleles is so remarkably high in regions of Armenia, Turkey and Morrocco that a possible heteozygote advantage has been postulated (Aksentijevich et al., 2001; Tunca et al., 2002). Indeed, positive selection appears to have acted on the pyrin protein during primate evolution (Schaner et al., 2001). Interestingly, the known human mutations in the pyrin protein are predicted to be wild type sequences in primate ancestors. These amino acid sites must therefore be sites that can change the functional character of the pyrin protein in response to some unknown environmental pressure. However, the nature of the functional changes caused by the mutations is not yet clear.

FMF attacks are characterized by a massive influx of neutrophils into the inflamed site. Pyrin, the protein product of the *MEFV* gene, is expressed in neutrophils as well as in monocytes/macrophages and dendritic cells (Centola et al., 2000; Diaz et al., 2004). Pyrin is also expressed, although at lower levels, in tissue fibroblasts found at the sites of inflammatory attacks, including skin, synovial and peritoneal fibroblasts (Centola et al., 2000; Diaz et al., 2004; Matzner et al., 2000). These localizations, combined with the evolutionary data indicating that pyrin is functionally evolving in response to some environmental pressure, suggest that pyrin may operate on the front line of the inflammatory response, either sensing or coordinating responses to pathogenic insults.

Recent work in several laboratories has documented the interaction of pyrin with five cellular proteins: PSTPIP1 (Shoham et al., 2003), 14.3.3 (Jeru et al., 2005), Caspase-1 (Chae et al., 2006), ASC (Apoptosis-associated Speck-like protein with CARD domain; Richards et al., 2001), and Siva (Balci-Peynircioglu et al., 2008). The interaction with ASC, is particularly interesting since multimers of ASC provide a central platform for the formation of several types of inflammasome, a multiprotein complex that efficiently activates Caspase-1, promoting the activation and release of Il-1β (Martinon et al., 2002). ASC also has a tendency to form large peri-nuclear aggregates called “specks,” structures that appear to presage the death of the cell (Masumoto et al., 1999; Richards et al., 2001). Pyrin binds to ASC via N-terminal pyrin domains (PyD), encoded by

exon 1 of both proteins. The interaction of pyrin with ASC modulates both the formation of ASC specks and the rate of apoptotic death following speck formation (Richards et al., 2001). Additional data suggest that pyrin also modulates ASC's inflammasome function (Dowds et al., 2003; Yu et al., 2007). Indeed, interactions between ASC and Caspase-1 (Srinivasula et al., 2002), between pyrin and Caspase-1 (Chae et al., 2006), and between ASC and pyrin (Richards et al., 2001) have all been documented, and analysis of the binding domains responsible for these interactions indicate that tri-molecular interactions are possible. That is, Caspase-1 binds via its N-terminal CARD domain to the C-terminal CARD domain of ASC; pyrin binds to the N-terminal PyD of ASC; and the C-terminal rfp/B30.2/SPRY domain of pyrin interacts with Caspase-1. Thus, this complex of proteins could play a regulatory role in inflammation and perhaps apoptosis.

Pyrin also interacts with PSTPIP1, the protein that is mutated in PAPA syndrome (Pyogenic Arthritis, Pyoderma gangrenosum, and Acne), another debilitating autoinflammatory disease. The direct link between pyrin and PSTPIP1 involves pyrin's B-box and Coiled-coil domains; the B-box alone is necessary, but not sufficient for this interaction (Shoham et al., 2003). PSTPIP1 is homologous to Cdc15, an actin-associated protein important for cytokinesis in yeast. Overexpression of PSTPIP1 results in the generation of filopodia by 3T3 cells (Spencer et al., 1997), suggesting that mammalian PSTPIP1 also affects actin organization.

Earlier studies of pyrin in transfected cells documented direct interactions between pyrin and elements of the cellular cytoskeleton (Mansfield et al., 2001). An interaction with tubulin was mapped to the N-terminal PyD of pyrin. A potential interaction with actin was also documented in that study, but not well characterized; in transfected cells, pyrin was observed to co-localize with actin in lamellar structures and in supranuclear ruffles, but not with stress fibers. The domain of pyrin responsible for this co-localization could not be determined (Mansfield et al., 2001). More recent work indicates that, through its interaction with Siva, a protein that functions in the apoptotic response to oxidative stress, pyrin is occasionally localized in actin stress fibers (Balci-Peynircioglu et al., 2008). These studies, and the interaction of pyrin with PSTPIP1, predict that pyrin plays a role in cytoskeletal signaling pathways. This is important because such pathways are regulators of cell migration as well as cell-cell signaling, critical processes that control the inflammatory response.

Here, we examine more closely the distribution of pyrin in native human monocytes. We document the co-localization of pyrin and ASC in membrane ruffles and show that, in migrating human monocytes, both ASC and pyrin are dramatically polarized to the leading edge, a site of active actin polymerization. We confirmed the association of both pyrin and ASC with polymerizing actin and further mapped the pyrin domains involved using the *Listeria monocytogenes* rocket tail assay (Welch et al., 1997). In addition, we identified low affinity

interactions between pyrin and three cytoskeletal proteins (Arp3, VASP, and actin) that may be involved in the recruitment pyrin to sites of active actin polymerization in the cell. The additional co-localization of ASC to the same dynamic actin-rich regions could provide a previously unrecognized means to connect cytoskeletal function to inflammatory signaling.

Materials and Methods

Cell Culture and Transfection. COS-7, HeLa, and 293T cells were cultured in Dulbecco's Modified Eagle Medium (Gibco by Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum (Gibco by Invitrogen, Carlsbad, CA). For experiments, cells were plated on sterile glass coverslips in 6-well culture plates. Cells were transfected as indicated in the text using FUGENE-6 (Roche Applied Science, Indianapolis, IN) and incubated for 24-48 hours before analysis. Transfection rates of 60-70% were observed.

Monocyte Isolation. Human blood was collected from healthy volunteers (IRB# 1992-0480) and mixed with an anti-coagulation solution containing 0.14M anhydrous citric acid, 0.20M citric acid trisodium salt, and 0.22M dextrose. Blood was then incubated at room temperature for 30 – 45 minutes after the addition of a solution containing 6% dextran to facilitate sedimentation of red blood cells. The upper, leukocyte-rich layer was then removed and any remaining red blood cells were destroyed by hypotonic lysis in distilled water. Leukocytes were

concentrated by centrifugation, then layered onto Ficoll/Paque (Pharmacia, Uppsala, Sweden) density gradient and centrifuged again. The monocyte/lymphocyte layer was removed, and monocytes were enriched by cell adhesion. Non-adherent lymphocytes were washed off 24 hours later, and the adherent monocytes were fixed and stained.

Plasmids and Antibodies. All myc- and FLAG-tagged constructs were cloned into pCMVTag3a and pCMVTag2b (Stratagene, La Jolla, CA) respectively. GFP tagged gelsolin, capping protein, Arp3, VASP, and α-actinin as well as YFP tagged actin constructs were obtained from Maria Diakonova, Ph.D. (University of Michigan, Ann Arbor, MI). HRP-conjugated α-myc primary antibody and goat α-mouse IgG secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse α-FLAG (M2 monoclonal), rabbit α-myc (polyclonal), and mouse α-tubulin (monoclonal) were obtained from Sigma (St. Louis, MO). Mouse α-GFP (monoclonal) was acquired from Clontech (Mountain View, CA). Goat α-mouse AF488, goat α-mouse AF568, goat α-rabbit AF488, and goat α-rabbit AF568 secondary antibodies as well as Phalloidin AF488 were purchased from Molecular Probes (by Invitrogen, Eugene, OR). Rabbit α-human pyrin polyclonal antibody was generously provided by Dr. Daniel Kastner (National Institutes of Health, Bethesda, MD). Mouse α-ASC monoclonal antibody was a kind gift of Dr. Junya Masumoto (Shinshu University School of Medicine, Nagano, Japan).

Listeria Rocket Assay. Single colonies of wild-type *Listeria monocytogenes* were picked from BHI agar plates and grown in BHI media at 25ºC overnight. The bacteria were then sub-cultured in fresh BHI media at 37ºC for 90 minutes. Bacterial cells were pelleted by centrifugation, washed in PBS three times, and then diluted to an OD of 0.05 in PBS. One mL of this bacterial solution was then added to each well containing COS-7 cells. The bacteria were allowed to infect cells for 2 hours, then the media was replaced with DMEM + 10% FBS containing gentamicin at 50μg/mL. Cells were incubated for an additional 5 hours, and then fixed with 4% parafomaldehyde in PBS and stained.

Cytochalasin D Treatment. A 2mM stock of cytochalasin D prepared in chloroform was diluted in cell culture medium to a working concentration of 10μM. Cells were treated for 30 minutes before fixation with 4% paraformaldehyde in PBS.

Immunofluorescence. Cells were permeablized using 0.2% Triton X-100 in PBS, and blocked with a solution containing 10% goat serum, 1% bovine serum albumin, and 0.1% Tween20 in PBS. Primary and secondary antibody application was carried out for 1 hour and for 20 minutes, respectively, and nuclei were counterstained with DAPI. Coverslips were mounted on slides using a ProLong Antifade Kit (Molecular Probes by Invitrogen, Eugene, OR) and allowed to dry in the dark for several hours. Slides were visualized using a Nikon E800 microscope or an Olympus FV-500 confocal microscope.

Immunoprecipitation and Western Blotting. 293T cells were transfected as described, and were lysed 48 hours later using a buffer containing 0.5% Triton-X 100. Protein lysates were tested for expression and proteins were precipitated using α-myc antibody and a mixture of protein A and protein G-coated sepharose beads (Invitrogen, Carlsbad, CA). The immunoprecipitation reactions were run out on gradient polyacrylamide gels (4-20%; BioRad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. Membranes were blocked in a buffered solution of nonfat milk and bovine serum albumin, and then treated with antibodies diluted in blocking solution. Membranes were washed extensively and treated with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL), then exposed to X-ray film.

Results

Pyrin and ASC colocalize with actin in membrane ruffles in human monocytes and in transfected cells. Freshly isolated human monocytes were examined after immunostaining with a polyclonal antibody previously documented to detect human pyrin (Diaz et al., 2004). As shown in Figure 2.1A-C, pyrin was found in filamentous structures at the top of the cell, above the nucleus. The same cells were also stained with a monoclonal antibody that recognizes ASC and a similar pattern was observed (Figure 2.1D-F). In cells co-stained for pyrin and filamentous actin (using phalloidin), the filamentous structures were revealed to be actin-rich membrane ruffles (Figure 2.1G-I).

Transfection of HeLa cells, which do not express pyrin, with myc-tagged pyrin confirmed the localization of pyrin to supranuclear ruffles (Figure 2.1J, arrow); pyrin was also observed in lamellapodial structures (Figure 2.1K, arrow). Co-staining with phalloidin confirmed the presence of actin in both ruffles and lamellapodia (Figure 2.1L). At higher magnification (Figure 2.1M-O), pyrin could be found in lamellapodia (arrows), but not in actin stress fibers (arrowheads).

The pattern of pyrin staining in human monocytes requires intact actin microfilaments, but does not coincide with the microtubular network. To test whether the pattern of pyrin localization in human monocytes depends on an intact actin network, we treated monocytes with cytochalasin D, which disrupts actin filaments. The reticular pattern of both pyrin and phalloidin staining was abolished in cytochalasin D-treated cells (Figure 2.2A-D).

Pyrin was previously shown to co-localize with microtubules, but those findings were based only on the examination of pyrin in transfected cells (Mansfield et al., 2001). We examined the relationship between pyrin and the microtubule network in human monocytes using confocal microscopy. Figure 2.2E and 2.2F show two different Z-planes of a field of human monocytes. In Figure 2.2E, a section near the base of the cells reveals the microtubule organizing network (green) in several cells. A section close to the top of the cells (Figure 2.2F) shows the supranuclear distribution of pyrin. In no case did we find

evidence of co-localization between pyrin and the microtubular network in human monocytes.

Pyrin and ASC concentrate in areas of actively polymerizing actin. In some monocytes stained with both phalloidin and anti-pyrin, we noted a clear-cut polarization of actin staining (Figure 2.3A). These cells exhibited the typical morphology of migrating monocytes (Fais and Malorni, 2003): the leading edge was actin-rich, the nucleus was pulled towards the leading edge and the trailing edge contained numerous spiky cell projections (the uropod). Strikingly, in these migrating cells, pyrin staining was polarized to the actin-rich leading edge (Figure 2.3B-C). In cells stained for actin and ASC, a similar polarized pattern of staining was observed (Figure 2.3D-F).

The leading edge of migrating cells is a region that is rich in polymerizing actin. A convenient assay to detect and further dissect the association of proteins with the actin polymerizing machinery is the *Listeria monocytogenes* actin rocket tail assay (Dramsi and Cossart, 1998; Welch et al., 1997). *Listeria monocytogenes* is an infectious bacterium that uses the actin polymerizing machinery of the host cell to build a comet-shaped actin-based tail (a “rocket”) that propels the bacterium throughout the cytoplasm and into neighboring cells. These tails are distinct structures, easily identified by phalloidin staining (Figure 2.3G).

We transfected myc-tagged pyrin into COS-7 cells and infected these cells with *Listeria*. Rocket tails were allowed to develop, and then were visualized by staining with phalloidin. The negative control protein lacZ was not recruited to *Listeria* rocket tails (Figure 2.3G-I). However, pyrin was consistently found in rocket tails (Figure 2.3J-L); the pyrin staining intensity inside the actin tails exceeded the staining intensity elsewhere in the cell, indicating that pyrin was being concentrated within the tail, in close association with the polymerizing actin. Likewise, ASC was drawn to Listeria actin tails (Figure 2.3M-O).

The association of pyrin with sites of active actin polymerization requires the B-box and coiled coil domains. To map the regions of pyrin that mediate its recruitment to *Listeria* tails, we used a series of deletion mutations, summarized in Figure 2.4. Exon 1, the region encoding the PyD of pyrin that is known to interact with ASC, was dispensable for pyrin localization to *Listeria* tails (Figure 2.5A-C), as was exon 2 (Figure 2.5D-F). However, exon 3 (Figure 2.5G-I) and exons 3-4 (data not shown) were weakly recruited. Importantly, the C-terminal rRp/B30.2/SPRY domain encoded by exon 10, where the majority of pyrin mutations are found, was not required for recruitment of pyrin to *Listeria* rocket tails (Figure 2.5J-L). Two minimal regions of pyrin were found to strongly and consistently localize to *Listeria* tails: exons 3-5, encoding the B-box and part of the coiled-coil region of pyrin (Figure 2.5M-O), and the exon 6-10 region, which includes additional coiled coil sequences as well as the Rfp domain (Figure 2.5P-R). Testing the exon 6-9 region alone was not feasible due to the

apparent instability of this protein fragment. In addition, testing the PyD and CARD domains of ASC was impossible due to the fact that these domains, when expressed alone, form filaments in cells (Moriya et al., 2005; Richards et al., 2001).

Exclusion of the known pyrin-interacting protein PSTPIP1 as the mediator of pyrin's recruitment to Listeria rocket tails. PSTPIP1 was previously isolated from a monocyte cDNA library using a yeast two hybrid screen with pyrin as the bait (Shoham et al., 2003). PSTPIP1 is the homolog of yeast cdc15, a yeast actin-binding protein (Fankhauser et al., 1995); overexpression of murine PSTPIP1 in 3T3 fibroblasts causes actin reorganization and extension of filopodia (Spencer et al., 1997). More recent work has shown that PSTPIP1 is recruited to the immunological synapse by WASP, a protein that is important for the initiation of actin polymerization (Badour et al., 2003). Thus, PSTPIP1 is an actin-associated protein that has the potential to modulate PEST phosphatase activity in actin-based cell functions. We hypothesized that since pyrin is known to interact with PSTPIP1, and since PSTPIP1 and pyrin are both found in monocytes, PSTPIP1 may recruit pyrin to the leading edge. We therefore tested this possibility in the *Listeria* rocket assay. We found that when PSTPIP1 is transfected into COS-7 cells, PSTPIP1 forms delicate filaments throughout the cytoplasm (data not shown). When these cells are further infected with *Listeria*, the filaments are often disrupted, but PSTPIP1 does not localize to *Listeria* actin

tails (Figure 2.6). Thus pyrin's recruitment to sites of active actin polymerization likely does not involve PSTPIP1.

Identification of cellular proteins that could recruit pyrin to Listeria rocket tails. Seven cellular proteins are minimally required for the formation of Listeria rocket tails in vitro: VASP, gelsolin, CapZ, α-actinin, Arp3, profilin, and actin (reviewed in Pollard et al., 2000). We tested, using immunoprecipitation assays, whether GFP-labeled forms of each of these proteins could bind to exons 3-5 or exons 6-10 of pyrin (regions sufficient for recruitment to rocket tails). Our initial experiments showed a co-precipitation of pyrin exons 3-5 with actin, VASP, and Arp3, while only actin and VASP were precipitated with exons 6-10 (data not shown). These interactions were rather weak, as immunoprecipitation was inefficient, but could be confirmed using full-length pyrin (Figure 2.7). These weak interactions were therefore subsequently confirmed using other strategies (see ASC speck binding assay, below).

Since ASC is also drawn to *Listeria* actin tails and to leading edge actin, we tested whether any of the seven GFP-tagged Listeria tail proteins described above interact directly with ASC. Previous studies had indicated that immunoprecipitations using ASC can be problematic due to its extreme insolubility (Richards et al., 2001). We therefore examined whether any of the seven proteins are recruited to ASC specks in transfected COS-7 cells. Cells co-transfected with ASC and the individual GFP-tagged proteins were examined 24

hours later, at a time when specks are clearly visible in transfected cells. We were not able to detect the binding of any of these seven proteins to ASC specks (data not shown). Thus, the nature of the cellular protein that is responsible for the association between ASC and *Listeria* rocket tails is unclear.

Confirmation of pyrin binding studies using an ASC-speck binding assay. Because the pyrin immunoprecipitation results suggested rather weak interactions, we wished to confirm the binding using a different approach. Thus, we took advantage of a visual protein:protein interaction assay that we previously devised to examine pyrin-interacting proteins (Balci-Peynircioglu et al., 2008). Pyrin is known to co-localize with ASC specks, and the binding of pyrin to ASC requires the PyD of both proteins (the PyD of pyrin is encoded by exon 1, a region that is dispensable for pyrin's recruitment to *Listeria* rocket tails, see Figure 2.4 and Figure 2.5). Since the studies above indicate that ASC alone does not recruit the seven *Listeria* actin tail proteins to ASC specks, we used cells transfected with three proteins (ASC, pyrin, GFP-tagged test protein) to examine whether pyrin can recruit the other seven candidate binding proteins to ASC specks. Figure 2.8A-F validates the assay, showing that pyrin can recruit wildtype PSTPIP1 to the ASC speck (Figure 2.8A-C), but is unable to recruit the W232A mutant form of PSTPIP1 that does not bind to pyrin (Figure 2.8D-F). As shown in Figure 2.8, both Arp3 (panels G-I) and VASP (panels J-L) were carried to ASC specks by pyrin, while all other proteins tested were not, further validating the immunoprecipitation results.

Mutations in pyrin do not appear to alter its association with polymerizing actin in human monocytes or in the Listeria rocket assay. In monocytes from patients with FMF, pyrin and ASC were seen in membrane ruffles in a pattern indistinguishable from that seen in monocytes of unaffected individuals (Figure 2.9A-C). Co-staining with phalloidin to identify polymerized actin and for pyrin revealed that pyrin co-localized with actin in membrane ruffles in these monocytes as well (Figure 2.9D-F). In addition, migrating monocytes in which a leading edge could be identified also displayed a polarized distribution in which mutant pyrin was dramatically localized to the leading edge (Figure 2.9G-I).

To further test the effect of FMF-associated pyrin mutations on the ability of pyrin to interact with polymerizing actin, we tested whether these mutations altered the ability of pyrin to concentrate in *Listeria* rocket tails. Several disease causing mutations, including M694V (Figure 2.10 A-C), M680I (Figure 2.10 D-F), R761H (Figure9 G-I), V726A (Figure 2.10 J-L), E148Q (Figure 2.10M-O), and P369S (data not shown) were tested. All mutations retained the ability to localize to Listeria tails. Next, we tested whether the M694V mutant form of pyrin was also able to bind to actin-associated proteins. We found that all forms of mutant pyrin showed binding to Arp3, VASP, and actin at levels comparable to wild-type pyrin (Figure 2.10P).

Discussion

In this report, we demonstrate a clear link between pyrin, its interacting partner ASC, and a specialized portion of the cellular actin cytoskeleton. While in freshly isolated monocytes, pyrin and ASC are both associated with membrane ruffles throughout the cell, they undergo a dramatic polarization to the leading edge of migrating cells, where they co-localize with the actively polymerizing actin network. The re-distribution appears nearly complete; little staining for either protein is detectable in other regions of the cell. This polarization suggests that both pyrin and ASC may play important roles in cytoskeletal signaling at the cell's leading edge.

Our data indicate that pyrin's affinity for the leading edge may be at least in part explained by the fact that it weakly interacts with actin, as well as with two proteins that play important roles in actin polymerization at the leading edge, VASP and Arp3. It is not possible to conclude at this time whether these interactions are co-dependent. That is, the association of pyrin with cellular actin could potentially depend partially on stabilization provided by its weak interactions with VASP and/or Arp3, and *vice versa*.

In human monocytes, the co-localization of pyrin and ASC at these specialized sites may be further reinforced by the previously recognized interaction between these two proteins. In contrast, in the *Listeria* assay in COS-

7 cells, which do not express either ASC or pyrin, it can be concluded that the association of ASC with polymerizing actin does not depend on its association with pyrin, and likewise, the association of pyrin with these regions does not depend on ASC. Since ASC (at least in the conformation that it assumes in specks) does not appear to interact with actin, VASP and Arp3, independent mechanisms must stabilize the two proteins in the actin compartment of *Listeria* tails.

One of the domains of pyrin important for its localization to sites of active actin polymerization (the B-box and coiled coil) is identical to the domain required for pyrin's interaction with PSTPIP1 (Shoham et al., 2003). Since PSTPIP1 is homologous to cdc15, an actin-associated protein in yeast (Li et al., 1998), we initially speculated that PSTPIP1 might be responsible for the recruitment of pyrin to the leading edge of migrating cells. However, infection of cells with *Listeria* does not result in the recruitment of PSTPIP1 to *Listeria* tails. When pyrin and PSTPIP1 are co-transfected into cells, pyrin co-localizes with PSTPIP1 filaments (data not shown). Yet, when these co-transfected cells are infected with *Listeria*, PSTPIP1 filaments are disrupted, but only pyrin is localized to tails. Thus, under these conditions, pyrin appears to prefer to associate with polymerizing actin rather than with PSTPIP1. It is possible that *Listeria* infection affects the phosphorylation status of PSTPIP1, as its binding to pyrin is highly dependent upon the level of PSTPIP1 phosphorlylation (Shoham et al., 2003).

Together, these data suggest that the association of pyrin with actively polymerizing actin is independent of pyrin's association with ASC and PSTPIP1 and that PSTPIP1 does not spontaneously associate with these actin structures. However, PSTPIP1 can be recruited to the immunological synapse in T-cells, another site of active actin polymerization; this recruitment depends on WASP, a known substrate of PEST phosphatases, and a binding partner of PSTPIP1. Thus, under some regulated conditions, wild type PSTPIP1 (independently of pyrin) is localized to sites of polymerizing actin.

Mutations in PSTPIP1 cause PAPA syndrome, a debilitating inflammatory disease that begins in childhood with a sterile arthritis and progresses after puberty to massive granulomas and severe cystic acne (Wise et al., 2002). Previous work from the Kastner laboratory revealed that mutations in PSTPIP1 increase its affinity for pyrin, suggesting a molecular link in the disease etiology of FMF and PAPA syndrome (Shoham et al., 2003). In accord with these data, we have seen that mutant forms of PSTPIP1 are drawn to actin tails by wild type pyrin (data not shown); this recruitment depends on pyrin, as mutant PSTPIP1 by itself is not drawn to *Listeria* tails. Since PSTPIP1 functions as an adaptor that links PEST phosphatases to their substrates, alterations in PSTPIP1 compartmentalization mediated by its increased binding to pyrin could potentially have important consequences for the phosphorylation status of important regulatory molecules that are known to bind to PSTPIP1 (e.g., WASP, c-Abl).

It is striking that of the more than 40 mutations that have now been identified in *MEFV*, over 60% are found in the most C-terminal domain encoded by exon 10, a domain variously known as the RFP or SPRY or B30.2 domain (Mansfield et al., 2001; Rhodes et al., 2005). Though one of the regions of pyrin that is sufficient for its association with polymerizing actin is encoded by exons 6-10, our binding studies indicate that exon 10 is not necessary for this interaction. Indeed, mutations in exon 10 do not prevent recruitment of pyrin to *Listeria* rocket tails, nor do they cause a detectible reduction in the ability of mutant pyrin to bind both Arp3 and VASP. However, since binding to both Arp3 and VASP is quite weak, and may even be co-dependent, it is possible that there is an alteration in binding affinity that is simply too slight to be detected by immunoprecipitation. But, precisely because of this low affinity, minimal alterations have the potential to have major consequences for function.

The binding of pyrin to VASP and Arp3 explains the localization of pyrin to regions of polymerizing actin, and suggests a role in actin polymerization. Both Arp3 and VASP are components of the leading edge and *Listeria* rocket tails. Arp3 works in conjunction with Arp2 to nucleate actin filaments at the minus end, allowing rapid growth at the plus end. Additionally, the Arp2/3 complex can nucleate a branch from an existing actin filament, allowing the formation of a web of actin filaments. The Arp2/3 complex is consistently overexpressed in invasive colorectal cancers and contributes greatly to the cell's increased motility and thus its ability to metastasize (Otsubo et al., 2004). VASP protects the growing ends

of actin filaments from capping protein, which would stop their growth. VASP is associated with focal adhesions and is thought to be critical for cell motility. It has recently been proposed that an increase in the expression of VASP is involved in the progression and invasion of lung adenocarcinomas, and that this is likely due to an increase in cell motility (Dertsiz et al., 2005). Additionally, cardiac fibroblasts from VASP -/- mice display increased cell spreading, but impaired migration and reorientation ability in a wounding assay (Garcia Arguinzonis et al., 2002). Thus, an attractive hypothesis, currently being tested, is that pyrin might modulate cell migration through its interaction with these proteins. In any case, the studies described here provide evidence that the connection between pyrin, ASC and the active actin cytoskeleton is more extensive than previously appreciated.

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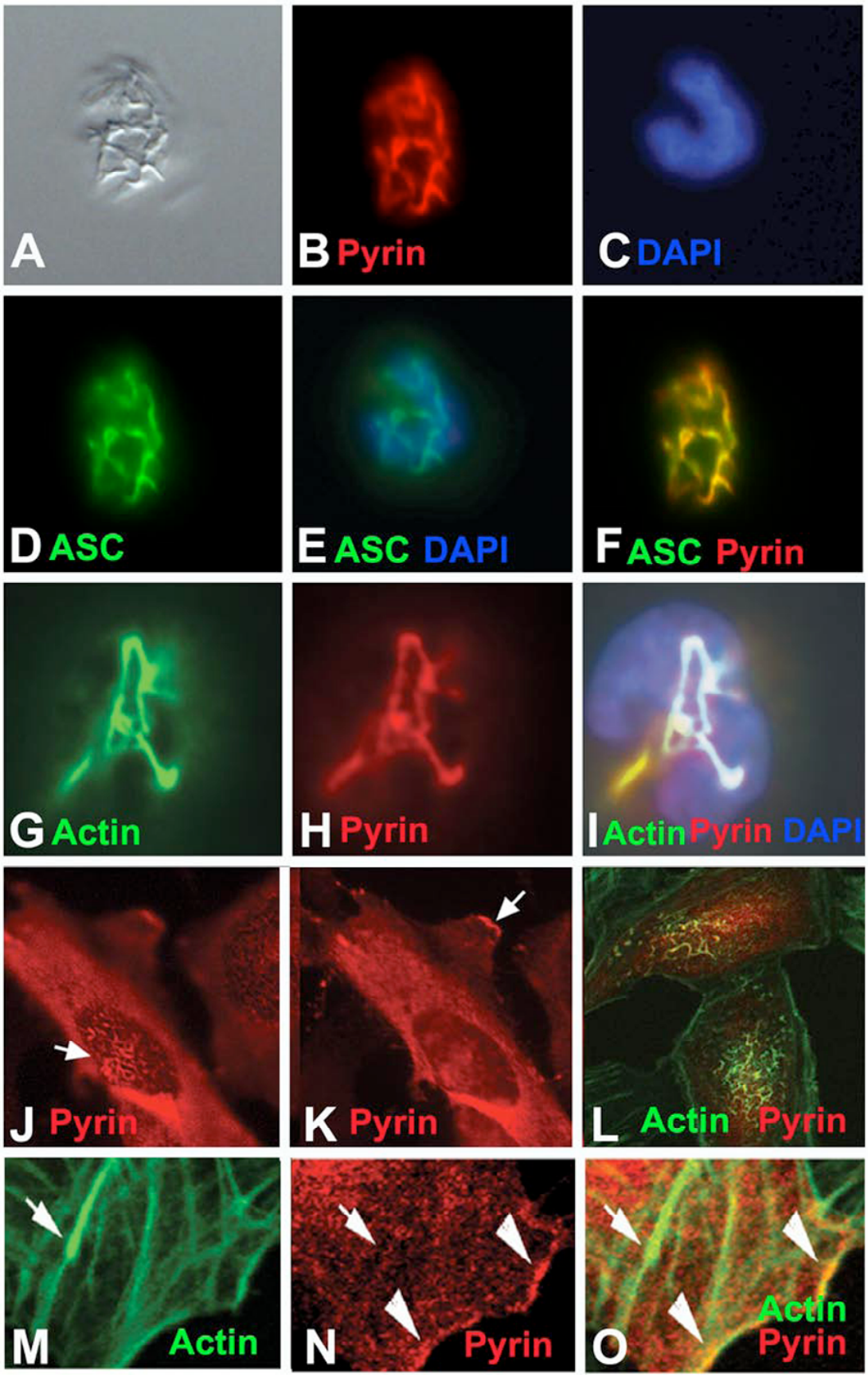
Figure 2.1: Pyrin, ASC, and actin expression in native and transfected cells. In monocytes, pyrin and ASC localized to supranuclear ruffles (A-F). Staining with phalloidin revealed that these ruffles were actin-based (G-I). In transfected HeLa cells, myc-tagged pyrin can be seen concentrated in membrane ruffles (J, L) and at the edge of cells (K). At increased magnification, it is possible to see that while pyrin co-localizes with actin at the edge of cells, it does not co-localize with actin stress fibers (M-O). Photographs taken at 600X by Philip Schaner.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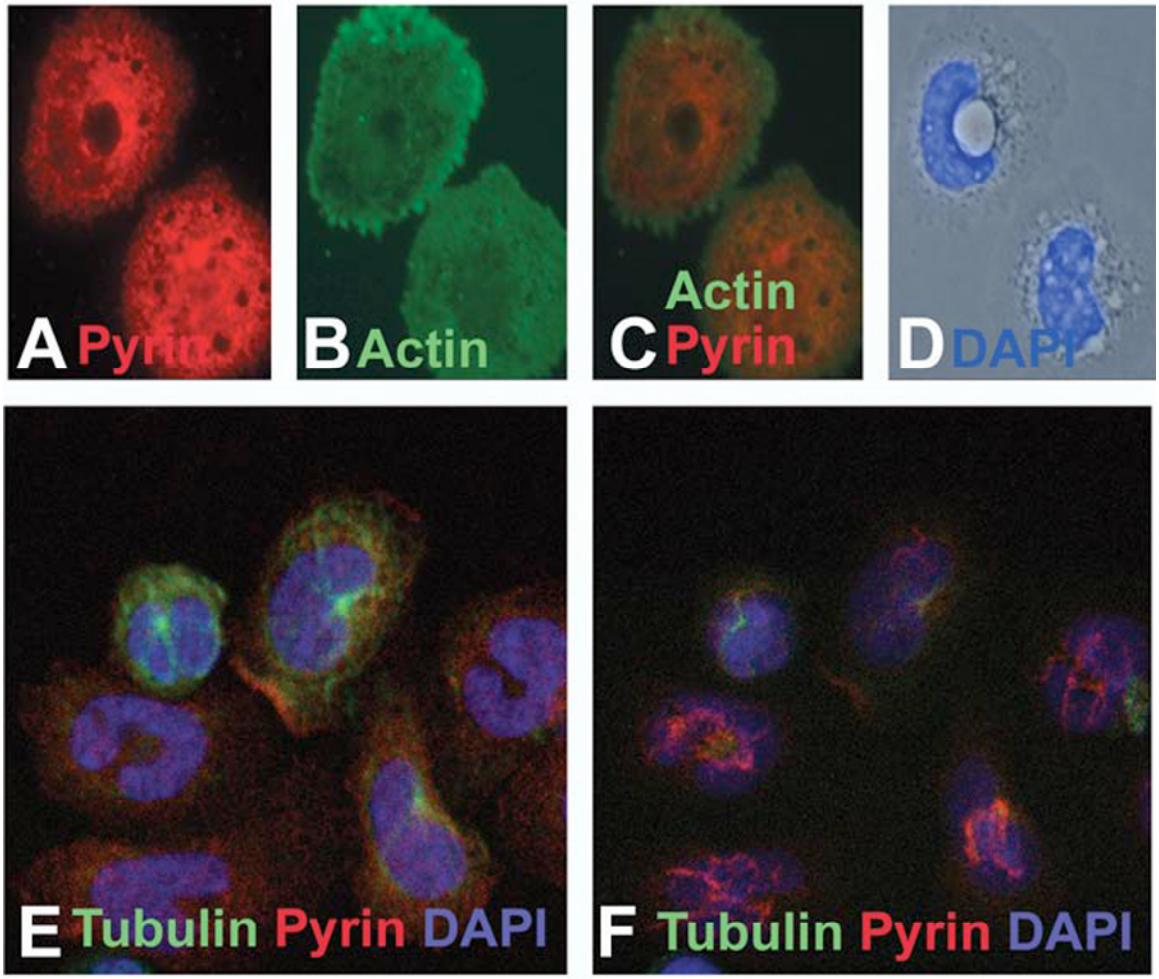
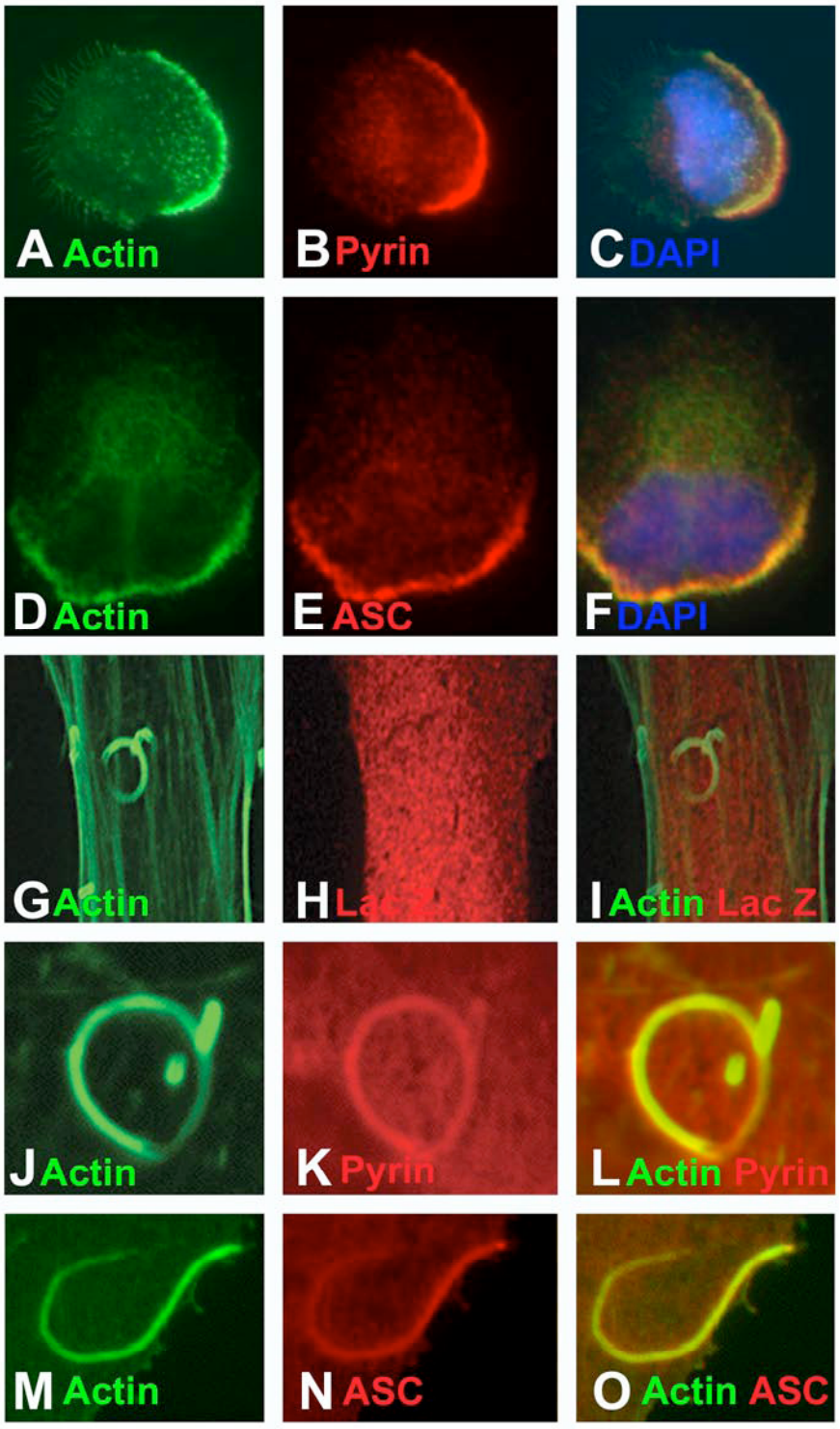


Figure 2.2: Pyrin distribution in monocytes. Treating cells with cytochalasin D to disrupt actin distribution also resulted in alteration of pyrin distribution (A-D). Confocal images of monocytes co-stained for pyrin and tubulin reveal that pyrin is located in supranuclear ruffles and does not co-localize with microtubules (E-F). Photogrraphs taken at 600X by Philip Schaner.

Figure 2.3: Pyrin co-localizes with regions of polymerizing actin. In migrating monocytes, distribution of pyrin (A-C) and ASC (D-F) is polarized. Both proteins are concentrated in the actin-rich leading edge of migrating cells. In the *Listeria* rocket assay, cells were infected with *Listeria monocytogenes* and their actin-based tails were used to recruit actin polymerizing machinery. *Listeria* tails do not recruit the negative control protein myc-LacZ (G-I), but does recruit both myc-tagged pyrin (J-L) and myc-tagged ASC (M-O). Panels A-I photographed at 600X by Philip Schaner. Panels J-O photographed at 600X by Andrea Waite.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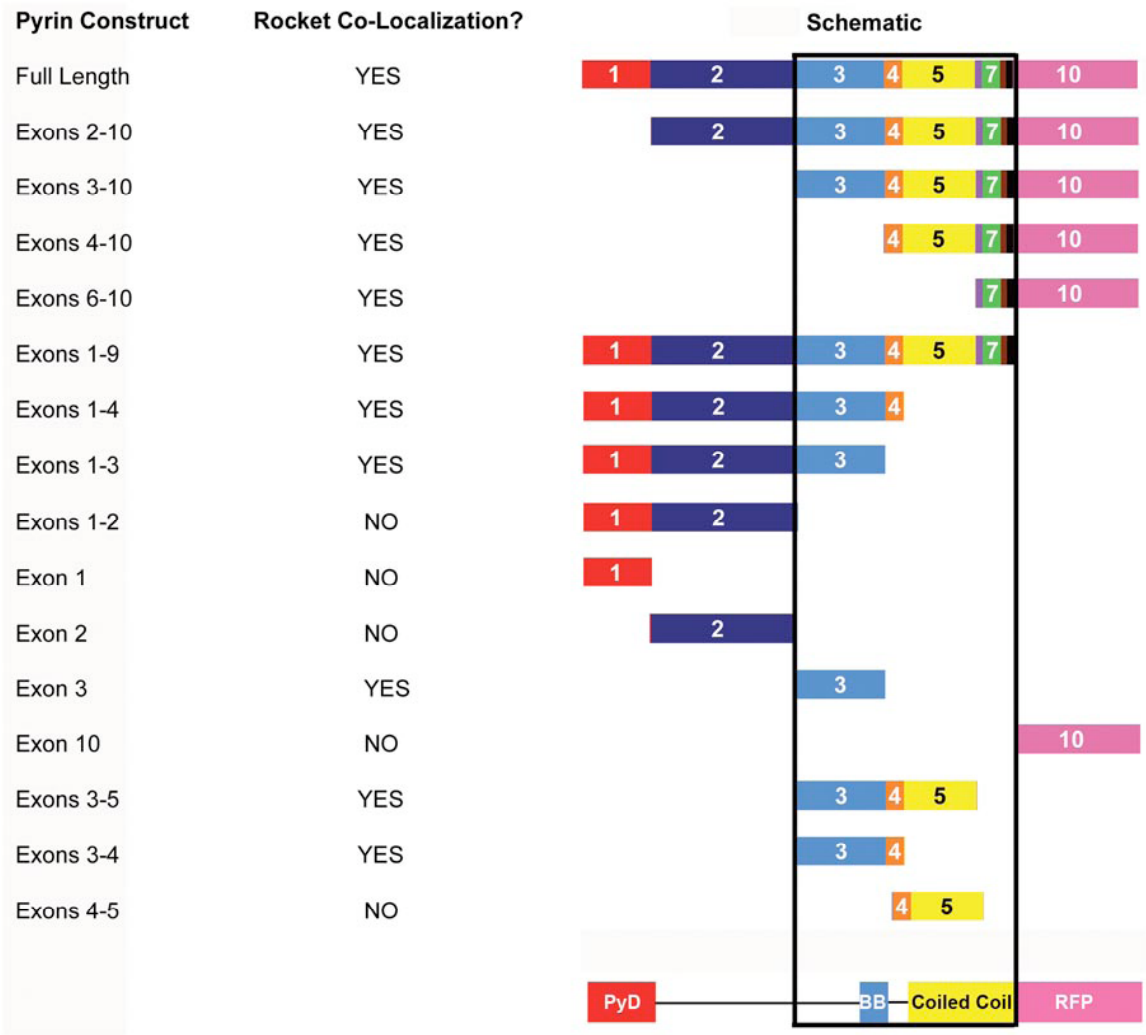
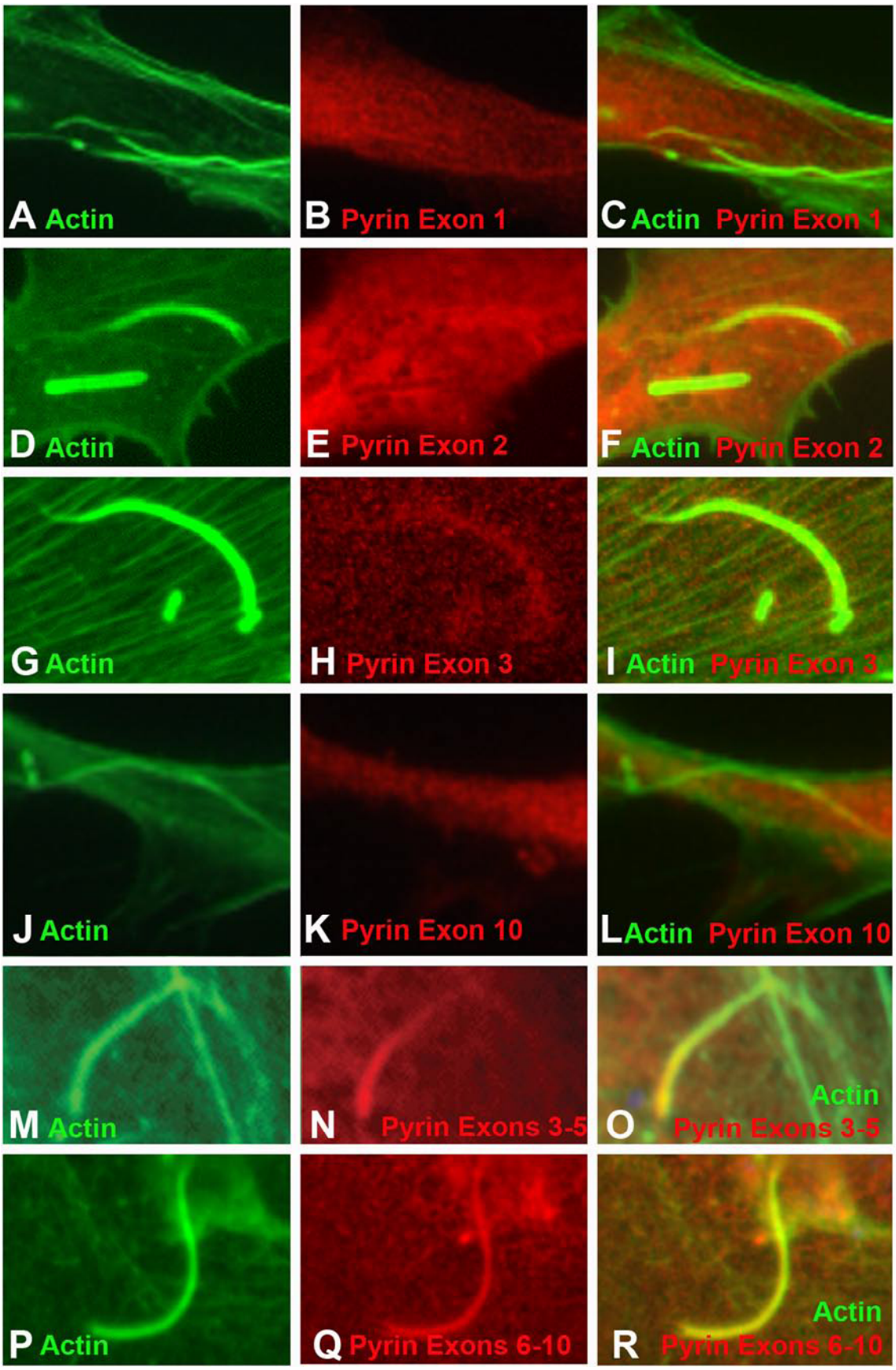


Figure 2.4: Regions of pyrin required for recruitment to *Listeria* rocket tails. Several myc-tagged regions of pyrin were tested for their ability to localize to *Listeria* rocket tails. Two minimal regions of pyrin were found to be sufficient for this recruitment: exons 3-5 and exons 6-10. Since these regions encompass the Bbox and coiled-coil domains of pyrin, we determined that these domains were critical for pyrin's recruitment to regions of polymerizing actin.

Figure 2.5: Regions of pyrin that are recruited to *Listeria* rocket tails. Myc-tagged versions of pyrin were transfected in cells that were then infected with *Listeria*. Cells were stained with an α-myc antibody to detect pyrin constructs, and with phalloidin to detect filamentous actin. Both the PyD encoded by exon 1 of pyrin (A-C) and the region encoded by exon 2 (D-F) were not recruited to rocket tails. Exon 3 of pyrin, which encodes the Bbox, was consistently but weakly recruited to rocket tails (G-I). Exon 10 of pyrin, which encodes the Rfp domain, was not recruited to tails (J-L). Two minimal regions of pyrin for rocket tail recruitment were determined to be exons 3-5 (the Bbox and part of the coiled-coil; M-O) and exons 6-10 (the rest of the coiled-coil and the Rfp domain; P-R). Photographs taken at 600X.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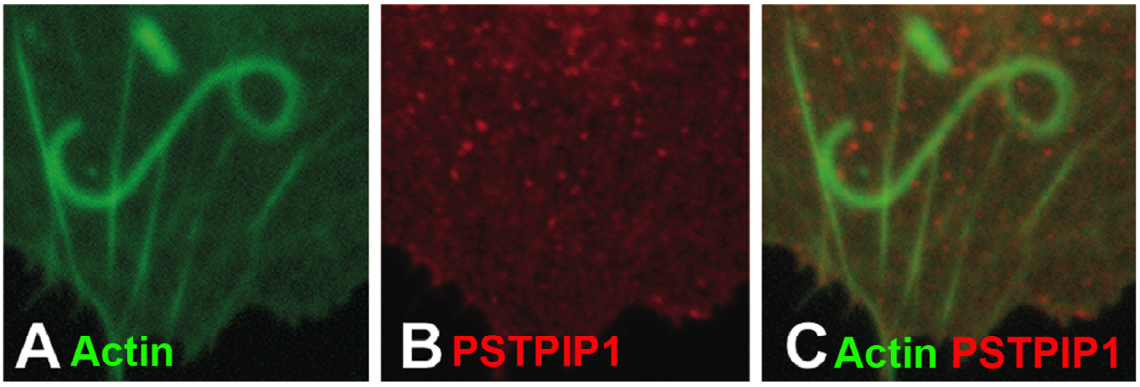


Figure 2.6: PTPIP1 is not recruited to *Listeria* rocket tails. PSTPIP1 is a known actin-associated protein that also binds to pyrin. Despite this known interaction, FLAG-tagged PSTPIP1 is not recruited to *Listeria* rocket tails. Photographs taken at 600X.

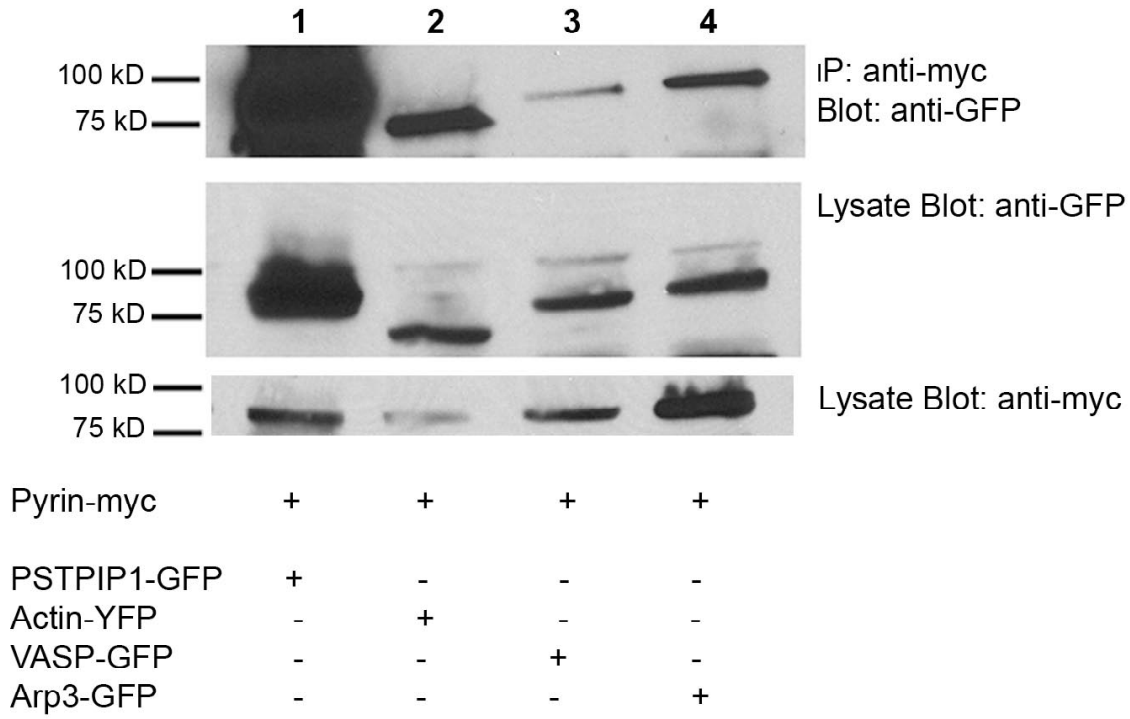
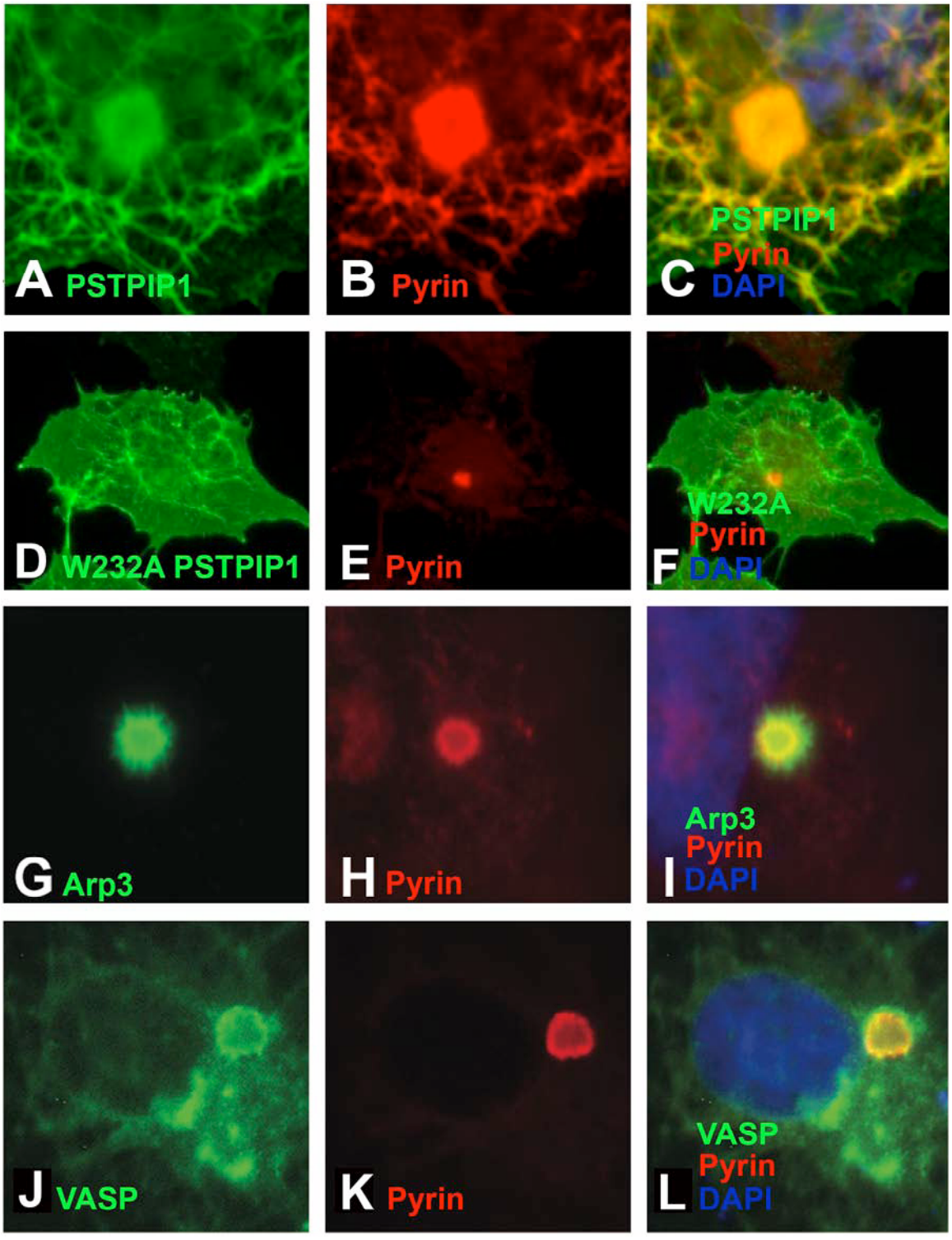


Figure 2.7: Pyrin interacts with actin, VASP, and Arp3 by imunoprecipitation. Immunoprecipitation experiments revealed that myc-tagged pyrin precipitates YFP-tagged actin, as well as GFP-tagged VASP and Arp3. These interactions were weak as compared with the positive control protein PSTPIP1-GFP, but were repeatable.

Figure 2.8: Pyrin recruits VASP and Arp3 to ASC specks. The ASC speck assay is a novel protein:protein interaction assay. The validity of this assay is demonstrated by myc-pyrin's ability to recruit wildtype FLAG-PSTPIP1, which is normally not seen in specks, to ASC specks (A-C). However, a mutated version of PSTPIP1, FLAG-W232A, that does not interact with pyrin is not recruited to specks (D-F). Myc-pyrin recruits GFP-tagged versions of both Arp3 (G-I) and VASP (J-L) to specks, verifying the interaction between these proteins. Panels A-C, G-L photographed by Andrea Waite at 600X. Panels D-F photographed by Neil Richards at 400X.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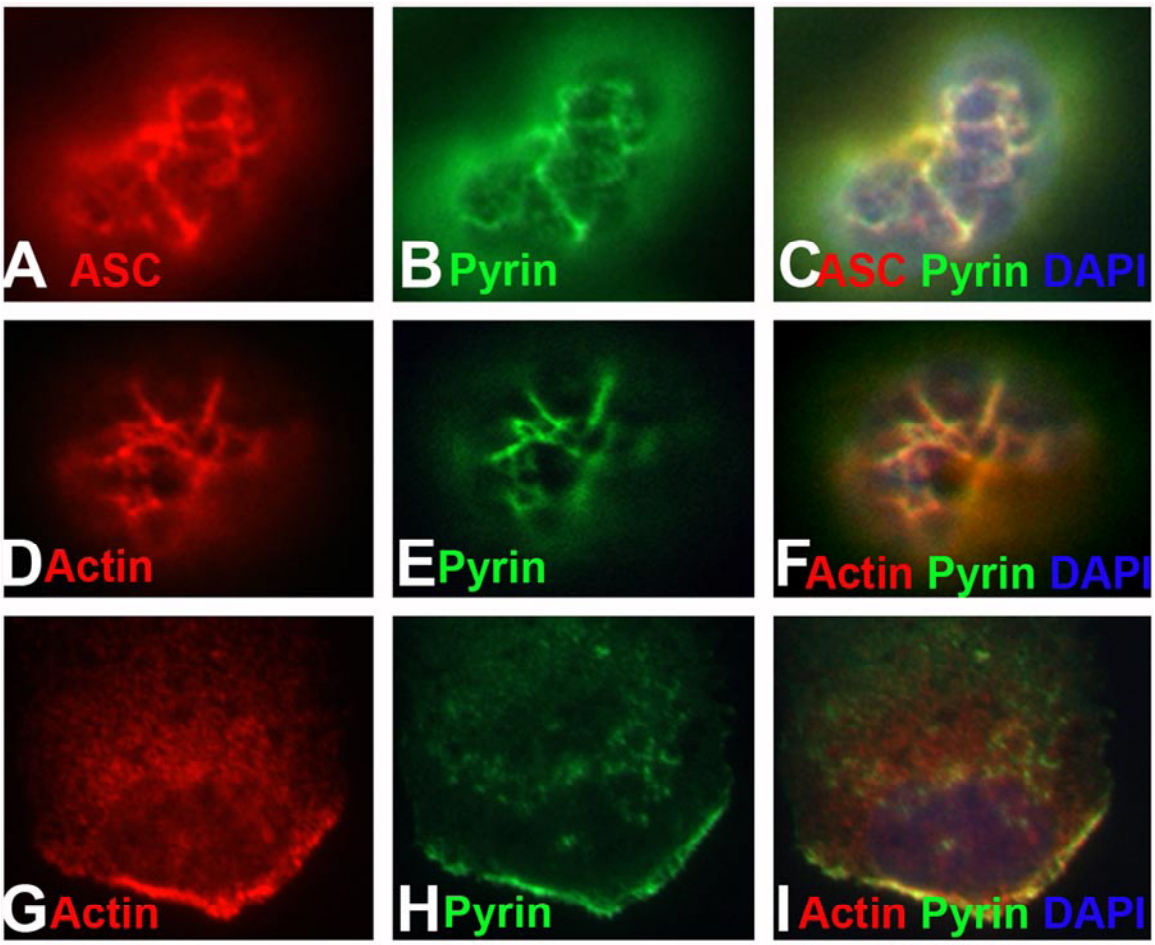
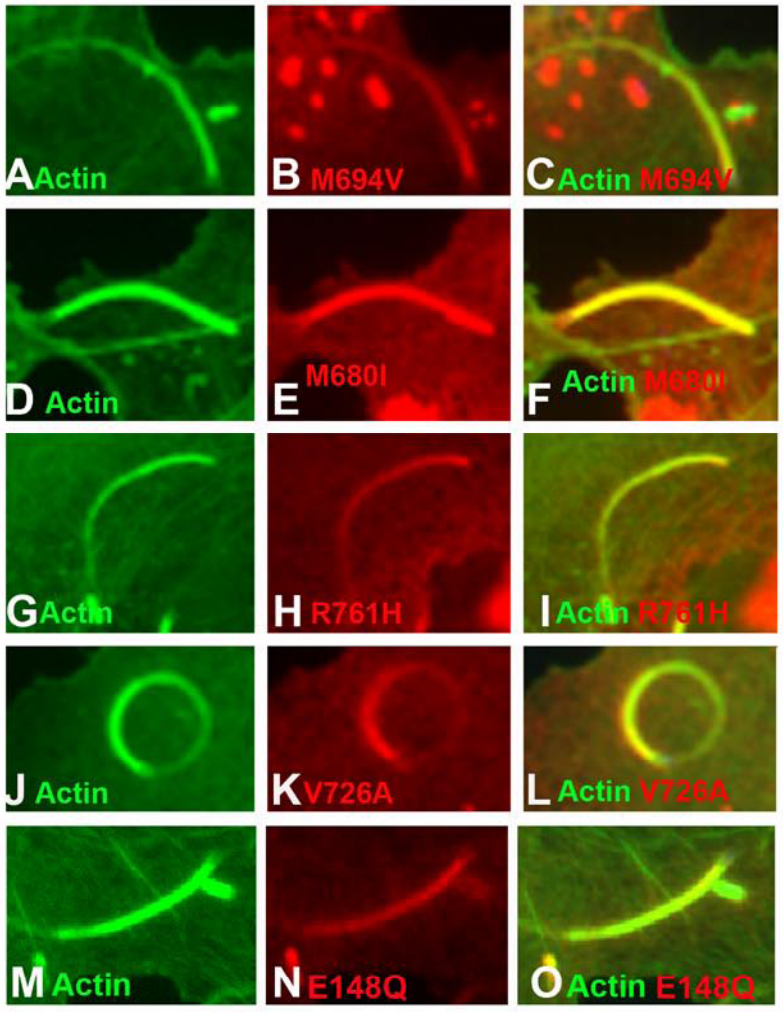


Figure 2.9: Effect of pyrin mutations on pyrin distribution. Monocytes isolated from an FMF patient homozygous for the M694V mutation were examined for pyrin, ASC, and actin distribution. Both ASC and pyrin were found in supranuclear ruffles (A-C) in a pattern indistinguishable from that of wildtype monocytes. Co-staining for actin (using phalloidin) and pyrin revealed that the two proteins co-localize in these ruffles (D-F). In migrating monocytes, pyrin was again seen concentrated in the leading edge (G-I). Photographs taken at 600X by Philip Schaner.

Figure 2.10: Effect of pyrin mutations on protein:protein interactions. Mutant versions of myc-tagged pyrin were tested in the *Listeria* rocket assay. Here we show four of the mutations tested: M694V (A-C), M680I (D-F), R761H (G-I), and V726A (J-L), and E148Q (M-O). In all cases, mutant pyrin was recruited to *Listeria* rocket tails. The ability of M694V mutant pyrin to bind to actin-associated proteins was then tested using immunoprecipitation. M694V bound to GFP-tagged PSTPIP1, actin (YFP-tagged), VASP, and Arp3 at levels comparable to wild-type pyrin (P). Photographs taken at 600X.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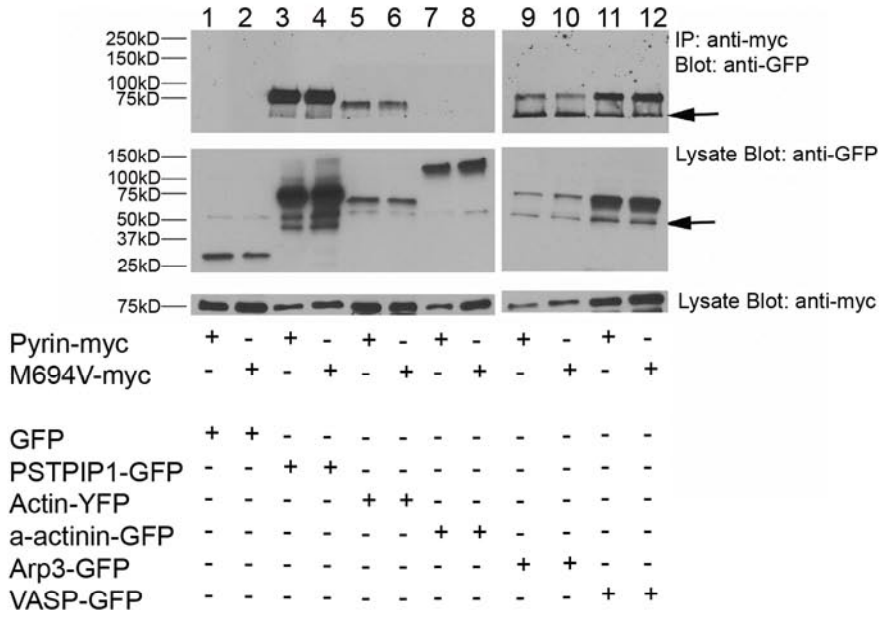
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Chapter 3

Pyrin, product of the *MEFV* locus, interacts with the proapoptotic protein, Siva[2]

Abstract

Mutations in pyrin cause the autoinflammatory disorder familial Mediterranean fever (FMF), a syndrome characterized by sporadic and unpredictable attacks of fever and localized severe pain. Currently, it is not clear how attacks are triggered, nor why they spontaneously resolve after two or three days. In fact, the cellular function of the pyrin protein and the molecular underpinnings of its malfunction in FMF have so far eluded clear definition. The identification of pyrin-interacting proteins has the potential to increase our understanding of the cellular networks in which pyrin functions. Previous reports have established that pyrin interacts with the apoptotic protein ASC, the cytoskeletal adaptor protein PSTPIP1, the inflammatory caspase, Caspase-1 and certain forms of the cytosolic anchoring protein 14.3.3. Here, we report that pyrin also interacts with Siva, a pro-apoptotic protein first identified for its interaction with the cytosolic tail of CD27, a TNF family receptor. The interaction between pyrin and Siva involves the C-terminal B30.2/rfp/SRPY domain of pyrin and exon 1 of Siva. We show that Siva and pyrin are indeed co-expressed in human neutrophils, monocytes and synovial cells. Furthermore, using a novel protein/protein interaction assay, we demonstrate that pyrin can recruit

Siva to ASC specks, establishing a potential platform for intersection of ASC and Siva function. Finally, we show that pyrin modulates the apoptotic response to oxidative stress mediated by Siva. Thus, the Siva-pyrin interaction may be a potential target for future therapeutic strategies.

Introduction

Familial Mediterranean fever (FMF) is classified as an autosomal recessive disease that is characterized by sporadic attacks of fever and localized inflammation (Livhen, 1997). Although FMF is prevalent among eastern Mediterranean populations, mainly non-Ashkenazi Jews, Armenians, Turks and Arabs, it is observed throughout the world due to extensive population movements of the 20th century (Schaner and Gumucio, 2005). The FMF gene, which is located on the short arm of chromosome 16 and symbolized as '*MEFV*' (for MEditerranean FeVer), encodes a 781 amino acid protein named pyrin or marenostrin (International FMF Consortium; French FMF Consortium) herein referred to as pyrin. Pyrin is expressed primarily in neutrophils, eosinophils, cytokine-activated monocytes and some fibroblasts (Centola et al., 1998; Diaz et al., 2004). Based on the nature of FMF attacks, it has been speculated that wild-type pyrin acts as a regulator of the inflammatory response, though the precise nature of this regulatory activity remains to be identified.

Previous identification of several pyrin-interacting proteins, including ASC

(Richards et al., 2001) and PSTPIP1 (Shoham et al., 2003), has led to the conclusion that pyrin participates in at least three cellular pathways that are connected to inflammation: cell death, cytokine secretion and cytoskeletal signaling. ASC acts as an adaptor protein to promote the assembly of a variety of large multi-protein complexes that mediate IL-1β processing (Martinon et al., 2002). ASC also provides a central platform for a cell death pathway that involves the generation of a large cytosolic perinuclear aggregate called a "speck" (Masumoto et al., 1999) or pyroptosome (Fernandes-Alnemri et al., 2007). A second pyrin-interacting protein, PSTPIP1, is also thought to act as an adaptor. PSTPIP1 links PEST phosphatases to their substrates, cytoskeletal signaling proteins such as WASP, c-Abl and Crk (Cong et al., 2000). Importantly, mutations in PSTPIP1 cause PAPA syndrome (pyogenic arthritis, pyoderma gangrenosum and acne) (Wise et al., 2002), which like FMF, is an autoinflammatory syndrome involving severe reactive skin and joint inflammation. The PAPA mutations reduce PSTPIP1 binding to PEST phosphatases and increase PSTPIP1 binding to pyrin, suggesting that pyrin and PSTPIP1 are members of a common pathway that controls inflammatory reactions (Shoham et al., 2003). Recently, additional reports have emerged indicating that pyrin also interacts directly with Caspase-1 (Chae et al., 2006), the major activator of IL-1β, and with certain isoforms of 14.3.3 (Jeru et al., 2005), an interaction that has the potential to control the compartmentalization of pyrin in nucleus or cytoplasm.

Here, we report the identification of a fifth pyrin-interacting protein: the pro-apoptotic protein, Siva. Siva was previously characterized as a protein that interacts with the cytoplasmic tail of several TNF receptor family members (CD27, GITR, OX40 and 4-1BB) (Prasad et al., 1997; Spinicelli et al., 2002). Siva also interacts with and neutralizes some of the anti-apopototic BCL-2 family members (Chu et al., 2004; Xue et al., 2002). Siva has been implicated in cell death following Coxsackie virus infection (Henke et al., 2001) and has been identified as a p53 target gene (Fortin et al., 2004) that can enhance apoptosis in response to cisplatin and other cellular toxins (Chu et al., 2005). The establishment of a direct physical connection between Siva and pyrin further broadens the realm of pyrin's potential influence on cellular inflammatory and apoptotic signaling.

Materials and Methods

Yeast Two-Hybrid Screen. Screening for pyrin-interacting proteins was performed as previously described (Richards et al., 2001). Full-length human pyrin was used as the bait to screen a cDNA library (2 x 10^6 clones) prepared from human neutrophil RNA isolated from a gouty knee. A total of 132 clones were isolated and confirmed as bone fide pyrin interacting proteins using the protocol previously described (Richards et al., 2001). One of these encoded the entire coding region and 5'UTR of Siva-1.

Cell Culture and transfection. COS-7 and human embryonic kidney 293T cells were grown in DMEM supplemented with 10% (vol/vol) FBS. Cells were transiently transfected by using FuGENE 6 (Roche Molecular Biochemicals). COS-7 were used for immunofluorescence staining and 293T cells were used for immunoprecipitation. Transfection rates of 60-70% were observed.

For monocyte/neutrophil isolation, heparinized blood from healthy adult volunteers was mixed with 6% dextran in PBS at a ratio of 5:1 and was allowed to settle at 37° C for 30 min. The buffy coat was layered onto the Ficoll-Hypaque (Pharmacia) density gradient and was centrifuged for 15 min at 1500 rpm at room temperature. Neutrophils and monocytes were recovered from the pellet of the gradient by hypotonic lysis of contaminating erythrocytes using H_2O. Neutrophils were greater than 98% pure, as assessed by May Giemsa staining. Mononuclear cells ($2x10^6$) were seeded in six-well plates in 2 ml of serum-free RPMI medium 1640 for 1h. Nonadherent cells were removed by aspiration and wells were washed with PBS.

Human synovial cells (fifth to eighth passage) were kindly provided by Dr. David Fox (Division of Rheumatology, University of Michigan). Synoviocytes were stimulated by incubation with LPS (100u/ml).

Plasmids. Full-length Siva-1, image clone Siva-2 (open biosystems-clone ID 3860364) and deletion constructs were subcloned into the pEGFP-C2 (Clontech,

Mountain View, CA) vector. All myc-tagged pyrin constructs were cloned into the pCMVTag3a (Stratagene) vector.

Expression analysis. Semiquantitative PCR analysis was performed on total RNA isolated from neutrophils, synovial cells, monocytes, SW-13, SW-480, THP-1, J774, and COS-7 cells. Primer sequences used for PCR were; F 5'CTCCTCCACCAGAAGTCAG 3', R 5'AGCTCTGGAACATTGAACATT 3' for pyrin, F 5'GCCTCCTTTGACTCCTTGA AGC 3', R 5' TTCTGCCTCTCACGAAACTCCTC 3' for PSTPIP1, F 5' CTTCTACCTGGAGACCTACGGC 3', R 5' TCTTGCTTGGGTTGGTGGG 3' for ASC and F 5' CGAGAAGACCAAGCGACTCC 3', R 5' TGAGGAACAGGCAATGGACG 3' for Siva-1, F 5' CGAGAAGACCAAGCGACTCC 3', R 5' TGAGGAACAGGCAAT GGACG 3' for Siva-2. Primer sequences used for control HPRT PCR were; F 5' TGGCGTCGTGATTAGTGATG 3' and R 5' AATCCAGCAGGTCAGCAAAG 3'.

Antibodies. Rabbit anti-myc polyclonal antibody was obtained from Sigma, and mouse anti-GFP monoclonal antibody was obtained from Clontech (Mountain View, CA). Fluorescent secondary antibody AF 568 goat anti-rabbit was purchased from Molecular Probes. Horseradish peroxidase-conjegated antibodies used for Western blot (goat anti-mouse and mouse anti-myc) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoprecipitation and western blot analysis. 293T cells were transiently transfected with different pyrin, Siva-1 and Siva-2 constructs. After 24 h, cells were lysed in 10mM TrisHCl pH 7.4, 300mM NaCl, 2mM EDTA, 0.5% Triton X-100 lysis buffer in the presence of Complete Mini EDTA-Free Protease Inhibitor Cocktail (Roche). Cells were incubated with anti-myc antibody which was crosslinked to protein A-G sepharose beads for 16h at 4° C. After washing the beads, bound proteins were subjected to SDS/PAGE and transferred to nitrocellulose membranes for detection with appropriate antibodies.

Immunofluorescence staining. After transfection, cells were fixed in 4% paraformaldehyde in PBS, permeabilized by using 0.2% Triton X-100 in PBS and blocked in 10% goat serum, 1 % BSA in PBS for 1 hour. After antibody treatment, DAPI staining was performed and cells were visualized with a Nikon E800 immunofluorescence microscope.

ASC speck assay. COS-7 cells were co-transfected with ASC, pyrin and Siva constructs. After transfection (24 h) immunofluorescence and DAPI staining (to stain nuclei) were carried out. Co-localization of Siva constructs with pyrin in ASC specks was then observed.

Apoptosis assay. The hydrogen peroxide apoptosis sensitization assay was carried out using a slight modification of a protocol previously used to detect Siva-mediated apoptosis (Cao et al., 2001). COS-7 cells were plated in 24-well

plates and transfected with Siva-1, Siva-2, and/or various forms of wild type or mutant pyrin. Forty hours after transfection, cells were treated with 1mM H_2O_2 for 2 hours, then washed with culture medium (DMEM + 10% FBS) and cultured for an additional 20 hours. Adherent and non-adherent cells were collected and stained with trypan blue. Live and dead cells were counted using a hemacytometer, and the percentage of apoptotic cells was calculated. The transfections were repeated four times and all counting was done by an investigator blinded as to the identity of the samples.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism software. P values were calculated using an unpaired two-tailed t test, and values of ≤0.05 were considered to be significant.

Results

Identification of Siva as a pyrin binding protein. We previously reported the generation of a human neutrophil cDNA library which we employed in a yeast two hybrid screen to identify ASC as a pyrin-interacting protein (Richards et al., 2001). From the same screen, we isolated a single clone which, upon sequence analysis, proved to represent full length Siva-1. The human Siva gene is localized to chromosome 14q32-33 and is organized into four exons that code for the full-length Siva-1 transcript. An alternate splice form, Siva-2, has also been described that lacks the exon 2 coding sequence (Py et al., 2004). The full-length

form of Siva, Siva-1, is a 175 amino acid protein that contains a conserved amphipathic helical region at its N-terminus (partially missing in Siva-2) and a cysteine-rich C-terminus that binds zinc and may adopt a zinc finger-like conformation (Nestler et al., 2006).

Expression of Siva, pyrin and other pyrin-interacting proteins. Siva was first isolated from T-cells as a CD27 binding protein, but expression of Siva is more widespread (Jacobs et al., 2007). Since the expression of pyrin is restricted to myeloid cells and certain fibroblast cell types (Centola et al., 1998; Diaz et al., 2004), we examined these particular cells as well as several additional cell lines for co-expression of pyrin and Siva message. The expression of mRNA encoding two other pyrin-interacting proteins, ASC and PSTPIP1, were also queried. We confirmed that neutrophils, monocytes, and synoviocytes, as well as SW-13, SW-480, and THP-1 cells all express the relevant messages, supporting the potential for physical interaction of these proteins (Figure 3.1). In addition, we found that none of these transcripts can be found in 293T cells (data not shown), indicating that these cells are an appropriate line to use to investigate the binding of pyrin and Siva independently of other known interacting proteins.

Siva binds pyrin by immunoprecipitation. Once it was established that pyrin and Siva are expressed in several relevant cell types, we set out to confirm the interaction between Siva and pyrin by immunoprecipitation. Myc-tagged pyrin and GFP-tagged Siva-1 or Siva-2 were co-transfected into 293T cells.

Immunoprecipitation was accomplished with anti-myc and blots were probed with anti-GFP. Both Siva-1 and Siva-2 proteins were co-precipitated by antibodies to epitope-tagged pyrin (Figure 3.2, lanes 3 & 4 respectively) but not by the negative control protein lacZ-myc (Figure 3.2, lanes 2 & 5 respectively). Although expression of Siva-1 and Siva-2 in lysates was similar (Figure 3.2, center panel, lanes 2 & 3 for Siva-1, lanes 4 & 5 for Siva-2), Siva-2 was reproducibly precipitated more efficiently than Siva-1, suggesting a stronger binding interaction between pyrin and Siva-2.

Binding domains of pyrin and Siva. To identify the domains in pyrin that interact with Siva, immunoprecipitation was performed using a set of deletion constructs of myc-tagged pyrin and GFP tagged Siva co-transfected into 293T cells (Figure 3.3). Interestingly, the size of Siva varied slightly from lane to lane in the gel (Figure 3.3A-C, center panels), possibly indicating post-translational modification of the protein; indeed, Siva is known to be phosphorylated by Arg kinase (Cao et al., 2001), and this may be highly context-dependent. Siva-1 and Siva-2 were both immunoprecipitated using pyrin constructs lacking exon 1, which encodes the pyrin domain or PyD (Figure 3.3A & 3.3B, lanes 2 & 3). However, when IP was performed using pyrin constructs that lack other regions of the protein, a marked reduction in binding efficiency was seen. Of particular interest is the fact that the lowest binding levels were observed with pyrin exons 1-9, which is missing the C-terminal B30.2 domain (also called the SPRY or rfp domain) encoded by exon 10 (Figure 3.3A & 3.3B, lane 1). These data indicate

that the B30.2 domain plays an important role in the interaction of pyrin with Siva-1 and Siva-2, although binding may be stabilized by other regions of the pyrin molecule.

Conversely, to determine the binding domain of Siva responsible for its interaction with pyrin, IP was performed using deletion constructs of GFP-tagged Siva and myc-tagged full length pyrin in co-transfected 293T cells. GFP-Siva exon 1 was co-precipitated with pyrin (lane 2), while a construct encoding GFP-Siva exons 3-4 was much less efficiently co-precipitated (Figure 3.3C, lane 6). Interestingly, precipitation of the Siva-1 construct that included both exons 1 and 2 (Figure 3.3C, lane 4) was less efficient than was Siva exon 1 alone (Figure 3.3C, lane 2), suggesting a potential inhibitory effect of Siva's exon 2 on this interaction. This may explain why Siva-2, which lacks exon 2, appears to bind pyrin with higher affinity than Siva-1 (Figure 3.2). These data together indicate that the exon 1 coding domain of Siva is the primary region involved in the interaction of Siva proteins with pyrin.

Co-localization of pyrin and Siva in cells. To further confirm and explore the interaction between Siva and pyrin, we used immunofluorescence in co-transfected cells. Transfection of COS-7 cells with full-length pyrin linked to different epitope tags (myc-pyrin and GFP-pyrin) revealed a cytosolic distribution of the transfected pyrin protein (Figure 3.4A, pyrin-myc shown), as previously demonstrated (Diaz et al., 2004; Richards et al., 2001). However, GFP-tagged

Siva-1 was predominantly nuclear, frequently localizing to discrete aggregates within the nucleus (Figure 3.4B-C). GFP-tagged Siva-2 was again observed in the nuclear compartment, but was also detectable in the cytoplasm, often in stress fiber-like structures (Figure 3.4D-E). To test whether Siva constructs might localize predominantly to the cytosol in the early stages after transfection, when protein concentrations are lower, we transfected cells with GFP-tagged Siva-1 and Siva-2 constructs and examined the cells at 6, 9 and 12 hours after transfection. In all cases, Siva-1 was found to be nuclear, while Siva-2 was both cytoplasmic and nuclear (data not shown). Thus, at least in transfected cells, Siva prefers the nuclear environment.

When cells were co-transfected with pyrin and Siva-1, the two proteins appeared to maintain their distinct compartmentalization (nuclear Siva-1 and cytosolic pyrin; data not shown). When pyrin and Siva-2 were co-transfected, the two proteins showed a clear cytosolic co-localization on the stress fiber like structures and on the cellular membrane (Figure 3.4F-K). These studies confirm that pyrin and Siva-2 are expressed in the same cellular compartment, which makes binding possible. However, this experimental technique failed to confirm expression of Siva-1 and pyrin in the same cellular compartment.

Though our co-transfection experiments in COS-7 cells suggested that Siva-1 is exclusively nuclear, Siva-1 has been detected outside of the nucleus in other studies (Chu et al., 2005; Jacobs et al., 2007; Xue et al., 2002). We

suspected therefore, that the trafficking of Siva-1 may be highly regulated and that this protein may be capable of rapidly entering and leaving the cytosol in particular contexts. Therefore, we sought a method to detect small amounts of Siva-1 that might exit the nucleus and test whether, once in the cytosol, a Siva-1/pyrin interaction could be demonstrated. For this purpose, we took advantage of another pyrin-interacting protein, ASC. ASC and pyrin interact via an N terminal PyD present in both molecules (Richards et al., 2001). Importantly, when cells are transfected with ASC, characteristic cytosolic speck-like aggregates are formed within 24 hours (Masumoto et al., 1999; Richards et al., 2001). If pyrin is co-transfected with ASC, pyrin is also drawn to the speck compartment. This requires exon 1 of pyrin, a region of the pyrin molecule that appears to be dispensable for its interaction with Siva. We therefore examined whether speck-associated pyrin could trap cytosolic Siva-1 in cells triply transfected with myc-pyrin, untagged ASC, and EGFP-tagged Siva-1. Indeed, in triply transfected cells, both Siva-1 and Siva-2 proteins could be observed in ASC specks by immunofluorescence microscopy (Figure 3.5). In the absence of pyrin, neither Siva isoform was associated with specks, confirming pyrin-dependent attraction of Siva to ASC specks (data not shown). In addition, tests with another protein that does not interact with pyrin (α-actinin) showed that the attraction of Siva to pyrin-associated ASC was specific (data not shown). These findings not only provide further evidence for the binding of pyrin to both Siva-1 and Siva-2, but also indicate that the triple association of pyrin-ASC and Siva is possible, allowing potential crosstalk between Siva and ASC-mediated signaling pathways.

Interaction of Siva with pyrin constructs harboring FMF-associated mutations. Many of the major FMF-associated mutations are clustered in the rfp domain of pyrin, and they have been recently proposed to affect the interaction between pyrin and Caspase-1 (Chae et al., 2006). We therefore considered the possible effect of these mutations on the interaction between pyrin and Siva proteins. We examined the effect of the E148Q mutation in pyrin exon 2, as well as the M694V mutation in exon 10 on Siva's ability to bind pyrin. Both GFP-tagged Siva-1 and Siva-2 were precipitated with mutant forms of pyrin-myc at levels comparable to wild-type pyrin (Figure 3.6), indicating no discernable effect of these mutations on binding in this assay.

Effect of pyrin on Siva-induced apoptosis in response to oxidative stress. Siva has been shown to be an apoptosis-associated protein in a variety of contexts (Chu et al., 2004; Henke et al., 2001; Prasad et al., 1997; Xue et al., 2002). It has further been reported that the two splice isoforms of Siva differentially transduce apoptosis; Siva-2 triggers apoptosis in Rat-1 cells to a much lesser extent than Siva-1 (Yoon et al., 1999). As we sought to identify a potential functional relevance for the interaction between pyrin and Siva, we examined whether this interaction alters Siva-mediated apoptosis. COS-7 cells were transfected with myc-tagged pyrin and GFP-tagged Siva, alone or in combination. The cells were then placed under oxidative stress by brief exposure to hydrogen peroxide, and the apoptotic response was assessed after

20 hours as described in Methods. The results from five independent replicates of this assay confirmed that wild type pyrin, transfected alone, fails to induce a significant apoptotic response in control cells or in response to H_2O_2 (Figure 3.7). Though Siva-1 does not induce apoptosis in the absence of oxidative challenge, H_2O_2 exposure induces a clear apoptotic response, as described previously (Cao et al., 2007). However, co-transfection of Siva-1 with pyrin significantly diminishes this Siva-induced apoptotic response.

In contrast to the results with Siva-1, Siva-2, when transfected alone, is unable to induce apoptosis in response to H_2O_2 in agreement with previous reports (Cao et al., 2001). When Siva-2 and pyrin are co-transfected, a higher rate of apoptosis is observed than with either construct alone, but this effect, though reproducible, is not statistically significant.

We further explored these observations by using a disease-associated pyrin mutant (M694V) that contains a mutation in exon 10, the region shown to be important for the Siva-pyrin interaction. When M694V was transfected alone, it displayed a rate of apoptosis in response to H_2O_2 that was similar to that of wild type pyrin (Figure 3.7). Furthermore, when co-transfected with Siva-1, the M694V mutant pyrin, like wild type pyrin, reduced the apoptotic response of Siva-1 to H_2O_2 challenge, though in this case, the difference did not reach significance. As for wild type pyrin, M694V pyrin did not modulate the Siva-2

response to H_2O_2. Taken together, these observations suggest that pyrin may modulate apoptotic signaling transduced by Siva-1, but not by Siva-2.

Discussion

This work identifies and characterizes an interaction between pyrin and the proapoptotic protein, Siva. This brings the total number of known pyrin-interacting proteins to five: ASC, PSTPIP1, Caspase-1, 14.3.3 and Siva, further broadening the potential signaling role of pyrin in inflammation and apoptosis. The domain of pyrin that we find to be the most important for its interaction with Siva is the B30.2/SPRY/rfp domain that is encoded by exon 10. This is the same region of the pyrin molecule that mediates its interaction with Caspase-1. It is not clear at this time if the Caspase-1 and Siva interactions are competitive or involve different regions of the B30.2/SPRY/rfp domain. Interestingly, ASC and PSTPIP1 interaction domains of pyrin map to very different regions of the molecule (N-terminal PyD in the case of ASC and the B-box and coiled coil central region for PSTPIP1). We show here that pyrin can indeed bridge the co-localization of ASC and Siva, suggesting a mechanism by which ASC and Siva-mediated apoptotic signaling pathways could potentially intersect. Though it was not directly tested, the interaction of pyrin, Siva, and PSTPIP1 also seems potentially possible.

We also present in this report a novel type of assay for the sensitive detection of cellular protein/protein interactions: the ASC speck recruitment assay. This assay relies on the efficient recruitment of pyrin's PyD to ASC specks. In this case, the fact that the PyD of pyrin seems dispensable for the pyrin/Siva interaction allowed us to use speck-associated pyrin as a cytosolic magnet to which Siva was attracted. Potentially, this system could be exploited for other protein/protein interaction tests, by: a) linking the PyD of pyrin (approximately 90 amino acids) onto one of two potential interaction partners; b) demonstrating effective recruitment of that PyD-tagged protein to ASC specks; and c) testing whether triple transfection of ASC, the PyD tagged bait and its prey results in recruitment of the prey to specks. Since the speck represents a relatively large structure with a highly concentrated localization of ASC protein, the assay appears to be quite sensitive in detecting small amounts of potential prey proteins (in this case, Siva-1).

Our finding that pyrin's C-terminal rfp domain is important for its interaction with Siva is particularly interesting. The rfp domain is found in >500 different proteins of various functions; it has been proposed that this domain mediates protein/protein interactions or binds particular ligands (Papin et al., 2007; Woo et al., 2006). This domain has a particular importance for pyrin and FMF disease since the majority of FMF-associated mutations are localized to this region. When we examined the effect of two common mutations, we failed to find evidence that these mutations affect the binding interaction or the functional

interaction in an oxidative stress assay. However, it is possible that the strength of the interactions is only mildly affected and cannot be measured with approaches or conditions used here. Since both pyrin and Siva interact dynamically with multiple other proteins, slight changes in binding affinity have the potential to alter the dynamics of cellular processes in which either protein participates.

In this regard, it is interesting that Siva also interacts with Arg kinase, a member of the c-Abl family of kinases. Arg phosphorylates both Siva-1 and Siva-2 and this phosphorylation event is associated with the induction of apoptosis (Cao et al., 2001). Upon integrin ligation or cytokine exposure, both Abl and Arg become activated and localize to areas of p103-CAS/Crk assembly including focal adhesions and membrane ruffles (Kain and Klemke, 2001). Both Abl and Arg interact with the SH3 domain of Crk via their conserved polyproline region, and promote tyrosine phosphorylation of Crk-Y221. Thus, Abl family kinases have an important biological role in the regulation of CAS/Crk coupling, an event that can be a switchpoint in the cell, determining whether the cell migrates or dies (Bouton et al., 2001). Remarkably, both c-Abl and Crk bind PSTPIP1 and are PEST phosphatase substrates (Cong et al., 2000). These observations suggest the possibility that a broader network of interactions between PSTPIP1 and Siva pathways, potentially modulated by pyrin binding to both proteins, could affect both apoptosis and cell migration. It is interesting in this regard that pyrin and Siva-2 appear to co-localize at membrane ruffles. Since FMF is characterized by

a massive migration of neutrophils into target inflammatory sites, an important next step will be to explore more fully the potential regulatory interactions between pyrin, Siva and PSTPIP1 as well as the status of the Cas/Crk coupling switch in FMF patients between and during attacks.

Another previous observation concerning Siva and pyrin may be of clinical importance given the physical and functional interactions between these two proteins documented here. It has been reported that the anti-cancer drug, cisplatin, can induce an FMF attack (Toubi et al., 2003). Interestingly, cisplatin induces a potent p53 response and Siva is one of only a few genes that are induced specifically during p53-mediated apoptosis (Jacobs et al., 2007). In fact, over-expression of Siva alone is sufficient to mimic p53-dependent cell death in the absence of p53 itself (Jacobs et al., 2007). Induction of p53 by Siva requires Caspase-8 and involves the association of Siva with the plasma membrane (Jacobs et al., 2007), a compartment in which we also observe pyrin (Figure 3.5). Further, the knockdown of Siva in cells treated with the DNA damaging toxin, camptothecin, attenuates the cell death response normally seen after treatment with this toxin. These findings together suggest the interesting hypothesis that induction of an FMF attack through cisplatin treatment might be Siva-dependent. The reduction in Siva-induced apoptosis in seen in the presence of pyrin (Figure 3.7) could potentially permit a prolonged period of cellular inflammatory signaling through cytokine release that could trigger an attack. If this hypothesis is

confirmed, the Siva-pyrin interaction might emerge as an important target for future therapeutic strategies.

[2] Balci-Peynircioglu B , Waite AL (co-first author), Hu C, Richards N, Staubach-Grosse A, Yilmaz E, Gumucio DL. 2008. Pyrin, product of the MEFV locus, interacts with the proapoptotic protein, Siva. *J Cell Physiol*. In press.

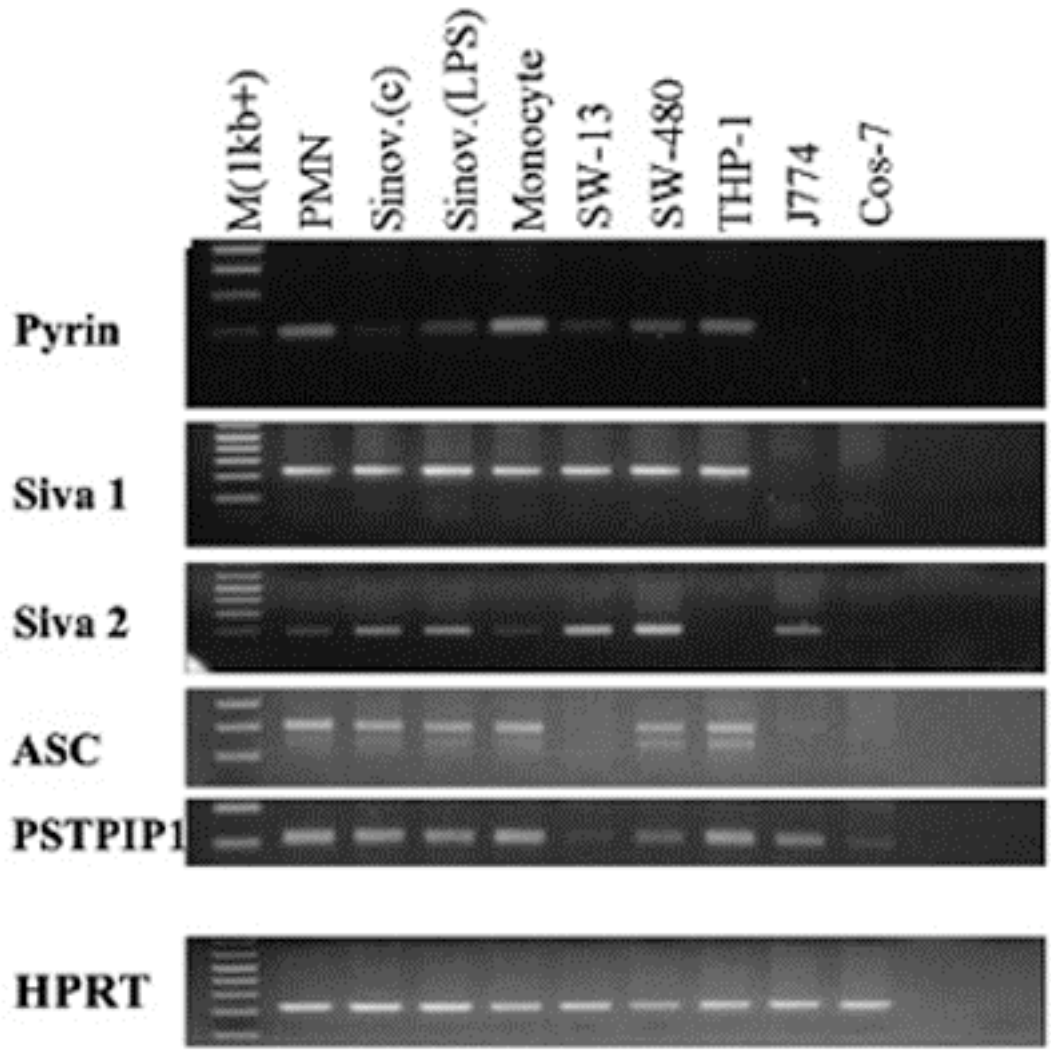


Figure 3.1: Endogenous expression of pyrin and pyrin-binding proteins. Native expression of pyrin, Siva-1, Siva-2, ASC, PSTPIP1 and the housekeeping gene HPRT was examined in a variety of cell types. RT-PCR analysis was performed on total RNA from freshly isolated PMNs, synoviocytes (both unstimulated and stimulated with LPS), and monocytes, as well as SW-13, SW-480, THP-1, J774, and COS-7 cells. Pyrin and Siva-1 were detected in all cells with the exception of J774 and COS-7. Siva-2 expression was found in all cell lines except THP-1 and COS-7. ASC was expressed in all cell lines except SW-13, J774, and COS-7, while PSTPIP1 was expressed in all cell lines.

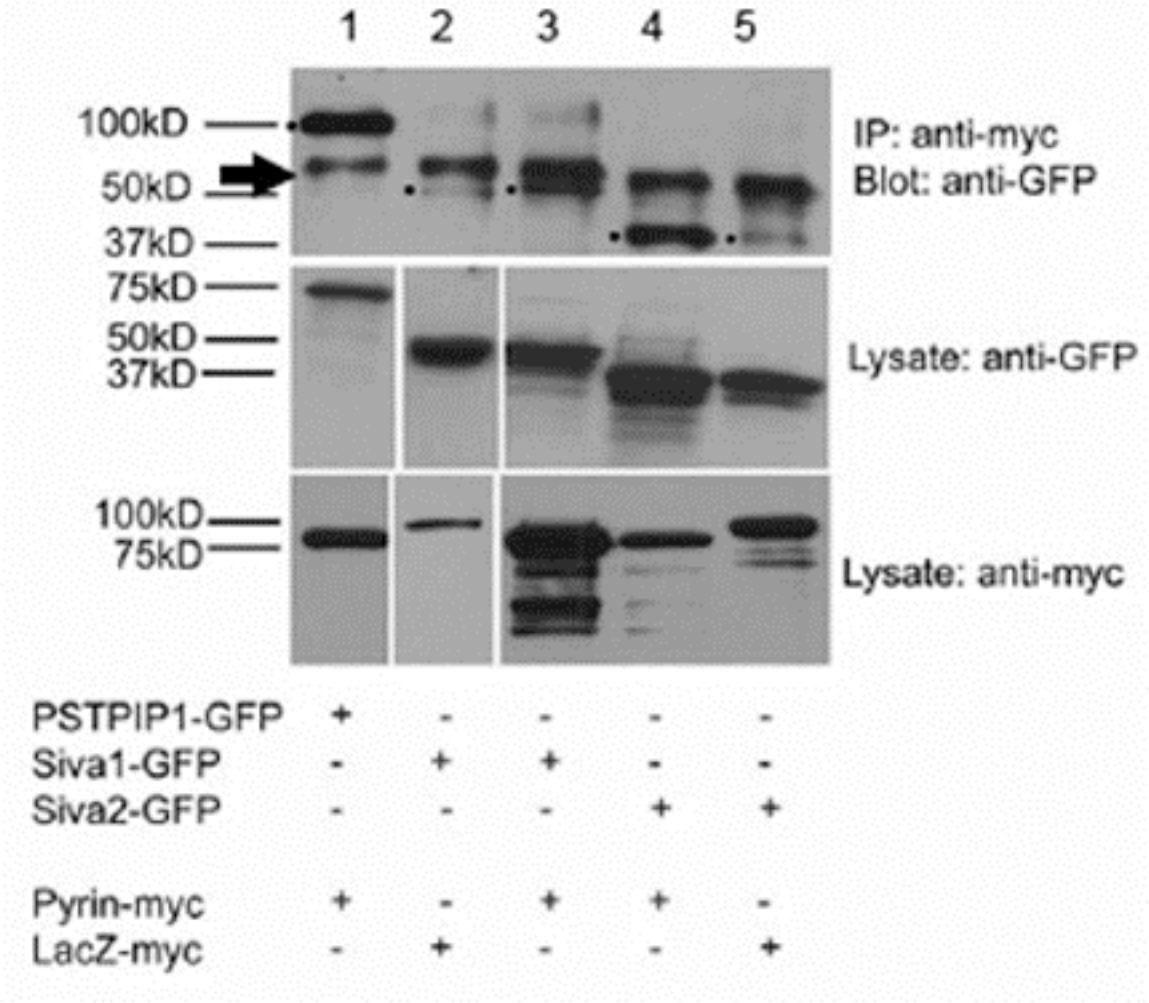
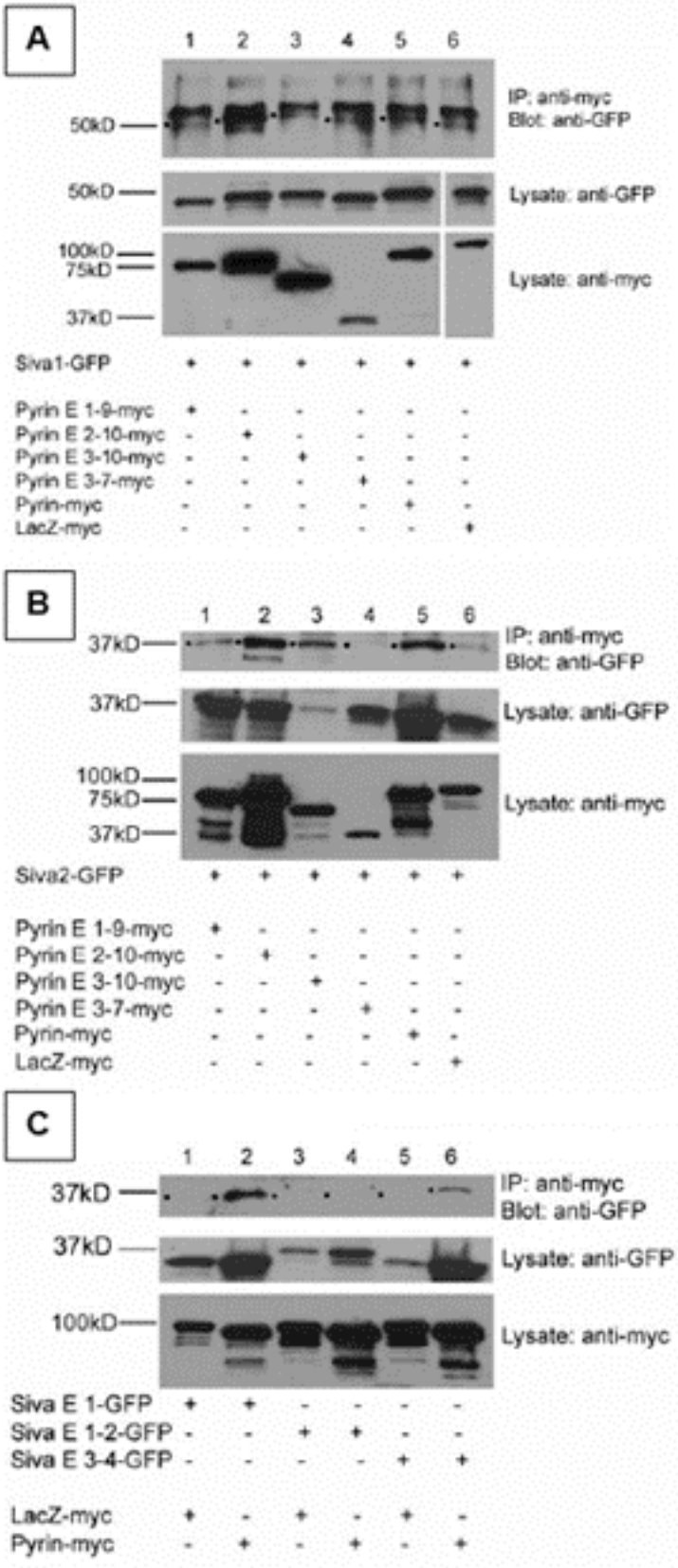


Figure 3.2: Pyrin binds to Siva-1 and Siva-2. 293T cells were co-transfected with GFP-tagged versions of Siva-1 and Siva-2 and myc-tagged pyrin. Cell lysates were tested for expression of transfected proteins, and then immunoprecipitated using α-myc polyclonal antibody (Sigma). Proteins were detected by western blot using α-GFP monoclonal antibody (Clontech). Small black dots to the left of the lanes indicate the expected location of co-precipitated proteins. Co-precipitation of pyrin and PSTPIP1 was reconfirmed as a control (lane 1). Full length pyrin-myc precipitated both Siva-1-GFP (lane 3) and Siva-2-GFP (lane 4) but not the control protein lacZ-myc (lanes 2 & 5 respectively). The band seen in all lanes of the top panel (arrow) is a background band due to the α-myc antibody used for immunoprecipitation.

Figure 3.3: Regions of pyrin involved in binding to Siva-1 and Siva-2. (A) Siva-1 was immunoprecipitated with several different portions of pyrin. Strong binding was observed with pyrin exons 2-10-myc (lane 2), but binding to pyrin exons 1-9 was very weak (lane 1). (B) Siva-2 precipitated strongly with myc-tagged pyrin exons 2-10 (lane 2) and 3-10 (lane 3), but not with exons 3-7 (lane 4) and very weakly with pyrin exons 1-9 (lane 1). (C) Myc-tagged pyrin was co-transfected with GFP-tagged deletion constructs of Siva. Pyrin precipitated Siva exon 1 (lane 2), but binding was inhibited by the addition of Siva exon 2 (lane 4). Siva exons 3-4 were also weakly precipitated by pyrin (lane 6). Dots to the left of the lanes indicate expected sizes of precipitated proteins.


A
1 2 3 4 5 6
50kD
IP: anti-myc
Blot: anti-GFP
50kD
Lysate: anti-GFP
100kD
75kD
37kD
Lysate: anti-myc
Siva1-GFP + + + + + +
Pyrin E 1-9-myc + - - - - -
Pyrin E 2-10-myc - + - - - -
Pyrin E 3-10-myc - - + - - -
Pyrin E 3-7-myc - - - + - -
Pyrin-myc - - - - + -
LacZ-myc - - - - - +
B
1 2 3 4 5 6
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IP: anti-myc
Blot: anti-GFP
37kD
Lysate: anti-GFP
100kD
75kD
37kD
Lysate: anti-myc
Siva2-GFP + + + + + +
Pyrin E 1-9-myc + - - - - -
Pyrin E 2-10-myc - + - - - -
Pyrin E 3-10-myc - - + - - -
Pyrin E 3-7-myc - - - + - -
Pyrin-myc - - - - + -
LacZ-myc - - - - - +
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Blot: anti-GFP
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Siva E 1-GFP + + - - - -
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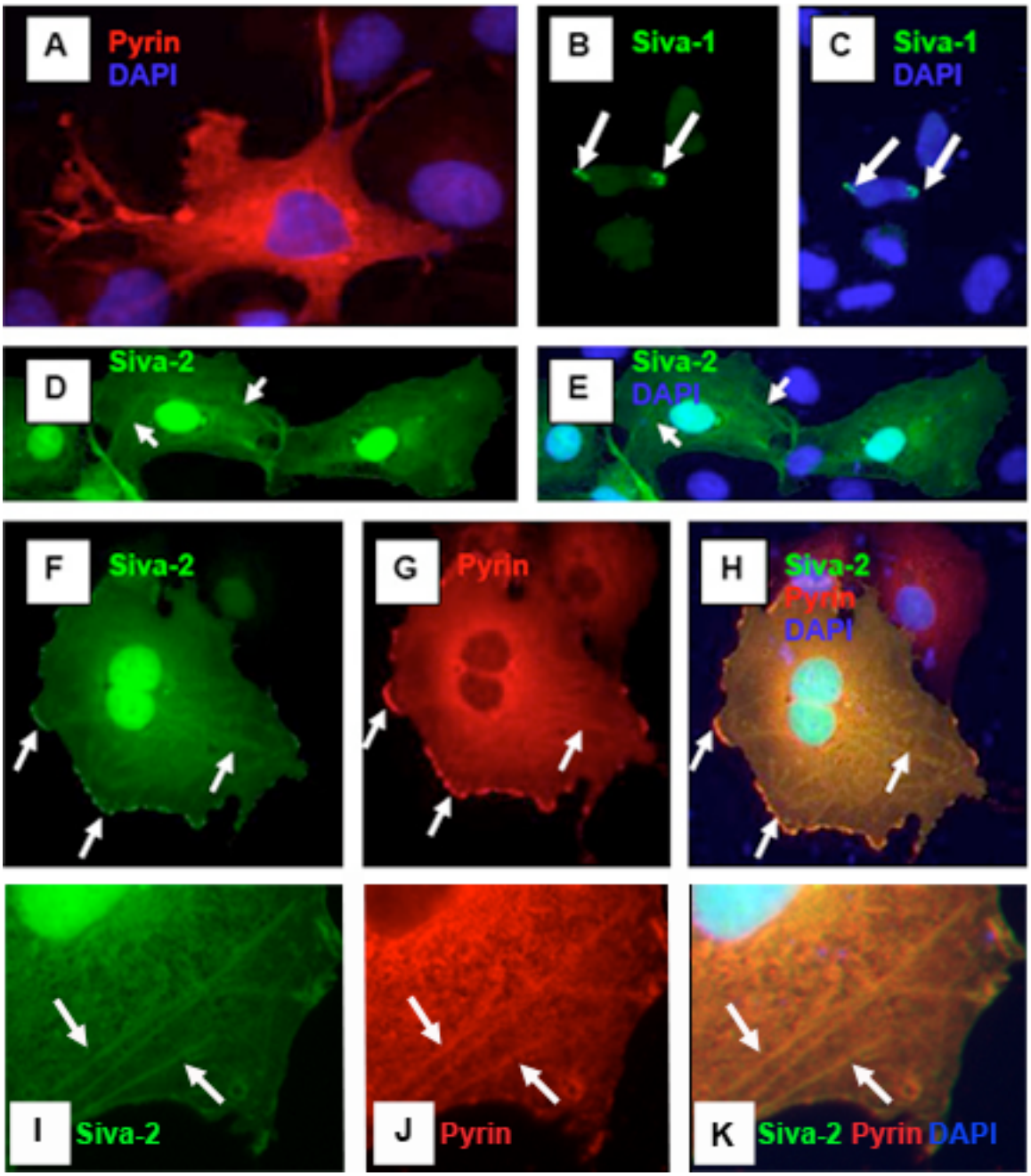


Figure 3.4: Cellular localization of Siva and pyrin in co-transfected COS-7 cells. (A) Myc-tagged pyrin is exclusively cytoplasmic. (B-C) GFP-tagged Siva-1 is nuclear; small nuclear aggregates are often seen (arrows). (D-E) GFP-tagged Siva-2 is both nuclear and cytoplasmic. Cytoplasmic Siva-2 appears to be in stress fiber-like structures (arrows). (F-H) Co-transfection of pyrin with Siva-2 results in a clear cytoplasmic co-localization of the proteins on the stress fiber-like structures in the cytoplasm and in lamellipodia-like structures at the membrane edge (arrows). Panels A-1 photographed at 400X by Banu Balci-Peynircioglu. Panels I-K photographed at 600X by Ann Staubach-Grosse.

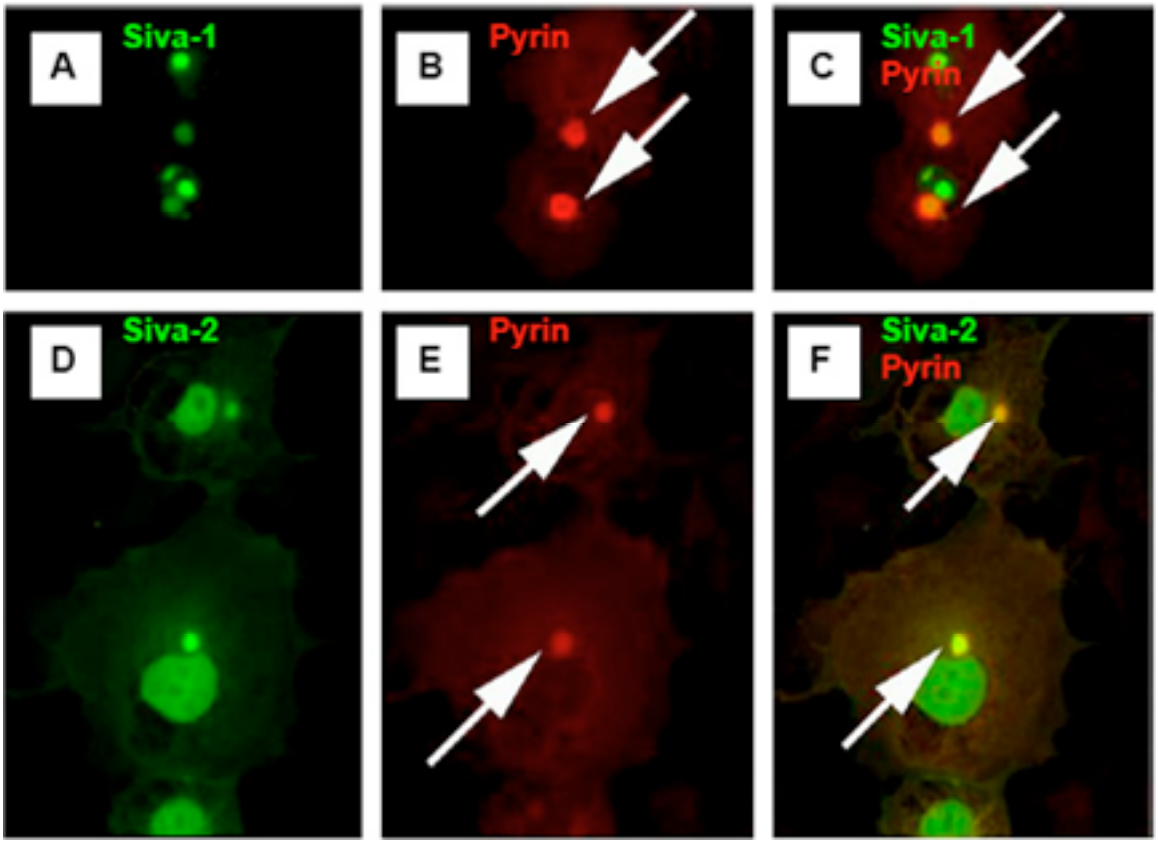


Figure 3.5: Siva and pyrin co-localization in the ASC speck assay. (A-C) COS-7 cells were co-transfected with GFP-tagged Siva-1 (green, panel A), myc-tagged pyrin (red, panel B), and ASC (untagged). Panel C is a merged picture of the red and green channels. Arrows point to ASC specks that contain pyrin in panel B. Arrows in panel C indicate that Siva-1 is also located in these specks (yellow). (D-F) Cells were co-transfected with GFP-tagged Siva-2 (green, panel D), myc-tagged pyrin (red, panel E), and ASC (untagged). Panel F is a merged picture of the red and green channels. Arrows in panel E point to ASC specks containing pyrin. Arrows in panel F indicate that Siva-2 is also located in these specks (yellow). Photographs taken at 400X by Banu Balci-Peynircioglu.

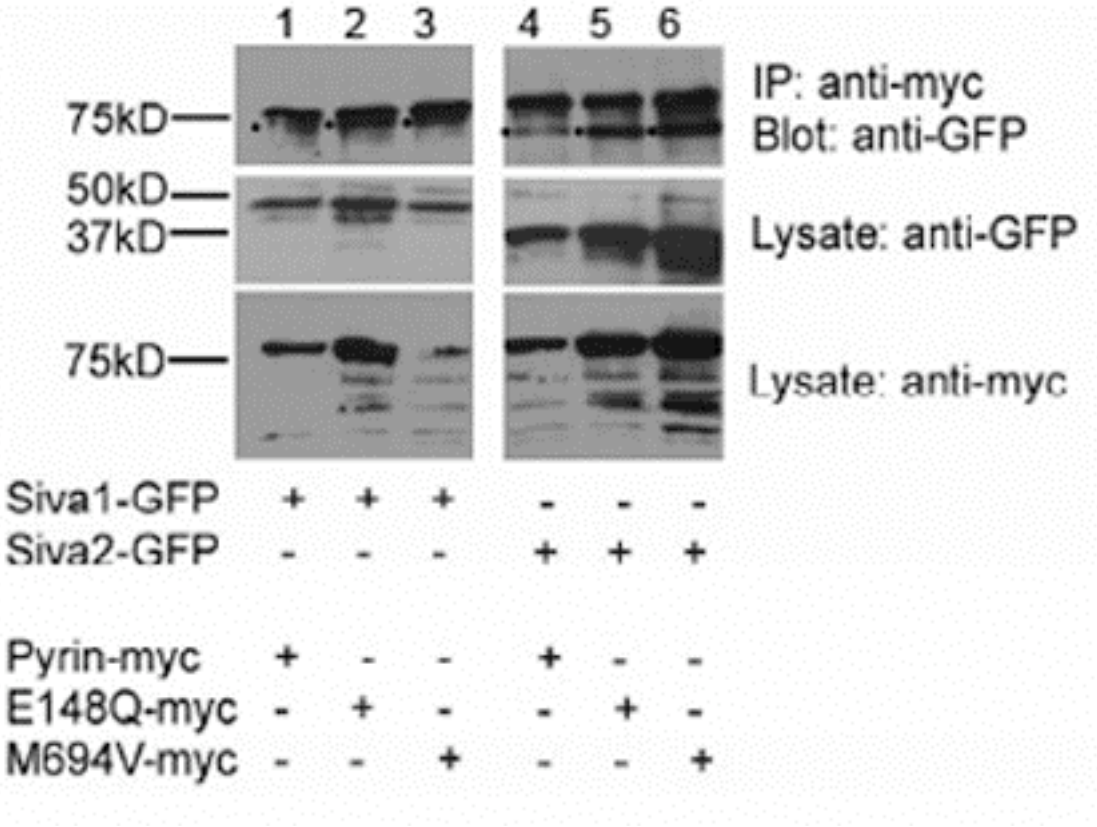
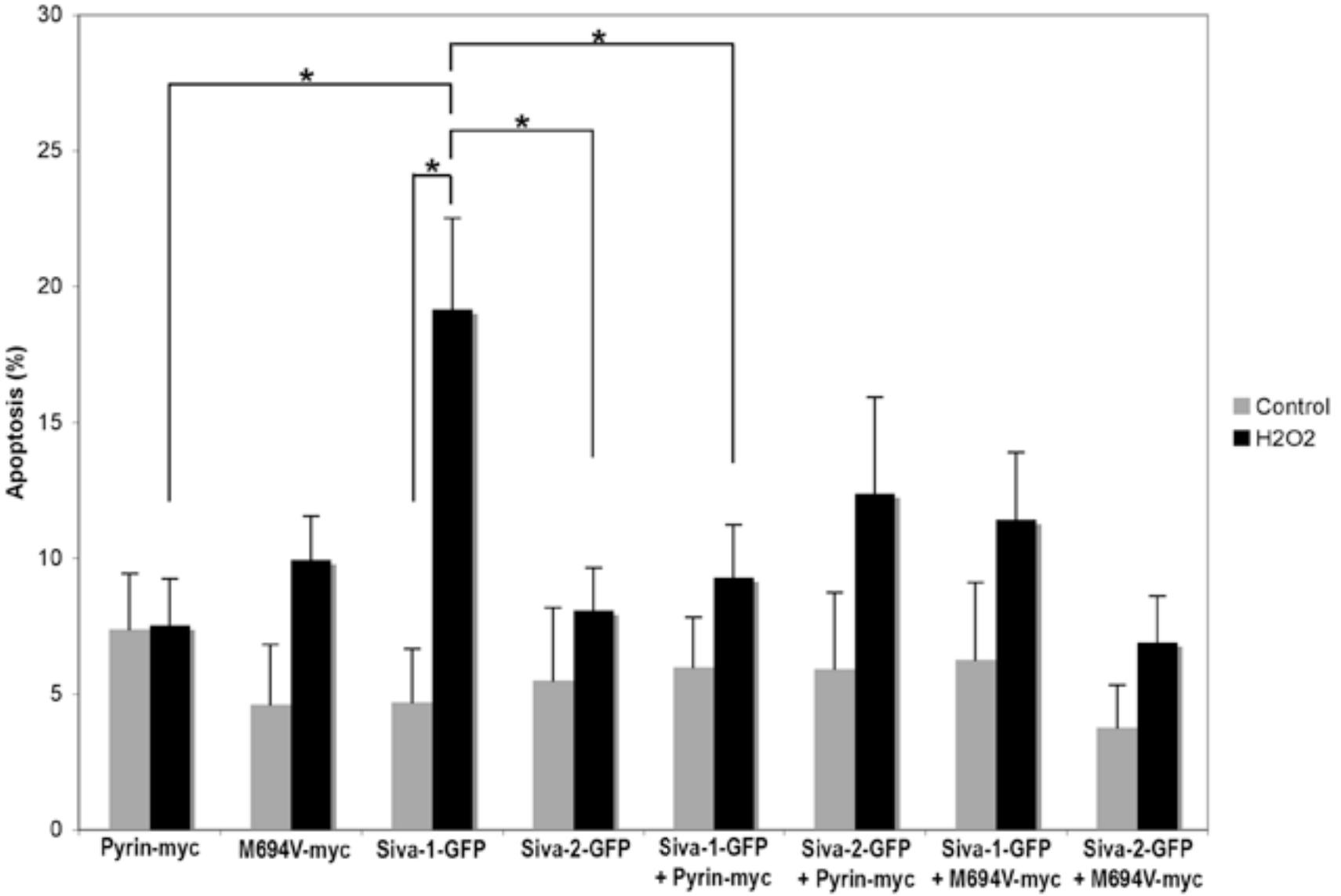


Figure 3.6: Effect of pyrin mutations on the binding of Siva-1 and Siva-2. The FMF-causing mutations E148Q and M694V were tested by immunoprecipitation for their ability to bind to Siva-1 and Siva-2. No appreciable difference was discerned between the binding of wild-type and mutant forms of pyrin to Siva-1 (lanes 1-3) or Siva-2 (lanes 4-6). Dots to the left of the lanes indicate expected sizes of precipitated proteins.

Figure 3.7: Apoptotic response to oxidative stress. COS-7 cells were transfected with GFP-tagged versions of Siva and myc-tagged versions of pyrin, alone and in combination. The cells were then treated with hydrogen peroxide and examined for apoptosis. Siva-1 caused a significant level apoptosis compared with control cells, while Siva-2 did not. When pyrin was co-transfected with Siva-1, the apoptotic activity of Siva was diminished. Asterisks indicate a significant change as determined by a p value of less than 0.05.


30
25
20
15
10
5
0
Apoptosis (%)
*
*
*
*
Control
H2O2
Pyrin-myc
M694V-myc
Siva-1-GFP
Siva-2-GFP
Siva-1-GFP + Pyrin-myc
Siva-2-GFP + Pyrin-myc
Siva-1-GFP + M694V-myc
Siva-2-GFP + M694V-myc

References

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Chapter 4

Membrane associated-PSTPIP1 filaments are remodeled by pyrin[3]

Abstract

PSTPIP1 is a cytoskeleton-associated protein that acts as an adaptor to link PEST-type phosphatases to their substrates. Mutations in PSTPIP1 are responsible for PAPA syndrome (Pyogenic sterile Arthritis, Pyoderma gangrenosum, and Acne), a rare, severe autoinflammatory disease marked by periodic attacks of fever and intense pain and inflammation. The inflammation is marked by a massive influx of neutrophils to the affected tissue, resulting in significant tissue damage. PSTPIP1 binds to pyrin, a protein with links to cytoskeletal signaling, cytokine secretion, and cell death. Interestingly, mutations in pyrin result in familial Mediterranean fever (FMF), another autoinflammatory disorder with a similar, although less severe, clinical presentation. This has led to the hypothesis that PSTPIP1 and pyrin are components of a common inflammatory pathway. The studies outlined here describe several new aspects of PSTPIP1 and pyrin biology. We confirm that PSTPIP1, which has homology to membrane-deforming BAR proteins, self-associates into filaments that are membrane associated; an extended FCH (Fes-Cip4 homology) domain of PSTPIP1 is responsible for its self-aggregation. Organization of PSTPIP1

filaments requires an intact tubulin cytoskeleton. We also demonstrate that pyrin binding alters the caliber and distribution of PSTPIP1 filaments. This requires the coiled coil region of PSTPIP1 and B-box and coiled coil of pyrin. Finally, we demonstrate that, in cells expressing pyrin, PSTPIP1 and ASC (a pyrin-interacting protein that participates in inflammasome and apoptosome signaling), the PAPA syndrome mutations in PSTPIP1 alter the efficiency of pyrin-mediated PSTPIP1 recruitment to the ASC speck-like aggregate.

Introduction

The systemic autoinflammatory disorders include a set of heritable human diseases that are characterized by periodic attacks of fever, pain, and inflammation in the apparent absence of an infectious trigger (Galon et al., 2000). The most common of these diseases, Familial Mediterranean Fever (FMF), is caused by mutations in the *MEFV* locus, which encodes the protein pyrin (1997a; 1997b). Recently, the adaptor protein, PSTPIP1 (proline serine threonine phosphatase-interacting protein 1) was identified as a pyrin-interacting protein (Shoham et al., 2003). This finding is of interest because mutations in the *PSTPIP1* gene result in PAPA Syndrome (Pyogenic sterile Arthritis, Pyoderma gangrenosum, and Acne OMIM #604416 (Wise et al., 2002)), another autoinflammatory disease with severe skin and joint involvement. Mutations in PSTPIP1 increase its binding affinity to pyrin, suggesting that pyrin and PSTPIP1

are functionally linked in an unknown pathway connected to inflammatory signaling (Shoham et al., 2003).

PSTPIP1 is closely related to PSTPIP2 (also called Mayp, for macrophage actin-associated tyrosine-phosphorylated protein (Wu et al., 1998a; Yeung et al., 1998). Interestingly, a locus that is associated with susceptibility to chronic multifocal osteomyelitis, another autoinflammatory disorder that affects bone and occasionally, skin and bowel, has been mapped to human chromosome 18q21.3-22 (Golla et al., 2002), a region that includes the *PSTPIP2* gene. In accord with a potential direct link between PSTPIP2 and inflammatory disease is the recent finding that a missense mutation in the murine *PSTPIP2* gene segregates with the osteomyelitis phenotype in a mouse model for chronic multifocal osteomyelitis (CMO) (Ferguson et al., 2006). Thus, both PSTPIP1 and PSTPIP2 appear to be involved in inflammatory signaling.

PSTPIP1 and PSTPIP2 share an N-terminal Fer-CIP4 homology (FCH) domain and a central coiled coil region, through which they bind to PEST-type phosphatases (Cong et al., 2000; Wu et al., 1998b). PSTPIP1, but not PSTPIP2, also contains a C-terminal SH3 domain that is important for the binding of several PEST phosphatase substrates, including c-abl and WASP (Tian et al., 2000; Wu et al., 1998a). PSTPIP1 is the vertebrate orthologue of the yeast protein Cdc15p, a phosphorylation-regulated modulator of cytoskeletal organization that is required for cytokinesis (Fankhauser et al., 1995). PSTPIP1, PSTPIP2 and

several related proteins (including FBP-17, CIP4, Toca-1, PACSIN/syndactin/FAP52 and NOSTRIN) share not only the FCH domain, but also an extended region downstream of this domain. These shared domains comprise the F-BAR (FCH-BAR) or EFC (extended FCH) class of the BAR domain superfamily (named for Bin-Amphiphysin-Rvs). The BAR domain proteins function to link cellular membranes to the actin cytoskeleton and are involved in endocytosis (Dawson et al., 2006).

Early work suggested that PSTPIP1 homo-dimerizes via its FCH and coiled coil domain (Wu et al., 1998b), although one recent study suggested that the functional unit is a trimer (Yu et al., 2007). The ability of these dimers to further associate into filaments is shared by several members of the F-BAR family. Recently, it was demonstrated that the filaments formed by the PSTPIP1-related protein FBP-17 are tubular membrane-associated structures that appear to represent extended endocytotic vesicles that have not undergone scission (Itoh et al., 2005; Kamioka et al., 2004; Tsujita et al., 2006). Analysis of the structure of the BAR domain homology region (amino acids 1-300) of the F-BAR proteins FBP-17 and CIP4 reveal a five helical structure that forms a banana-shaped dimer with an extended interaction face. Conserved, positively charged residues on the concave face of the dimer bind to phospholipids and are likely important for membrane binding and deformation (Itoh and De Camilli, 2006; Shimada et al., 2007).

In this report, we confirm that cytosolic filamentous structures formed by the PSTPIP1 protein are membrane associated and identify the minimal domains of PSTPIP1 that are required to form such filaments. This analysis indicates that an extended region of the FCH domain is necessary for filament formation, consistent with predictions based on the F-BAR structure. Though PSTPIP1 filaments do not appear to co-localize directly with intermediate filaments or the microtubule or actin cytoskeleton, the integrity of PSTPIP1 filaments depends on an intact microtubular system, a finding that could potentially have implications for the efficacy of colchicine as a treatment for FMF. We also show that the FMF protein pyrin alters the conformation of the filamentous network, and directly test the binding relationships between PSTPIP1, pyrin, and the pyrin-binding protein ASC. The data suggest that the balance of these interactions is dramatically altered by disease-causing mutations in PSTPIP1, but not by FMF-associated mutations in pyrin.

Materials and Methods

Plasmids and antibodies. All FLAG-tagged constructs were cloned into pCMV-Tag2B and myc-tagged constructs were generated using pCMV-Tag3A (both from Stratagene, La Jolla, CA). A QuikChange II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate PSTPIP1 and pyrin mutants. Fluorescent labeled constructs were expressed using pE-YFP and pE-GFP-C2 vectors from Clontech (Mountain View, CA). Anti-myc (rabbit polyclonal) and

anti-FLAG, anti-tubulin, and anti-vimentin (all mouse monoclonal) antibodies were obtained from Sigma (St. Louis, MO). Actin filaments were visualized using AlexaFluor488 Phalloidin from Molecular Probes by Invitrogen (Eugene, OR). Fluorescent-labeled secondary antibodies (AlexaFluor488 goat anti-mouse and AlexaFluor568 goat anti-rabbit) were also obtained from Molecular Probes (Eugene, OR). The PSPIP1 antibody was a kind gift of Dr. Ellis Reinherz (Harvard University).

Cell culture and transfection. COS-7 and HeLa cells were grown in DMEM (Gibco by Invitrogen, Carlsbad, CA) supplemented with 10% FBS (vol/vol). Cells were transfected using FUGENE-6 (Roche Applied Science, Indianapolis, IN). Cells were fixed 24-36 hours after transfection. Transfection rates of 60-70% were observed.

Monocyte and neutrophil isolation. Human blood was collected from healthy volunteers (IRB# 1992-0480) and mixed with an anti-coagulation solution containing 0.14M anhydrous citric acid, 0.20M citric acid trisodium salt, and 0.22M dextrose. Blood was then incubated at room temperature for 30 – 45 minutes after the addition of a solution containing 6% dextran to facilitate sedimentation of red blood cells. The upper, leukocyte-rich layer was then removed and any remaining red blood cells were destroyed by hypotonic lysis in distilled water. Leukocytes were concentrated by centrifugation, then layered onto Ficoll/Paque (Pharmacia, Uppsala, Sweden) density gradient and

centrifuged again. The neutrophil layer was removed, fixed, and stained. The monocyte/lymphocyte layer was removed, and monocytes were enriched by cell adhesion. Non-adherent lymphocytes were washed off 24 hours later, and the adherent monocytes were fixed and stained.

Immunofluorescence. Cells were fixed for 30 minutes in a 4% parafomaldehyde solution in PBS. After permeablization in 0.2% Triton-X in PBS for 10 minutes, cells were exposed to blocking solution (10% goat serum, 1% BSA, and 0.1% Tween-20 in PBS) for 1 hour. After antibody staining, 10mM DAPI was applied to the cells for 1 minute for nuclear visualization. Coverslips were mounted with ProLong Gold Anti-Fade Reagent (Molecular Probes by Invitrogen, Eugene, OR). Slides were visualized using a Nikon E800 fluorescence microscope.

Nocodazole Assays. Twenty-four hours after being transfected with PSTPIP1, HeLa cells were treated with 4.15μM nocodazole and incubated on ice for 10 minutes to depolymerize microtubules. Cells were then rinsed 3 times in media and observed at 37ºC in order to watch microtubules re-form.

Cytochalasin-D Assays. A 2mM stock of cytochalasin D was prepared in chloroform. At the time of experiment this stock solution was diluted in cell culture medium to a working concentration of 10μM. Cells were treated for 30 minutes and then fixed with 4% paraformaldehyde in PBS.

$DiIC_{16}$ Membrane Staining. The $DiIC_{16}$ membrane staining protocol was carried out using a method previously described (Kamioka et al., 2004). Twenty-four hours after COS-7 cells were transfected, cells were washed 3 times with DMEM without phenol red. DiI_{C16} (Invitrogen, Carlsbad, CA) was diluted to a concentration of 1.0mg/mL in DMSO, then further diluted to 1μg/mL in DMEM without phenol red. DiI_{C16} was applied to the cells, and cells were incubated at 37ºC for 10 minutes. After incubation, cells were rinsed 2 times in PBS and fixed for 30 minutes in 2% paraformaldehyde in PBS.

Results

PSTPIP1 is distributed in a filamentous network. It has been previously reported that PSTPIP1 is expressed hematopoetic cells and in the lung (Li et al., 1998; Spencer et al., 1997). To closely examine the sub-cellular localization of natively expressed PSTPIP1, we carried out immunolocalization studies in freshly isolated human blood cells. We verified that PSTPIP1 is highly expressed in the cytoplasm of both monocytes and neutrophils (Figure 4.1). In monocytes, PSTPIP1 is distinctly filamentous with long, straight fibrils extending edge of the cell toward the center (Figure 4.1A). Previous studies have established that PSTPIP1 forms filaments (Tsujita et al., 2006), although it is the first time that filament formation has been confirmed in natively-expressing cells. In

neutrophils, filamentous staining is suggested, but less distinct; PSTPIP1 often appears concentrated on the cell's leading edge (Figure 4.1B).

To further explore the nature of PSTPIP1 filaments, we transfected FLAG-tagged versions of PSTPIP1 into COS-7 cells. We observed that transfected PSTPIP1 also forms filaments (Figure 4.2A); often these elements appear to radiate from a common point near the nucleus and, as in monocytes, these filaments travel all the way to the cell's edge. In several related proteins of the F-BAR family, the EFC region, which includes the FCH domain and the adjacent CC along with the intervening sequence, is required for filament formation (Itoh and De Camilli, 2006; Shimada et al., 2007). We therefore tested whether this region would be important in forming PSTPIP1 fibrils. COS-7 cells were transfected with truncated versions of PSTPIP1-FLAG, either alone or in combination with full-length PSTPIP1-myc. The C-terminal CC-SH3 region (in the absence of the FCH domain) was not able to form filaments (data not shown), and could not bind to filaments formed by full-length PSTPIP1 (Figure 4.2B-C). In contrast, the EFC region encompassing FCH, intervening sequence and CC, could bind to filaments formed by full-length PSTPIP1, but was not able to form fibrils when transfected alone (Figure 4.2D-G). However, with addition of the sequence between the CC and the SH3 domain to the EFC, filaments were observed in the presence as well as the absence of full-length PSTPIP1 (summarized in Figure 4.2H). Thus, while the SH3 domain is dispensable for filament formation, the remainder of the molecule is required.

Both of the known PAPA-causing mutations (A230T and E250Q) are contained within the region of PSTPIP1 that is important for filament formation. Thus, we tested whether these mutations alter the ability of PSTPIP1 to form fibrils. Expression of flag-tagged versions of both mutant forms of PSTPIP1 resulted in filaments that were similar to those formed by wild type PSTPIP1 (Figure 4.2I-J).

PSTPIP1 fibrils are dependent on an intact tubulin cytoskeleton. The organization of PSTPIP1 filaments, with long, straight fibrils that often appear to radiate from a common origin near the nucleus, is reminiscent of the orderliness of the tubulin cytoskeleton. This observation, combined with the fact that the homologous FCH domain of CIP4 interacts with microtubules (Tian et al., 2000), suggested the possibility that the PSTPIP1 pattern may be due to an interaction with tubulin. We used nocodazole as a reversible means of depolymerizing microtubules in transfected cells, and examined filament structure after nocodozole treatment as well as during recovery induced by nocodozole washout. Destruction of the tubulin cytoskeleton by nocodozole eliminated PSTPIP1 filaments (Figure 4.3A, B). However, within one minute post-nocodazole washout, the microtubules began to rebuild, and as the tubulin structure was restored, a coordinated reconstruction of PSTPIP1 filaments ensued (Figure 4.3C, D). Both microtubules and PSTPIP1 filaments were almost completely restored by 30 minutes post-washout (Figure 4.3E-G). While these

data provide evidence that the structure of PSTPIP1 filaments depends on an intact tublin network, other cell structure proteins such as the intermediate filament vimentin also depend on tubulin for its organization within the cell, and may also contribute to the structure of PSTPIP1 filaments. Indeed, co-staining experiments revealed that PSTPIP1 filaments are near, but not directly associated with, vimentin filaments (Figure 4.3H-J).

Previous reports on PSTPIP1 have revealed additional connections with the cytoskeleton, including an association with cortical actin (Wu et al., 1998b). We confirmed that PSTPIP1 is associated with cortical actin (data not shown) and established that it does not co-localize with actin stress fibers (Figure 4.3K-M), We next used cytochalasin-D, a cell-permeable mycotoxin that both disrupts actin filaments and inhibits actin polymerization, to test the role of actin in the formation of PSTPIP1 filaments. Although cytochalasin-D clearly disrupted actin filaments, it did not destroy PSTPIP1 fibrils (Figure 4.3N, O). Thus, though PSTPIP1 is associated with cortical actin, the organization of PSTPIP1 filaments is not dependent upon the structural integrity of the actin cytoskeleton.

Pyrin expression alters PSTPIP1 distribution. Since pyrin is a known PSTPIP1-interacting protein, we examined whether co-expression of pyrin with PSTPIP1 would result in recruitment of pyrin to PSTPIP1 filaments. Indeed, co-transfection of myc-tagged pyrin with FLAG-tagged PSTPIP1 caused the normally diffuse pattern of pyrin to become filamentous and to associate with

PSTPIP1 filaments (Figure 4.4A-B). However, it was also observed that the long, relatively straight fibrils characteristic of PSTPIP1 had become reticularized, indicating that pyrin alters the distribution of PSTPIP1 filaments. De-convolution of these images revealed a network of branching fibrils extending throughout the cytoplasm, with pyrin particularly concentrated at the branching points of the filaments (Figure 4.4C-E). This indicates a role for pyrin in remodeling of the PSTPIP1 architecture.

The binding of PSTPIP1 and pyrin had been previously reported, and the SH3 domain of PSTPIP1 was demonstrated to be necessary but not sufficient for this interaction (Shoham et al., 2003). Additionally, a mutation in the CC region of PSTPIP1, W232A, has been shown to abolish PSTPIP1 binding both to pyrin (Shoham et al., 2003) and to PTP-HSCF (Wu et al., 1998a). We therefore examined the consequences of both removal of the SH3 domain and the W232A mutation on pyrin's recruitment to and reticularization of PSTPIP1 fialments. Figure 4.5A-C shows that the fibrils formed by PSTPIP1 lacking the SH3 domain effectively recruited pyrin, and were also reticularized by pyrin in a pattern similar to that seen with full-length PSTPIP1. However, the W232A mutation abolished both pyrin binding and consequently, reticularization (Figure 4.5D-F), indicating that reticularization of PSTPIP1 is a direct consequence of pyrin binding. We conclude that the SH3 domain is dispensible for pyrin binding.

We next examined smaller fragments of the pyrin molecule to determine which regions are required for PSTPIP1 binding and filament reticularization. Protein fragments encoded by exons 2-3 (Figure 4.6A-B) as well as exons 4-5 (Figure 4.6C-D) of pyrin did not bind to PSTPIP1 filaments. Pyrin exons 2-4 produced a protein capable of aligning with PSTPIP1 fibrils, but the PSTPIP1 filament network was not reticularized (Figure 4.6E-F). Only when the entire B-box/Coiled-coil region of pyrin was expressed (exons 3-5, Figure 4.6G-I) with PSTPIP1 did we observe a pattern similar to that of full-length pyrin expression, with pyrin localized to reticularized PSTPIP1 filaments. Thus, while the B-box of pyrin is sufficient for PSTPIP1 binding, the B-Box and Coiled-coil region together are required for altering PSTPIP1 filament pattern.

Mutations in pyrin or PSTPIP1 do not alter the pattern of PSTPIP1 filaments. It has been proposed that pyrin and PSTPIP1 are functionally linked in an unknown pathway that is connected to the innate immune system (Shoham et al., 2003). Thus, we tested whether FMF-causing mutations in pyrin would modulate the interaction between these two proteins or alter the distribution of PSTPIP1 filaments. Myc-tagged versions of pyrin mutants were co-transfected with FLAG-tagged PSTPIP1 in COS-7 cells. The first pyrin mutant that was tested was P369S, as this mutation is located in exon 3, one of the exons critical for PSTPIP1 binding. Despite the location of this mutation, P369S bound to PSTPIP1 and reticularized PSTPIP1 filaments in a manner similar to that of wildtype pyrin (Figure 4.7A-C). We then tested several other FMF-causing

mutations in other exons of pyrin, including E148Q (Figure 4.7D-F), M694V (Figure 4.7G-I), M860I (Figure 4.7J-L), V726A (Figure 4.7M-O), and A744S (Figure 4.7P-R). In all cases, the distribution of PSTPIP1 in the presence of mutant pyrin was similar to that seen with wildtype pyrin.

To explore the effect of PAPA mutations on the PSTPIP1:pyrin interaction, we next tested whether the filaments formed by mutant PSTPIP1 could be reticularized by pyrin. We found that both PAPA-causing mutations, A230T (Figure 4.7S-U) and E250Q (Figure 4.7V-X), were reticularized by pyrin in a manner similar to that of wildtype PSTPIP1. We therefore conclude that disease-causing mutations in either of these proteins do not alter the reticular pattern seen with expression of the wildtype proteins, though alterations in the function of the filament system cannot be ruled out.

PSTPIP1 filaments are associated with plasma membrane. PSTPIP1 is highly related structurally to several filament-forming proteins known as F-BAR proteins; these proteins contain an N-terminal FCH domain, a central coiled-coil, and a C-terminal SH3 domain. The region that contains the FCH, CC domains and intervening sequence has weak homology to a BAR domain, a domain that can bind and bend cellular membranes (Shimada et al., 2007). FBP-17 and CIP4, both F-BAR proteins, have been crystallized (Shimada et al, 2007; Frost et al., 2008). Both proteins form dimers with gently curved surfaces. Conserved, positively charged amino acid residues on the convex surface of the dimer are attracted to negatively-charged plasma membrane, and alter membrane shape to

cause tubular invaginations (shimada). In fact, the cellular filaments formed with FBP-17 stain with lipophilic dyes, indicating that they are membrane associated (Kamioka et al., 2004). The FBP-17 filaments are similar in appearance to the PSTPIP1 filaments shown here.

To determine whether the PSTPIP1 filaments that we detect in COS-7 cells are also membrane-associated, we transfected COS-7 cells with GFP-tagged PSTPIP1 and then exposed the cells to fluorescently labeled DiI_{C16}, a lipophilic dye that associates with cellular membranes. PSTPIP1 filaments were bound by DiI_{C16} (Figure 4.8A-C), indicating that PSTPIP1 fibrils are indeed membrane-associated. We next tested whether all or part of the reticular filaments produced by pyrin/PSTPIP1 co-transfection are also lipophilic. Figure 4.8D-F shows that found that DiI_{C16} staining overlapped directly with pyrin in the reticular pattern. Furthermore, mutations in pyrin (Figure 4.8G-I) or in PSTPIP1 (Figure 4.8J-L) did not affect the co-localization of filaments and DiI_{C16} staining. Taken together, these data indicate that PSTPIP1 filaments are membrane-associated, suggesting that PSTPIP1 shares the membrane-altering properties of several other EFC domain-containing proteins. Indeed, Tsujita et al. demonstrated that both PSTPIP1 and PSTPIP2, like the related F-BAR proteins, bind strongly to PI(4,5)P2 as well as to lipids extracted from brain (Tsujita et al., 2006).

Cellular interactions of PSTPIP1, ASC and pyrin; effect of mutations in PSTPIP1 and pyrin. Both of the known PAPA syndrome mutations in PSTPIP1, A230T and E250Q, are located within the coiled-coil domain and result in hyperphosphorylation by c-abl kinase (Shoham et al., 2003). It has previously been reported that hyperphosphorylation of PSTPIP1 increases the affinity of pyrin:PSTPIP1 binding (Shoham et al., 2003). Here, we use a recently described protein:protein interaction assay known as the ASC speck assay (Balci-Peynircioglu et al., 2008) to test the strength of the pyrin:PSTPIP1 interaction. The apoptotic speck protein (ASC) contains an N-terminal pyrin domain (PyD) and a C-terminal Caspase recruitment domain (CARD). ASC is normally distributed throughout the cytoplasm and nucleus (Figure 4.9A), but will spontaneously form a large perinuclear aggregate known as a "speck" (Figure 4.9B). Through its PyD, ASC binds to the PyD of pyrin and recruits it to the speck (Figure 4.9C, D). Any protein that binds to pyrin through regions other than the PyD (such as PSTPIP1 which binds to pyrin's B-box and Coiled-coil domain), could then also be recruited to the speck.

In cells co-transfected with PSTPIP1-FLAG and ASC-myc, ASC specks were formed, but PSTPIP1 was not recruited to the speck (Figure 4.9E-G). However, triple co-transfections of ASC (untagged), PSTPIP1-FLAG and pyrin-myc resulted in recruitment of PSTPIP1 to the speck in approximately 70% of transfected cells (Figure 4.9H-J). In the remaining 30% of cases, pyrin was either found in both speck and filaments (Figure 4.9K-M), or exclusively in the

speck (Figure 4.9N-P). In no case did we see pyrin binding to PSTPIP1 filaments without localizing to the speck. Two explanations are possible. In the first scenario, pyrin binds preferentially to ASC over PSTPIP1 and pyrin is able to recruit PSTPIP1 to the speck compartment. In the second, PSTPIP1 promotes the delivery of pyrin to ASC specks, but PSTPIP1 itself does not remain in the speck compartment. This second explanation is consistent with a previous model of ASC/pyrin/PSTPIP1 interaction, which posits that autoinhibited pyrin molecules require unfolding by PSTPIP1 in order to interact with ASC (Yu et al., 2007). If this is the case, then pyrin should not be recruited to ASC specks if it is prevented from interacting with PSTPIP1. Therefore, we transected cells with the FLAG-tagged W232A PSTPIP1 mutant that does not bind to pyrin, along with pyrin and ASC. In this case, pyrin bound readily to ASC specks, but PSTPIP1 was not recruited to the speck (Figure 4.9Q-S). Together, these data establish that pyrin interacts more readily with ASC than with PSTPIP1 and that recruitment of PSTPIP1 to the pyrin-coated ASC speck is pyrin-dependent.

When PAPA syndrome-causing mutants of PSTPIP1 were transfected with ASC and pyrin, PSTPIP1 appeared in the speck with pyrin 95% of the time (A230T Figure 4.9T-V; E250Q Figure 4.9W-Y). This verifies earlier reports that PSTPIP1 mutants bind to pyrin with higher affinity, and confirms that PSTPIP1 recruitment to the speck compartment depends on the PSTPIP1-pyrin interaction. This finding also validates the ASC speck assay as a sensitive method of detecting differences protein:protein interaction affinity. In contrast,

the use of mutant forms of pyrin did not alter the frequency of recruitment of PSTPIP1 to the ASC speck (data not shown), though as shown in earlier studies, mutant pyrin did increase the rate of speck formation (Balci-Peynircioglu et al., 2008; Richards et al., 2001).

Discussion

Both PSTPIP1 and pyrin are linked to autoinflammatory diseases (1997a; 1997b; Wise et al., 2002). The interaction between these proteins, and the previous finding that mutations in PSTPIP1 increase its binding affinity for pyrin led to the speculation that these two proteins function in the same inflammatory pathway (Shoham et al., 2003). Yu et al. (2007) suggested a model for this pathway. These authors provided evidence that both pyrin and PSTPIP1 are homotrimers in their basal state and that an internal interaction of pyrin's B-box and coiled-coil with its own PyD results in auto-inhibition of the pyrin trimer (Yu et al., 2007). The auto-inhibited state prevents pyrin's interaction with ASC (which occurs via the PyD of both proteins) thereby prevents ASC and Caspase-1 induced pyroptosis (Yu et al., 2007). According to this model, the binding of pyrin to PSTPIP1 is required to release this autoinhibition, freeing pyrin to bind to ASC and participate in inflammatory signaling. The greater affinity of the PAPA-associated mutants for pyrin was posited to release pyrin autoinhibition more efficiently, resulting in increased inflammatory signaling through ASC (Yu et al., 2007).

This sequestration model provides a potential explanation for the inflammatory episodes seen in PAPA syndrome. However, our co-transfection studies suggest that pyrin efficiently finds the speck compartment even in the absence of an interaction with PSTPIP1. It appears from our data as if pyrin is the molecule that recruits PSTPIP1 to ASC inflammasomes or pyroptosomes. The exact functional consequence of this recruitment is not clear, but the PAPA-associated mutations clearly increase the distribution of PSTPIP1 to the speck compartment by virtue of their increased binding to pyrin.

Our studies also confirm and extend previous findings that PSTPIP1 filaments are membrane-associated. Both PSTPIP1 and PSTPIP2 resemble the F-BAR proteins, FBP-17 and CIP4 (Shimada et al., 2007). These proteins can bind and tubulate phospholipid membranes; they function to couple membrane deformation to actin cytoskeletal polymerization during endocytosis (Tsujita et al., 2006). The crystal structures of these proteins reveal extended dimerization faces that are promoted by several conserved amino acid residues; they are therefore predicted to be stably and constitutively dimerized (Shimada et al., 2007). Mutation of these residues prevents dimer formation and reduces protein stability (Shimada et al., 2007). Alignment of the extended FCH domains of PSTPIP1 and PSTPIP2 with those of FBP-17 and CIP4 reveal a high degree of conservation. Indeed, all of the previously identified amino acids that function in dimerization are also present in the two PSTPIP proteins (Shimada et al; Frost et

al). In addition, the positively charged patches that have been shown to bind to phospholipids are also conserved (Shimada et al., 2007; Frost et al., 2008). Indeed, we show that PSTPIP1 filaments, both in the presence and absence of pyrin, attract $DiIC_{16}$ indicating that PSTPIP1 filaments are membrane lipid-associated.

PSTPIP1 has previously been linked to the actin cytoskeleton (Wu et al., 1998b), and here we report additional links to both the tubulin cytoskeleton and to the plasma membrane. Our data indicate that the integrity of PSTPIP1 filaments is dependent upon an intact tubulin cytoskeleton. While binding of PSTPIP1 to microtubules was not directly tested, the FCH domain of the related protein, CIP4, interacts with microtubules (Tian et al., 2000). The PSTPIP1 filament structure is rapidly regenerated as the microtubular structure is re-built following nocodozole treatment and washout. This suggests that the microtubular network might provide a stabilizing or nucleating base for the assembly of PSTPIP1 filaments.

Like other F-BAR proteins, PSTPIP1 is connected in a dynamic way to both the cytoskeleton and to the plasma membrane. Pyrin's association with PSTPIP1 means that both of these molecules have the potential to modulate a number of processes that involve the reorganization of these cellular compartments, including: cell migration, endocytosis and signaling via the immunological synapse. All of these processes have important implications for

PAPA syndrome and for FMF. For example, both diseases involve a robust mobilization of neutrophils in response to some unknown inflammatory stimulus. Our recent studies have shown that pyrin localizes to sites of active actin polymerization, including the leading edge of migrating monocytes (Waite, et al., manuscript submitted). Interestingly, our studies of native cells indicate that PSTPIP1 has a predilection for the leading edge of human neutrophils (Figure 4.1).

A functional link between migration and PSTPIP1 could potentially involve the molecular coupling mechanism between the proteins p130Cas, a PTP-PEST binding protein (Angers-Loustau et al., 1999), and Crk. This molecular switch regulates a cell's decision between migration and apoptosis in an ARG kinase-dependent manner (Bouton et al., 2001); ARG binds to PSTPIP1 via the SH3 domain and is a PEST phosphatase substrate (Kain and Klemke, 2001). Mutations in PSTPIP1 might push the balance in the direction of migration, leading to the robust migratory response and the delay in apoptosis seen in PAPA attacks. Additionally, recent work has shown that blocking the action of WASP, a cytoskeletal-associated protein that also binds the PSTPIP1 SH3 domain, prevents monocyte chemotaxis (Tsuboi, 2006). Although the primary cell involved in PAPA attacks is the neutrophil, PSTPIP1 may also exert control over cell migration through this pathway.

Another process that involves extensive cytoskeletal and plasma membrane remodeling is the formation of the structure known as the

immunological synapse, a region of increased actin polymerization at the interface of a T-cell and an antigen-presenting cell (APC). PSTPIP1 was originally identified in humans as a binding partner of the T-cell protein CD2 (Li et al., 1998). CD2 is a cell-surface protein that binds to CD58 on APCs, and leads to the formation of the immunological synapse. Within the T-cell, PSTPIP1 is able to simultaneously bind CD2 as well as CD2AP and WASP, two proteins that are critical in the formation of the synapse (Badour et al., 2003). It has been proposed that PSTPIP1 may serve to modulate the activity of these three proteins, and thus to control the formation, and perhaps the longevity, of the immunological synapse (Badour et al., 2003; Li et al., 1998). Whether this is also true in myeloid cells has not yet been demonstrated, but our finding that filamentous PSTPIP1 is present in myeloid cells and that the filamentous structures are associated with the plasma membrane in both monocytes and neutrophils (Figure 4.1), suggests a possible functional connection.

Cytoskeletal and membrane function are also tightly linked in the context of endocytosis. Both clathrin dependent and clathrin-independent endocytosis require actin polymerization, mediated by WASP, a binding partner of PSTPIP1 (Dawson et al., 2006; McGavin et al., 2001). A related process that is highly relevant to myeloid cells is phagocytosis, an activity that involves extensive cytoskeletal and membrane reorganization (reviewed in (Yeung and Grinstein, 2007). While the phagosome itself is actin-based, the initiation of its formation depends on membrane remodeling that results in a clustering of negatively-

charged phospholipids. These negative charges serve to recruit key signaling molecules that are required for phagosome formation. Recent findings have pointed to the PSTPIP1-associated protein WASP as a key regulator of the initial formation of the phagocytic cup in macrophages (Tsuboi and Meerloo, 2007). Preliminary observations in our laboratory suggest that overexpression of PSTPIP1 inhibits phagocytosis, although the underlying mechanism is unclear. One potential explanation may lie in the limitations PSTPIP1 dimers would place on the diameter of plasma membrane deformation. The curvature of the ECH domain of PSTPIP1 could restrict the size of the membrane invaginations to a size much smaller than the phagosome. Alternatively, the tubulated membrane structures induced by PSTPIP1 may stiffen the cell. Indeed, polymerized F-BAR domains (such as the one found in PSTPIP1) are predicted to be much more rigid than those formed by other BAR-like domain proteins such as amphiphysin (Frost et al., 2008).

We show here that while pyrin is not necessary for the formation of PSTPIP1 filaments, co-expression of the two proteins results in remodeling of PSTPIP1 fibrils so that the filaments become branched and reticulated. PSTPIP1 forms filaments through its FCH and Coiled-coil domains, and binds to pyrin (and PEST phosphatases) via the same regions. Indeed, an amino acid change at 232 abolishes pyrin binding. Our data indicate that the SH3 domain of PSTPIP1 is dispensable for pyrin binding to PSTPIP1 filaments , in contrast to an earlier study (Shoham et al., 2003). Neither the conformation nor the

stochiometry of pyrin binding to PSTPIP1 are clear, but it is interesting that pyrin seems to accumulate at branch points that it induces in the PSTPIP1 filament structure. In addition, pyrin binding has the potential to alter the curvature of the PSTPIP1 dimer, and therefore to change the diameter of the filaments. This may explain the observation that fibrils in the reticular pattern are often thicker than those made up of PSTPIP1 alone. The functional consequences of both filament reticulation and changes in filament diameter are important subjects for future study.

[3] This manuscript is in preparation for submission to the *Journal of Cell Science* with the following authors: Andrea L. Waite, Philip Schaner (co-first author), Neil Richards, Banu Balci-Peynircioglu, Michelle Fox, Arthur Hong, and Deborah L. Gumucio

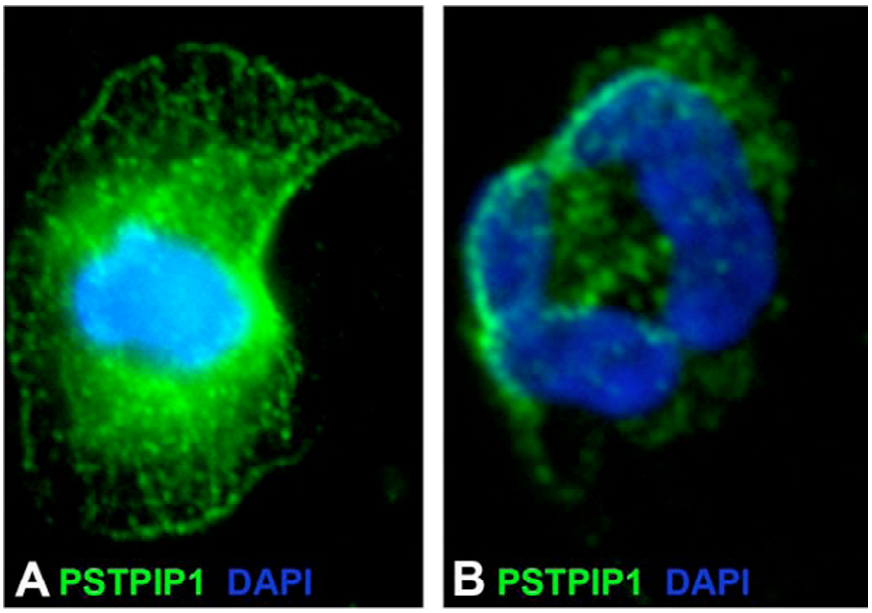


Figure 4.1: PSTPIP1 expression in native cells. (A) In human monocytes, PSTPIP1 is distributed in filaments extending throughout the cytoplasm from the edge of the cell to the center. (B) PSTPIP1 is also expressed in human neutrophils, the distribution is filamentous with a slight concentration at the edge of the cell. Photographs taken at 600X by Philip Schaner.

Figure 4.2: PSTPIP1 distribution in transfected cells. (A) PSTPIP1-FLAG expression in COS-7 cells. Straight filaments radiate from near the nucleus to the cell periphery. (B-J) Portions of PSTPIP1 (all tagged with myc) were transfected alone or in combination with full-length PSTPIP1-FLAG to determine the regions of the protein responsible for filament formation. B-C) A PSTPIP truncation containing the coiled-coil and SH3 region of PSTPIP1 was not able to form filaments, nor was it able to bind to filaments formed by full-length PSTPIP1. (D-G) The FCH and coiled-coil portion of PSTPIP1 bound filaments formed by full-length PSTPIP1 (D-F), but was not able to form filaments when transfected alone (G). (H) Summary of PSTPIP1 mutants tested. The FCH, coiled-coil, and surrounding regions (the EFC domain) are critical for filament formation, but the SH3 domain is dispensable. (I,J) The two PAPA-associated mutants, A230T (I) and E250Q (J) formed filaments similar to those of wildtype PSTPIP1. Panel A was photographed at 400X and panels B-G were photographed at 600X by Philip Schaner. Panels I-J photographed at 600X by Andrea Waite.

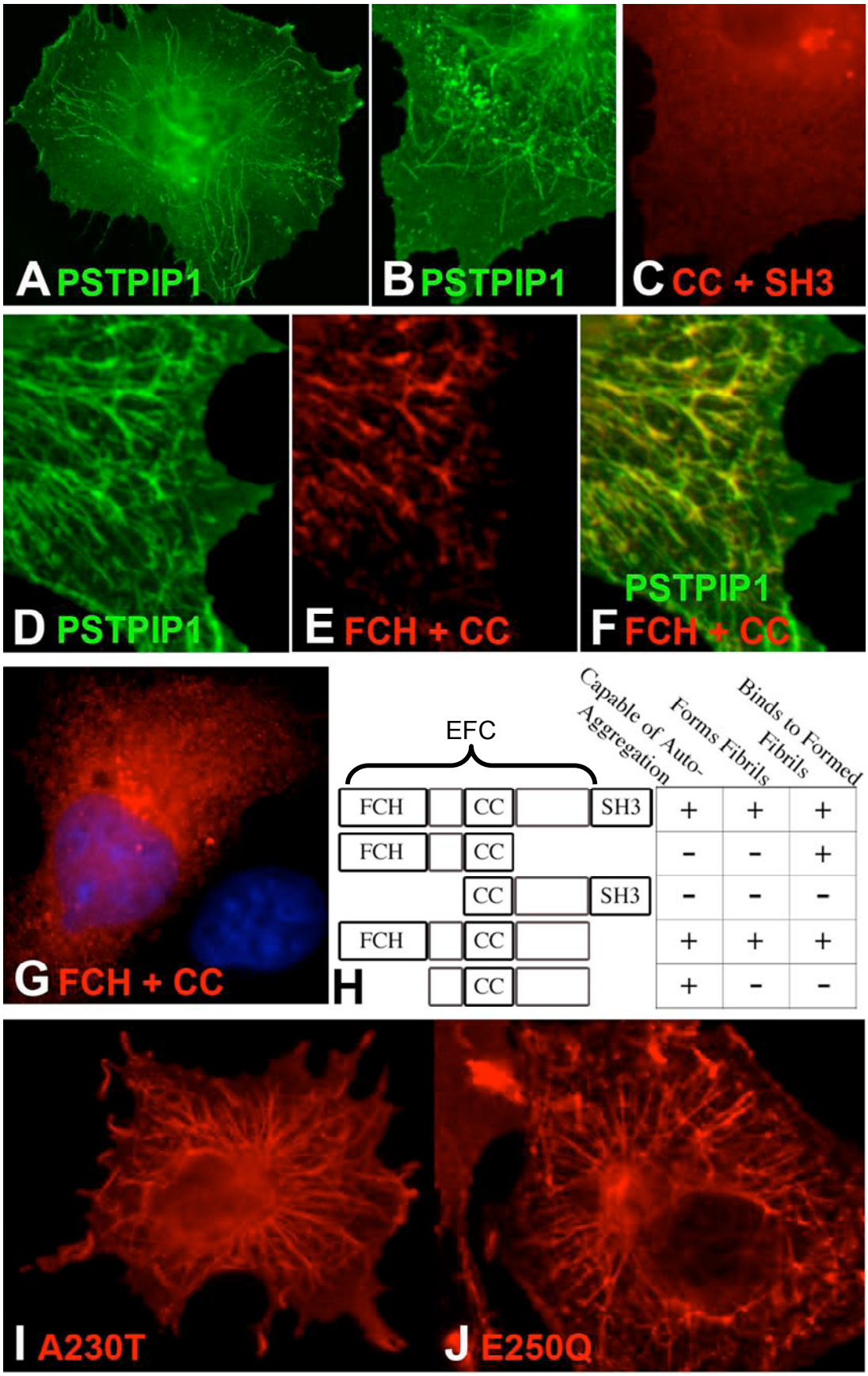
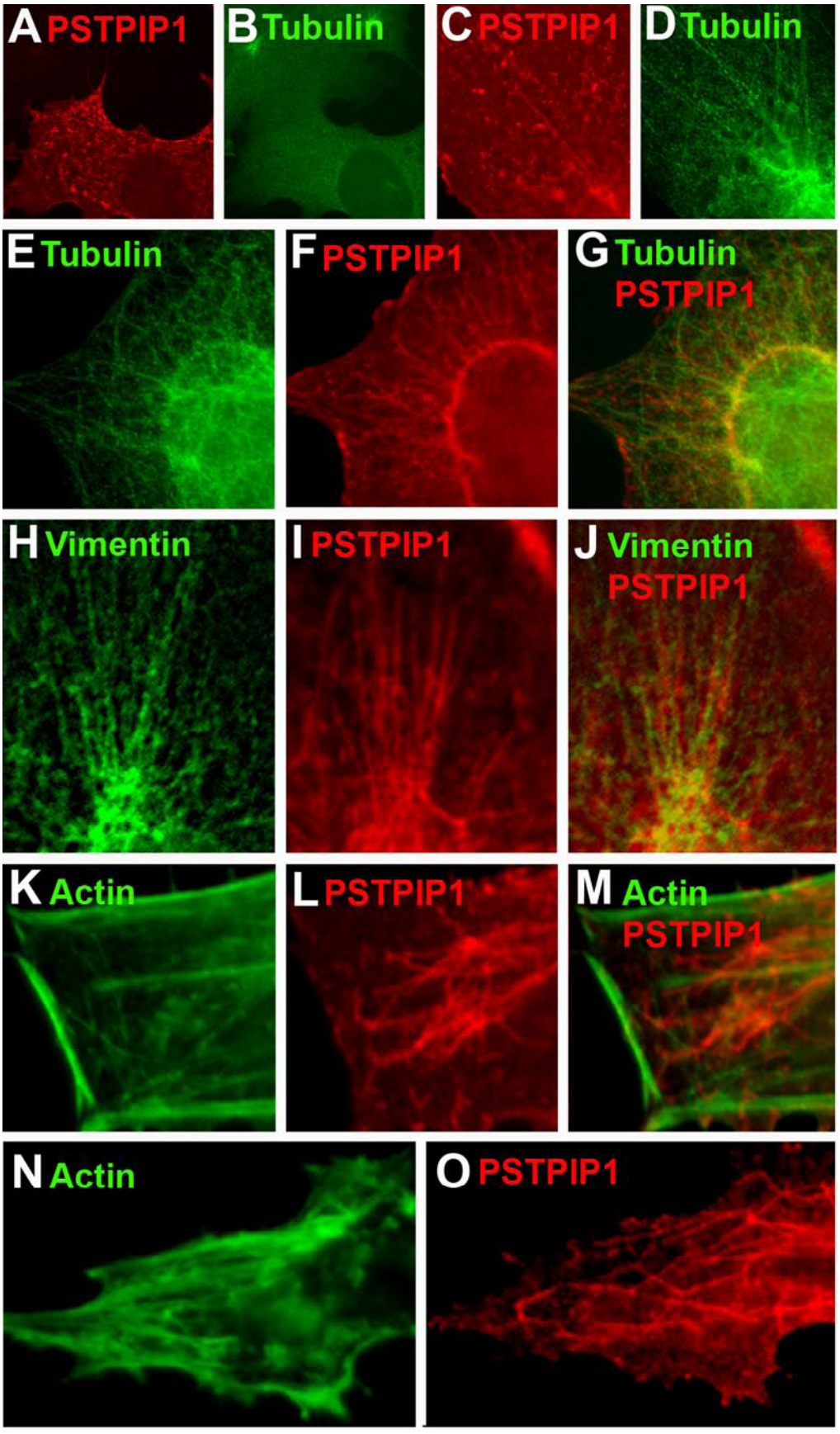

A PSTPIP1
B PSTPIP1
C CC + SH3
D PSTPIP1
E FCH + CC
PSTPIP1
F FCH + CC
G FCH + CC
EFC
Capable of Auto-Aggregation
Forms Fibrils
Binds to Formed Fibrils
FCH
CC
SH3
H
I A230T
J E250Q

Figure 4.3: PSTPIP1's interaction with the cytoskeleton. (A,B) Immediately after nocodazole treatment, microtubules were destroyed, as were PSTPIP1 filaments. (C-D) Within one minute of nocodazole removal, microtubules began to rebuild, and PSTPIP1 filaments began to organize. (E-G) Within 30 minutes of nocodazole removal, both microtubules and PSTPIP1 filaments were reconstituted. (H-J) PSTPIP1 fibrils aligned next to vimentin filaments, but did not directly overlap these filaments. (K-M) PSTPIP1 filaments did not co-localize with actin stress fibers as visualized by phalloidin staining (green). (N, O) In cells treated with cytochalasin-D to disrupt actin integrity, PSTPIP1 fibrils were still visible. Photographs taken at 600X by Philip Schaner.


A PSTPIP1
B Tubulin
C PSTPIP1
D Tubulin
E Tubulin
F PSTPIP1
G Tubulin
PSTPIP1
H Vimentin
I PSTPIP1
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PSTPIP1
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O PSTPIP1

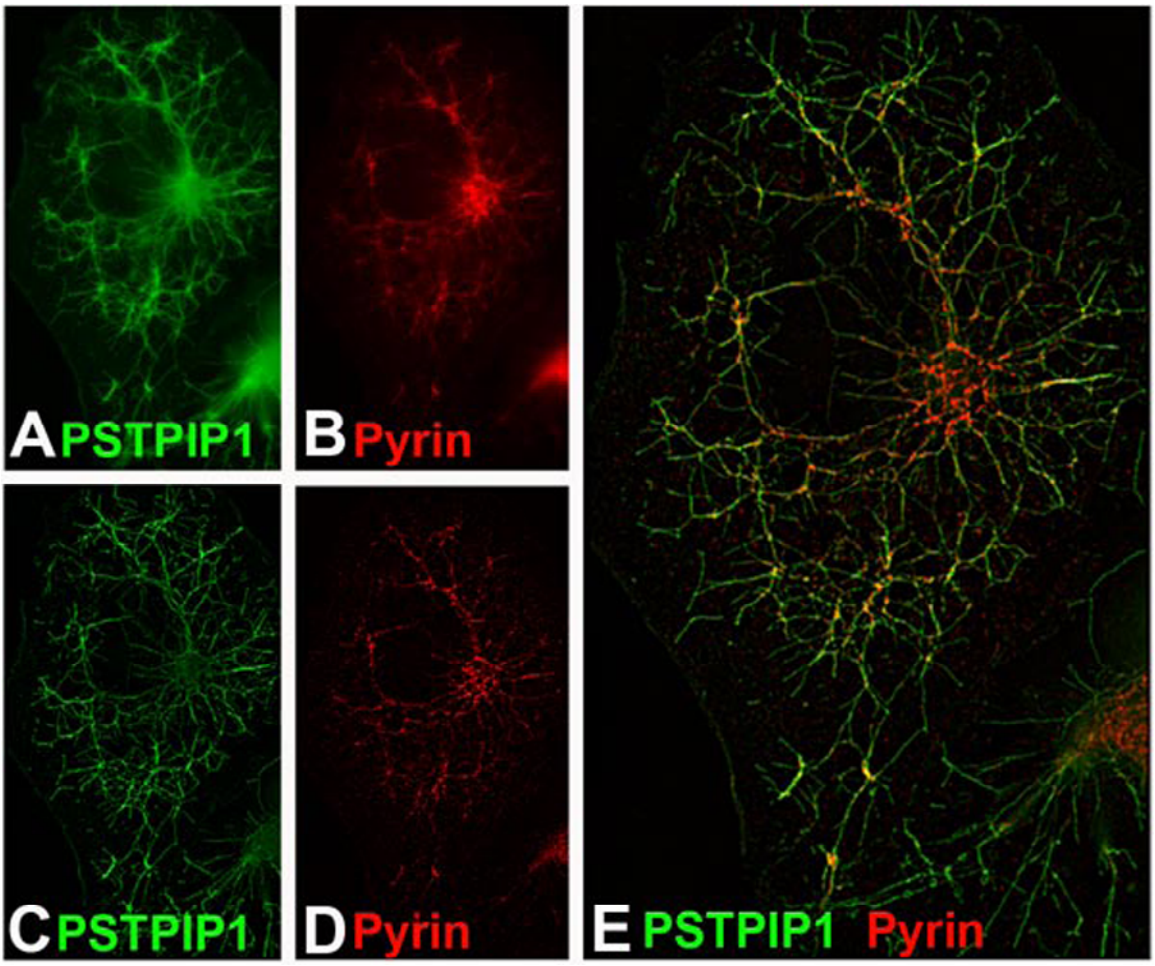


Figure 4.4: Co-expression of pyrin alters the distribution of PSTPIP1 in transfected cells. (A-B) In cells co-transfected with myc-tagged pyrin and FLAG-tagged PSTPIP1, PSTPIP1 filaments were branched and reticulated, and pyrin co-localized with these filaments (compare to Figure 4.2A). (C-E) Deconvolution of these images revealed an increase in the number of filament branching points; pyrin was concentrated at these nodes. Photographs taken at 600X by Philip Schaner.

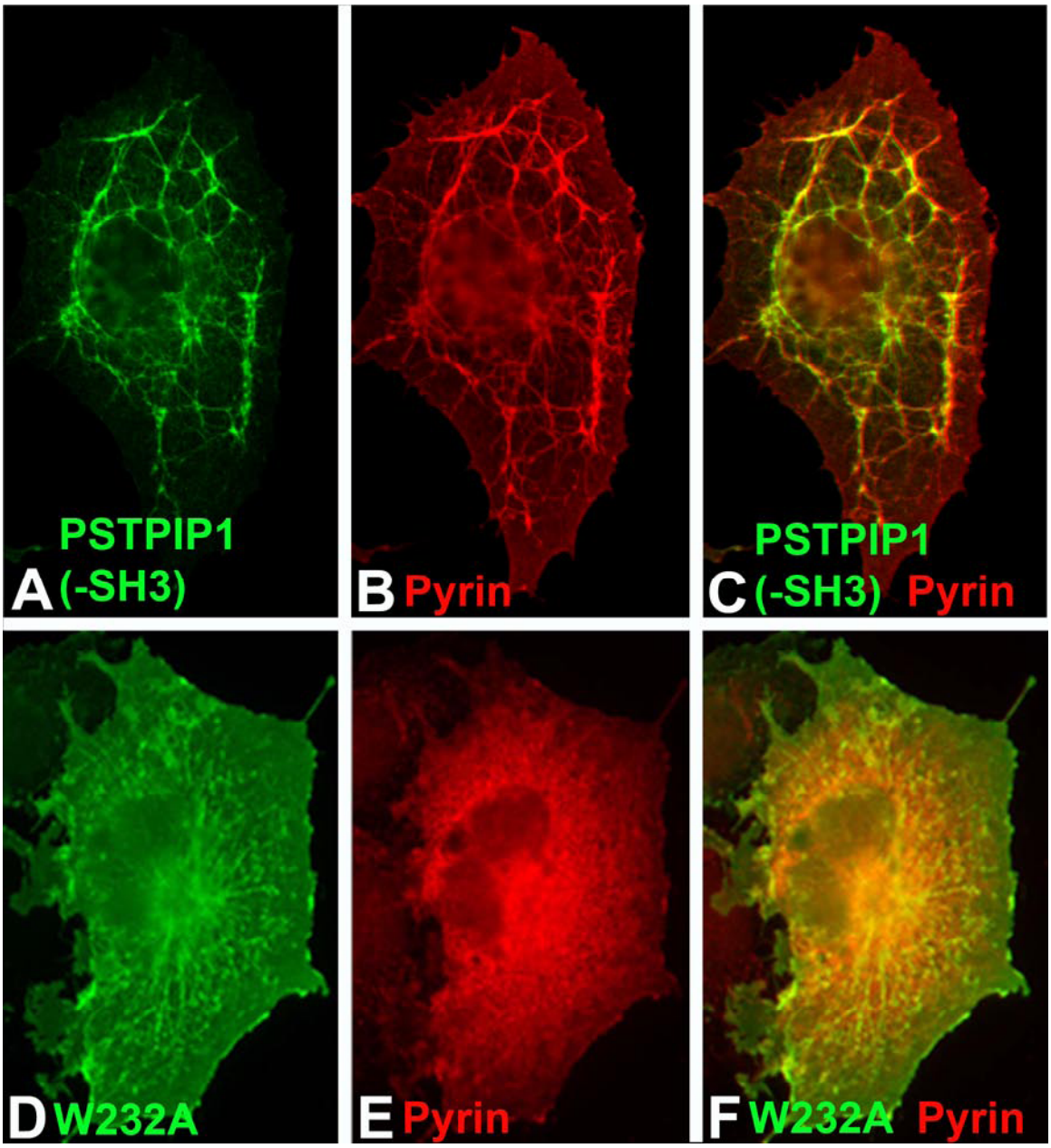


Figure 4.5: The coiled-coil of PSTPIP1 is critical for pyrin binding. (A-C) FLAG-tagged PSTPIP1 lacking the SH3 domain (PSTPIP1(-SH3)) was able to recruit myc-tagged pyrin to filaments, and was reticularized in a manner similar to that of the full-length protein. (D-F) The W232A mutation of PSTPIP1 formed filaments (green), but pyrin (red) did not decorate or reticularize these filaments. Panels A-C photographed at 600X by Philip Schaner. Panels D-F photographed at 400X by Neil Richards.

Figure 4.6: The B-box/coiled-coil region of pyrin is required for reticularization of PSTPIP1 fillaments. Portions of pyrin's B-box and coiled-coil region (all myc tagged), were co-transfected with PSTPIP1-FLAG. (A-B) Pyrin exons 2-3 or (C-D) exons 4-5 did not bind or reticulate PSTPIP1 filaments. (E-F) Pyrin exons 2-4 bound to PSTPIP1 filaments, but did not alter their distribution. (G-I) The B-box and coiled-coil region of pyrin, encoded by exons 3-5, both bound to and remodeled PSTPIP1 filaments. Photographs taken at 600X by Philip Schaner.

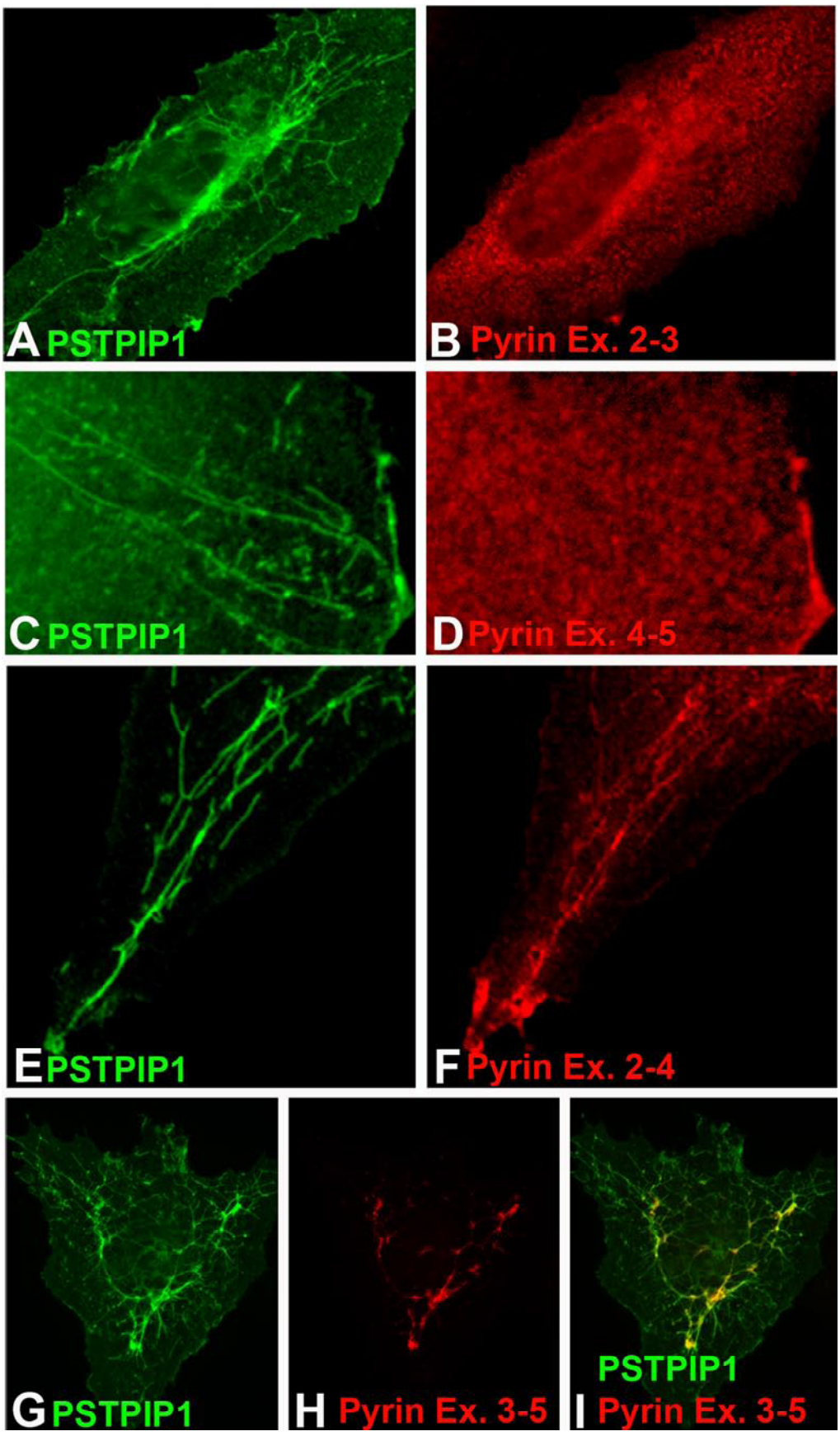
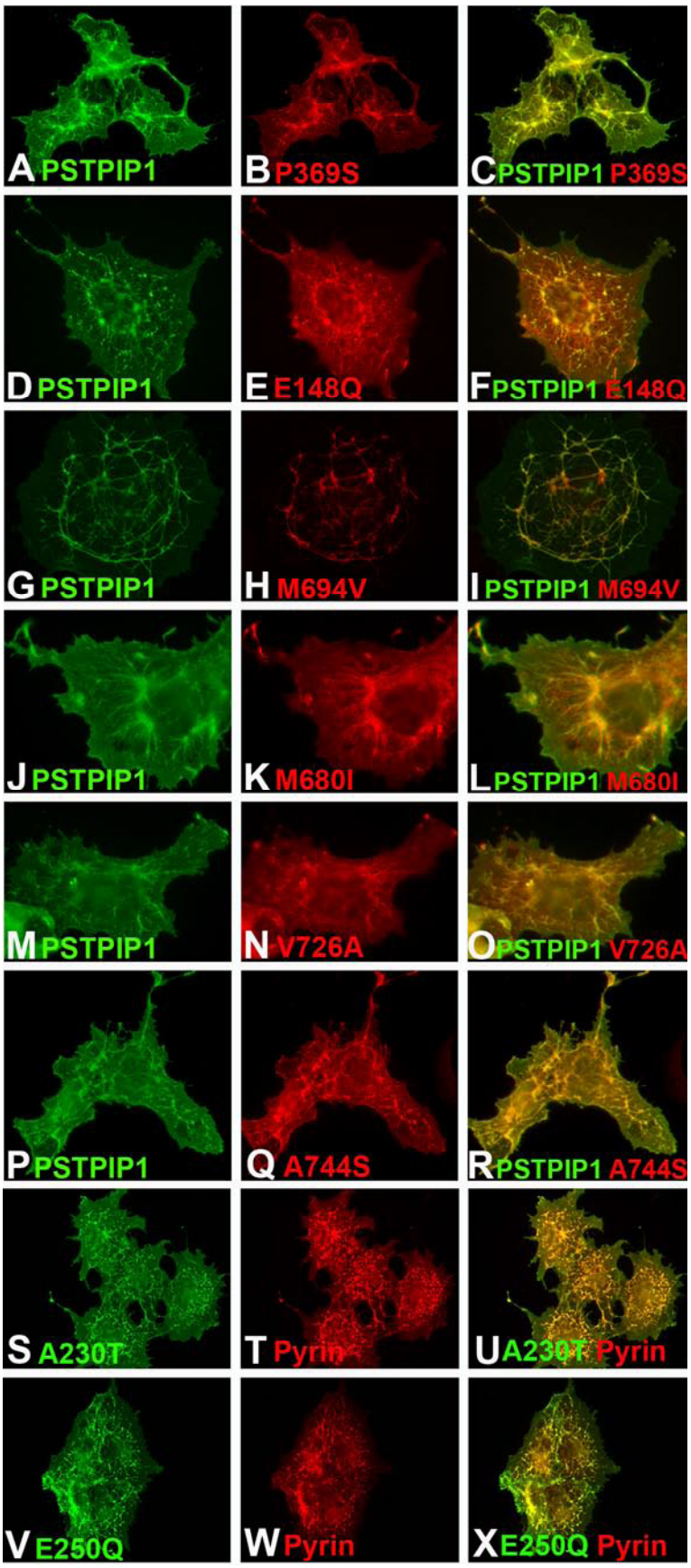

A PSTPIP1
B Pyrin Ex. 2-3
C PSTPIP1
D Pyrin Ex. 4-5
E PSTPIP1
F Pyrin Ex. 2-4
G PSTPIP1
H Pyrin Ex. 3-5
PSTPIP1
I Pyrin Ex. 3-5

Figure 4.7: FMF-causing mutations do not alter the appearance of reticular PSTPIP1 fibrils. (A-R) Myc-tagged versions of mutant pyrin were co-transfected with PSTPIP1-FLAG: (A-C) P369S; (D-F); E148Q; (G-I) M694V; (J-L) M680I; (M-O) V726A; (P-R) A744S. (S-X) PAPA syndrome-associated PSTPIP1 were co-transfected myc-pyrin: (S-U) A230T; (V-X) E250Q. Photographs taken at 600X.


A PSTPIP1
B P369S
C PSTPIP1 P369S
D PSTPIP1
E E148Q
F PSTPIP1 E148Q
G PSTPIP1
H M694V
I PSTPIP1 M694V
J PSTPIP1
K M680I
L PSTPIP1 M680I
M PSTPIP1
N V726A
O PSTPIP1 V726A
P PSTPIP1
Q A744S
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S A230T
T Pyrin
U A230T Pyrin
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X E250Q Pyrin

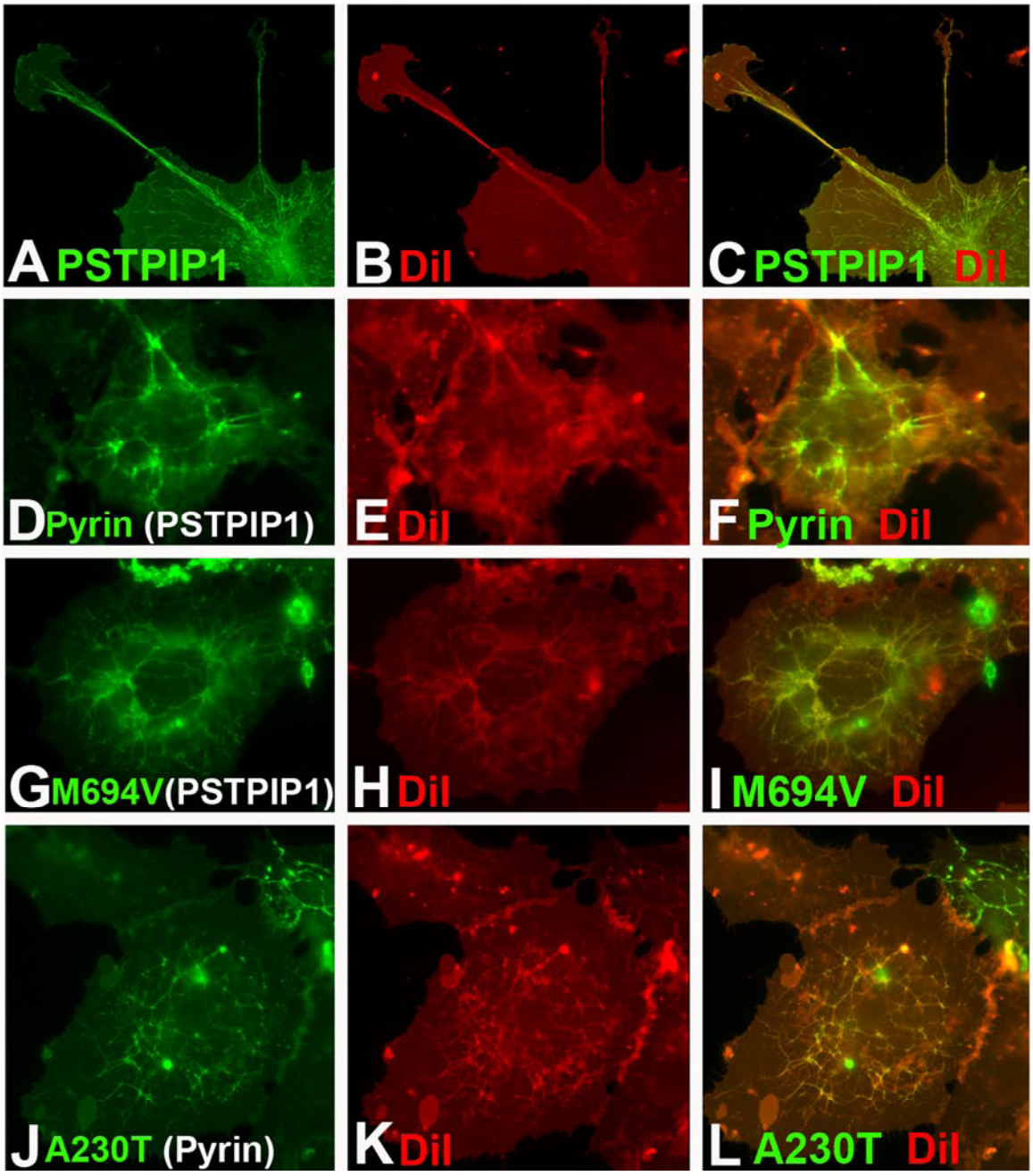
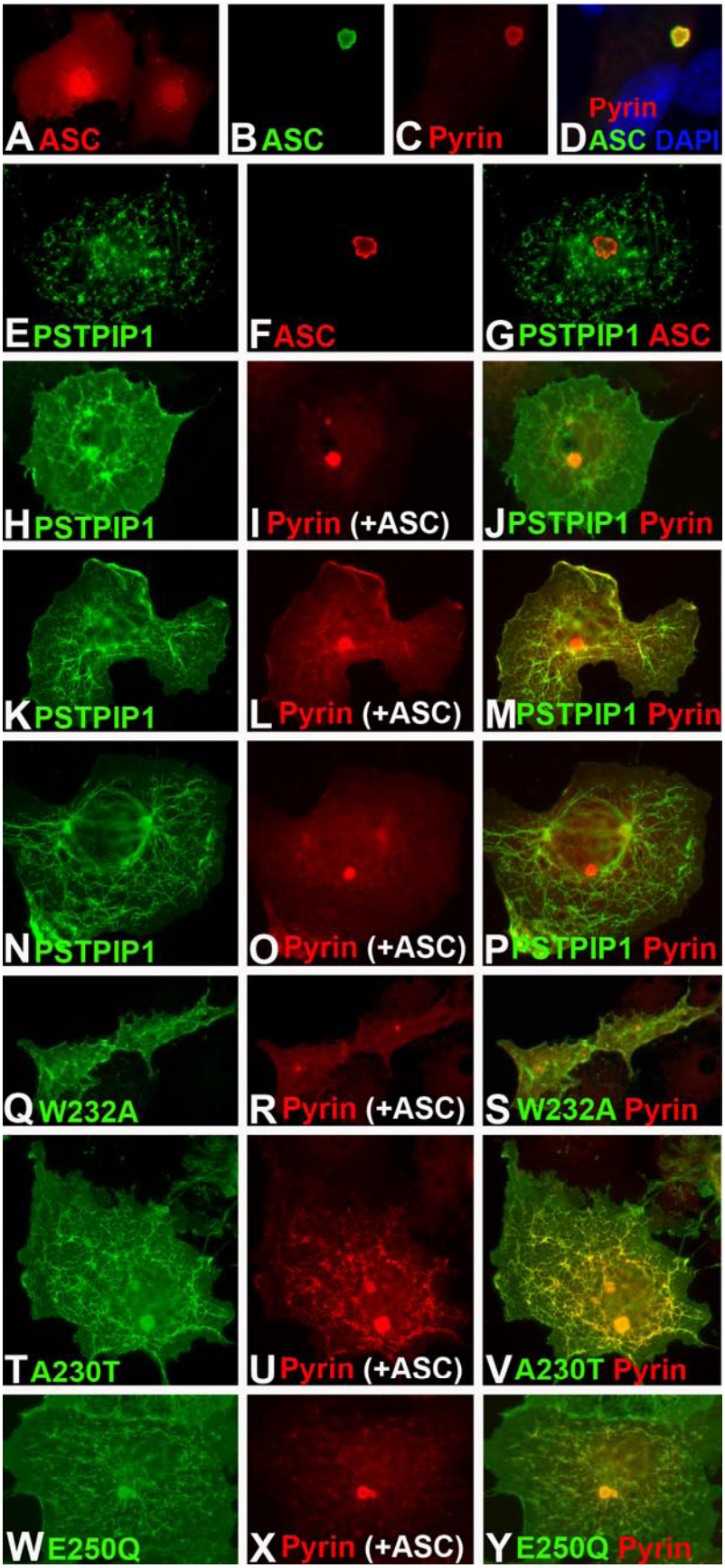


Figure 4.8: PSTPIP1 filaments are plasma membrane-based. (A-C) DiI_{C16} is taken up strongly by the PSTPIP1 filaments, indicating that the fibrils are membrane-based. (D-F) In cells transfected with pyrin and PSTPIP1, pyrin produced the typical reticular pattern, and DiI_{C16} staining was associated with the branched filaments. (G-L) In cells transfected with pyrin M694V-YFP and wild type PSTPIP1, filaments were visible and stained with DiI_{C16}. (J-L) In cells transfected with wild type pyrin and PAPA-associated A230T-GFP, $DiIC_{16}$ filament staining was preserved. Photographs taken at 600X.

Figure 4.9: Pyrin recruits PSTPIP1 to ASC specks. (A) The apoptotic speck protein ASC is normally distributed throughout the cell. (B) ASC can coalesce into a small perinuclear aggregate, the speck. (C-D) Pyrin is recruited to ASC specks via its PyD. (E-G) PSTPIP1 is not recruited to ASC specks in the absence of co-transfected pyrin. (H-J) In 70% of cells transfected with untagged ASC, PSTPIP1-FLAG and pyrin-myc, PSTPIP1 can be recruited to the speck through its interaction with pyrin. (K-P) In 30% of cases, transfection of the three proteins resulted in localization of pyrin in both PSTPIP1 fibrils and in the speck (K-M), or exclusively in the speck (N-P). (Q-S) FLAG-tagged W232A PSTPIP1 does not interact with pyrin, and is not recruited to specks. (T-Y) Recruitment of PAPA mutants by myc-pyrin to the ASC speck. (T-V) A230T-FLAG. (W-Y) E250Q-FLAG. Pyrin recruited these mutant forms to ASC specks in 95% of transfected cells. Photographs taken at 600X.


A ASC
B ASC
C Pyrin
D Pyrin ASC DAPI
E PSTPIP1
F ASC
G PSTPIP1 ASC
H PSTPIP1
I Pyrin (+ASC)
J PSTPIP1 Pyrin
K PSTPIP1
L Pyrin (+ASC)
M PSTPIP1 Pyrin
N PSTPIP1
O Pyrin (+ASC)
P PSTPIP1 Pyrin
Q W232A
R Pyrin (+ASC)
S W232A Pyrin
T A230T
U Pyrin (+ASC)
V A230T Pyrin
W E250Q
X Pyrin (+ASC)
Y E250Q Pyrin

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Chapter 5

Expression of ASC in renal tissues of FMF patients with amyloidosis; postulating a role for ASC in AA type amyloid deposition[4]

Abstract

Familial Mediterranean fever (FMF) is characterized by recurrent attacks of fever and serositis; in some cases, ensuing amyloidosis results in kidney damage. Treatment with colchicine reduces the frequency and severity of FMF attacks and prevents amyloidosis, though the mechanisms behind these effects are unknown. Pyrin, the protein product of the *MEFV* gene, interacts with ASC, a key molecule in apoptotic and inflammatory processes. ASC forms speck-like aggregates in cells that presage cell death. Here, we show that in non-myeloid cells, cell death after ASC speck formation is much slower than in myeloid cells. Additionally, we demonstrate that colchicine prevents speck formation and show that specks can survive in the extracellular space after cell death. Since we also find that ASC is expressed in renal glomeruli of FMF patients but not control patients, we posit that high local ASC expression may result in speck formation, and specks from dying cells may survive in the extracellular space where they have the potential (perhaps in association with pyrin) to nucleate amyloid. The fact that speck formation requires an intact microtubule network as shown here

could potentially account for the ability of prophylactic colchicine to prevent or reverse amyloidosis in FMF patients.

Introduction

The hereditary autoinflammatory diseases, including Familial Mediterranean fever (FMF), cryopyrin associated periodic syndromes (CAPS), tumor necrosis factor associated periodic syndrome (TRAPS) and Hyper IgD syndrome (HIDS) are associated with mutations in several proteins now believed to be involved in innate immune pathways (Werts et al., 2006). These diseases are characterized by systemic inflammation with high acute phase inflammatory markers and especially high serum amyloid A (SAA) levels. Secondary amyloidosis is a serious and life threatening complication for patients with FMF, TRAPS and CAPS (van der Hilst et al., 2005). In the case of HIDS, amyloidosis is much less common, but has been reported (van der Hilst et al., 2003).

Familial Mediterranean fever (FMF) is the most common of the autoinflammatory diseases and is characterized by recurrent attacks of fever with localized, painful inflammation. Attacks are often associated with high levels of acute phase reactants and inflammation typically involves the peritoneum, pleura, joints or skin (Aringer, 2004). More than in any of the other periodic fever syndromes, FMF patients are susceptible to multi-organ deposition of amyloid. In fact, prior to the use of colchicine as a prophylactic treatment for FMF,

amyloidosis was observed in up to 70% of FMF patients over 40 years of age, though the actual incidence was quite variable in different ethnic groups (Gafni et al., 1968). While the most serious impact of amyloidosis is seen in the kidneys, it can also affect the gastrointestinal tract, liver, spleen, heart and testes. Significant amyloid deposits found especially in glomeruli, tubules and interstitium in the kidney causes nephrotic syndrome and chronic renal failure, which may be fatal.

In the case of FMF, but not the other autoinflammatory diseases, the advent of colchicine treatment has had a remarkable effect on disease progression and outcome (Lidar et al., 2004; Zemer et al., 1986). Colchicine use reduces not only the frequency and severity of FMF attacks, but also prevents the development of amyloidosis. Even in the nephrotic stage of this disease, colchicine treatment may reduce kidney damage.

The FMF gene, which is located on short arm of chromosome 16 and symbolized as *MEFV* (ME diterranean Fe Ver), encodes a protein called pyrin or marenostrin (1997a; 1997b). While the function of pyrin is not clearly understood, it appears to play an important role in the inflammatory pathways that characterize the innate immune system. At least part of this function is mediated through pyrin's interactions with the apoptotic speck protein ASC (Richards et al., 2001), a 195-amino acid adaptor protein that contains an N-terminal pyrin domain (PyD) and C-terminal Caspase Recruitment Domain (CARD). These two

domains are related structures of the death domain family that act as protein-protein interaction interfaces. Through these two domains, ASC acts as an agglutinating platform for a wide variety of proteins involved in NF-kB activation, apoptosis and IL-1β processing (Gumucio et al., 2002). The multi-protein ASC-dependent platform associated with IL-1β processing has been termed the inflammasome (Mariathasan, 2007). Several different types of inflammasome have been documented, most of which contain ASC and Caspase-1 as well as a variety of accessory proteins, including NALP1, NALP2, NALP3 (or cryopyrin), and pyrin (Agostini et al., 2004; Martinon et al., 2002; Poyet et al., 2001; Srinivasula et al., 2002; Yu et al., 2006). ASC also forms a death-associated platform that has been termed a pyroptosome because of its role in rapid Caspase-1 mediated cell death (Fernandes-Alnemri et al., 2007).

On a cellular level, ASC can be found in two general patterns: either distributed throughout the cell (in nucleus and cytoplasm) or concentrated in a perinuclear aggregate that was initially called a ‘speck’ (Masumoto et al., 1999; Richards et al., 2001). The exact function of distributed ASC remains to be determined, but speck formation plays a role in Caspase-1 activation, IL-1β processing as well as cell death (Fernandes-Alnemri et al., 2007; Masumoto et al., 1999; Richards et al., 2001).

In myeloid cells, ASC specks that form in response to potassium efflux recruit Caspase-1. Agglutination of Caspase-1 causes rapid activation of this protease, causing cell death within minutes after speck formation. This process,

which is Caspase-1 dependent, is called pyroptosis and the speck in this case has been termed a pyroptosome (Fernandes-Alnemri et al., 2007).

In a variety of non-myeloid cultured cells transfected with ASC, specks form within 6 hours of transfection and speck formation is also associated with cell death (Masumoto et al., 1999; Richards et al., 2001). However, since these cells lack Caspase-1, the mechanism underlying cell death must be different than in myeloid cells. These differences are important since ASC is expressed in a variety of non-myeloid cells (Masumoto et al., 2001).

Pyrin has the potential to modulate the ASC death machinery via its direct interaction with ASC. Indeed, speck formation is accelerated in the presence of pyrin, but speck-positive cells that also contain pyrin succumb more slowly to death (Richards et al., 2001). How (or whether) the ASC-pyrin interaction fits into the clinical picture of FMF inflammatory attacks or explains the predisposition of FMF patients for amyloidosis is not yet clear.

Given the ability of pyrin to modulate ASC speck formation and subsequent apoptosis, and the known beneficial effects of colchicine in preventing FMF attacks, the initial aims of this study were to determine the effect of FMF-causing mutations in pyrin on speck formation and investigate whether speck assembly requires an intact microtubular network. We also explored the fate of the large ASC speck-like structures after cellular demise. Finally, we tested whether the expression or distribution of ASC is altered in kidneys of FMF patients with amyloid deposition. The results of these experiments lead us to

posit that ASC specks may be tied to amyloidosis and suggest a potential mechanism underlying the susceptibility of FMF patients to amyloid deposition, as well as a possible explanation for the efficacy of colchicine in treating amyloidosis.

Materials and Methods

Cell Culture and Plasmids. HeLa and COS-7 cells were grown in Dulbecco's modified Eagle medium plus 10% fetal bovine serum. For experiments, cells were plated onto glass coverslips placed in six-well culture plates. Cells were transfected using the FUGENE-6 transfection reagent (Roche Applied Science, Indianapolis, IN). Transfection rates of 60-70% were observed. Myc-ASC, myc-pyrin, and myc-M694V pyrin were expressed using the pCMV-Tag3A expression vector (Stratagene, La Jolla, CA). ASC-FLAG was generated using the pCMV-Tag2A vector (Stratagene, La Jolla, CA). ASC-YFP was generated using the pE-YFP vector from Clontech (Mountainview, CA).

ASC Speck Assay and Nocodazole Application. The rate of speck formation was examined in HeLa cells transfected with ASC-FLAG with either empty vector (pcDNA3.1), myc-pyrin, or myc-M694V pyrin. Six hours after transfection, vehicle (DMSO) or 4.15μM nocodazole was added to the cells and the cultures were placed on ice for 10 minutes to depolymerize microtubules. The cells were then returned to 37ºC and fixed twenty-four hours later using 4%

paraformaldehyde in PBS. The percentage of speck-positive cells was calculated as the number of specks divided by the total number of transfected cells.

Antibodies. Mouse-α-FLAG (M2 monoclonal), mouse α-tubulin (monoclonal), and rabbit α-myc (polyclonal) antibodies were obtained from Sigma (St.Louis, MO). Goat α-mouse AF488 and goat α-rabbit AF568 fluorescent secondary antibodies were purchased from Molecular Probes (Invitrogen, Eugene, OR). The ASC mouse monoclonal antibody was kindly provided by Dr. Junya Masumoto (Shinshu University, School of Medicine, Nagano, Japan).

Immunofluorescence. Cells were fixed for 30 minutes in 4% paraformaldehyde in phosphate-buffered saline, permeabilized using 0.2% Triton X-100 in phosphate-buffered saline, and blocked using 0.1% Tween20 in phosphate-buffered saline containing 10% goat serum and 0.01g/mL BSA. Primary and secondary antibody staining were carried out as described in the text, and nuclei were counterstained with DAPI. Cells were visualized using a Nikon E2000 Inverted fluorescent microscope, a Nikon E800 fluorescent microscope, or a Zeiss LSM 510 confocal microscope.

Patients. Patients were followed at the Hacettepe University, Faculty of Medicine, Ihsan Dogramaci Children's Hospital, Pediatric Nephrology and Rheumatology Unit in Ankara, Turkey. Fifteen amyloid positive FMF patients and

ten control cases (five biopsies taken from patients with a diagnosis other than FMF and five with focal segmental glomerular sclerosis (FGS)) were included in this study. Informed consents were obtained from patients. Five FMF patients eligible for mutation analysis were tested for MEFV mutations and all were homozygous for the M694V mutation. Biopsies of the other 10 patients did not yield sufficient material for mutation analysis.

Immunohistochemical staining of ASC. Immunohistochemical staining was performed with the anti-ASC monoclonal antibody using the streptavidin biotin peroxidase method (DAKO LSAB Kit, Dako, Carpenteria, CA). Culture fluids of the hybridoma producing anti-ASC monoclonal antibody were used as the primary antibody and tris-buffered saline (TBS) was used instead of MoAb for negative controls. 5μm thick sections of formalin-fixed, paraffin-embedded tissues were cut and deparaffinized. Endogenous peroxidase activity was quenched by incubation with 0.3% hydrogen peroxide in methanol for 30 min, then the sections were washed and blocked with 1% bovine serum albumin (BSA) in TBS for 1 hour. Sections were incubated with purified primary antibodies overnight at 4°C and then with biotinized anti-mouse secondary antibody for 60 min at room temperature. After washing 3 times with TBS, sections were incubated with horseradish-peroxidase conjugate streptavidin for 30 min. After washing 3 times with TBS the staining reaction was developed with 3,3-diaminobenzidine (Sigma Chemical, UK). Counterstaining was performed

with hematoxylin. Sections were visualized by using an Olympus BX51 light microscope.

Congo Red staining. 5μm thick sections of formalin-fixed, paraffin-embedded renal tissues were stained in congo red working solution for 10 minutes, rinsed in distilled water and differentiated quickly (5-10 dips) in alkaline alcohol solution. Sections were rinsed in tap water and counterstained with hematoxylin. Orange to red staining deposits that showed apple-green birefringence with polarizing light was accepted as amyloid deposition.

Video recordings of ASC speck formation. HeLa cells were transfected with ASC-YFP and allowed to form specks for 24 hours. Using phase contrast optics, a field of cells was selected containing speck-positive cells as well as non-transfected cells. A fluorescence image was obtained of this field to document the location of the speck containing cells. The cells were then maintained at 37°C under the microscope for 24 hours and phase contrast images were taken every five minutes. The complete image stack was assembled using NIH Image and converted to QuickTime movies. All cells were numbered and the fate of each cell was recorded. The procedure was repeated six times.

Results

Delayed cell death in non-myeloid cells transfected with ASC. Previous work in our lab (Richards et al., 2001) and others (Masumoto et al., 1999) established an association between the presence of ASC specks and increased cell death on a population level. Fernandes-Alnemri et al (Fernandes-Alnemri et al., 2007) were the first to examine this process on a single cell basis in a myeloid cell line (HL-60). Those studies revealed rapid formation of the speck (pyroptosome) within three minutes, followed by almost immediate cell death (Fernandes-Alnemri et al., 2007). However, our earlier studies in non-myeloid cells suggested that cell death was not an immediate consequence of speck formation (Richards et al., 2001). To examine this further, we used time-lapse video microscopy to follow cell death in transfected HeLa cells containing ASC specks. Cells were transfected with ASC-YFP and 24 hours after transfection, fluorescent images were captured to identify cells that contained specks. Once a field of speck-positive cells was identified, phase contrast images were captured every 5 minutes for 24 hours in order to determine cell fate.

Figure 5.1A shows a typical field of transfected and untransfected cells under phase contrast optics. A fluorescence image obtained of this same field is provided in Figure 5.1B. Figure 5.1C-F contains several frames extracted from the ensuing movie. Here, eight cells are tracked, four of which have specks (#1-4) and four of which are non-transfected (#5-8). The first of the speck-containing

cells to die (#4) succumbs in the first 12 minutes of observation (Figure 5.1C). By 5.5 hours, cell #2 also dies, and cells #1 and #3 are showing signs of imminent death (Figure 5.1D). By nearly 12 hours, all four speck-containing cells have died (Figure 5.1E). Continued observation reveals that cells #5-7 undergo mitosis, while cell #8 neither dies nor divides (Figure 5.1F). None of the four non-transfected cells undergo cell death.

These data demonstrate, on an individual cell basis, that once a speck forms, the speck-containing cell is destined for death, but death is not immediate. A total of 52 cells were individually imaged in this way, and 18 of these contained specks. Of the 18 speck-containing cells, 16 died during the course of the 24-hour observation period; cell death occurred as early as 12 minutes and as late as 22 hours after imaging was initiated. Of the 34 non-transfected cells, only one died. Many of the non-transfected cells completed mitosis, indicating that the observation conditions were not the cause of death in speck-containing cells. Thus, in non-myeloid cells (which lack Caspase-1), cell death following speck formation is delayed for up to several hours. As shown in our previous work, the presence of pyrin may further delay this cell death process (Richards et al., 2001).

ASC specks are cytosolic and perinuclear. The timelapse videos revealed that ASC specks are most often located very close to the nucleus; some appeared to be either in or on top of the nucleus. To further clarify whether

specks are located in the cytoplasm or nucleus, we used confocal microscopy. Figure 5.2B contains a tracing of the signal from the cell in Figure 5.2A that contains an ASC speck. The vector of the tracing is shown by the red line in Figure 5.2A. The speck is labeled with AlexaFluor568-conjugated anti-FLAG to detect FLAG-tagged ASC, while the nucleus is labeled with DAPI. The tracing clearly shows that the speck is located outside of the nucleus. This was true of at least 20 specks that were imaged in this way. The co-transfection of either wild type or mutant forms of pyrin with ASC did not change the location of ASC specks (data not shown).

ASC specks are located near the microtubule organizing center of apoptotic cells. Since the data above indicate that specks are perinuclear, we next tested whether specks co-localize with the microtubule-organizing center (MTOC), a structure that is also perinuclear. To investigate this, COS-7 cells were transfected with ASC-myc and after 24 hours, cells were co-stained for both myc and tubulin. In over 90% of cells, specks were found at or near the MTOC (Figure 5.3).

Microtubule disruption reduces speck formation. The consistent co-localization of ASC specks with the MTOC suggested that the transition from distributed, soluble ASC to aggregated specks might actually depend on an intact tubulin cytoskeleton. To test this, we exposed cells to nocodazole, a microtubule toxin with effects similar to that of colchicine. HeLa cells were transfected with

ASC-FLAG and treated with either nocodazole or vehicle (DMSO). When cells were treated with DMSO after transfection with ASC-FLAG, 6% of transfected cells formed specks within 24 hours, consistent with previous control experiments without DMSO (Richards et al., 2001). However after treatment with nocodazole, significantly fewer cells contained specks; 0.9% of transfected cells were speck-positive after 24 hours (Figure 5.4).

Our earlier studies indicated that the presence of pyrin increases the rate of speck formation (Richards et al., 2001). Therefore, we tested whether nocodazole also reduces the rate of speck formation in cells co-transfected with ASC and pyrin. In addition, we tested the effect of mutant pyrin on speck formation in the presence or absence of nocodazole. As shown in Figure 5.4, in the absence of nocodazole, co-transfection of cells with wild type pyrin and ASC resulted in increased speck formation as previously documented (Richards et al., 2001). Interestingly, a further increase in speck number was seen when mutant pyrin was co-transfected with ASC. In the presence of nocodazole, however, the number of specks was significantly reduced. Nocodazole reduced speck number by more than 6 fold in the absence of pyrin, by more than 4 fold in the presence of pyrin, and by more than 2.5 fold in the presence of mutant pyrin. The somewhat attenuated effect of nocodazole in the pyrin and mutant pyrin transfected cells may be a reflection of the fact that in the presence of pyrin, more specks were formed in the six hours prior to the administration of nocodazole. Indeed, when we lengthened the pre-treatment time to 16 hours,

the effect of nocodazole was even further attenuated by the presence of pyrin (data not shown). Together, these results demonstrate that: a) speck formation is increased by wild type pyrin and even more so by mutant pyrin; and b) speck formation is facilitated by an intact microtubular network.

ASC specks are stable in extracellular space. In the course of these experiments, we occasionally noted specks that appeared to be outside of cells (Figure 5.5). In addition, some dying cells appeared to extrude a speck. Such specks were stable in the extracellular space of the culture dish for prolonged periods of time (longest examination period was 72 hours). Though we have not confirmed that such "bare" specks also persist *in vivo*, the stability of these structures *in vitro* was remarkable.

ASC is aberrantly expressed in glomeruli of FMF patients with amyloid. The expression of *Pycard*, the gene for ASC, is induced under inflammatory conditions (Shiohara et al., 2002). Since FMF patients often experience kidney pathology, and since colchicine both prevents kidney pathology in these patients and modulates ASC speck formation, we tested whether ASC expression is associated with kidney pathology in FMF patients. We examined renal biopsies from these patients and from control patients (those with a diagnosis other than FMF). In the control group, we also included five patients with focal segmental glomerular sclerosis or FGS (FGS is associated with apoptosis of glomerular cells). In all of the control biopsies, ASC was detectable only in renal tubules. No

ASC expression was seen in glomeruli of any of the control patients (Figure 5.6A). However, in renal biopsies of amyloid positive FMF patients, ASC was detected in both renal tubules and in glomeruli (Figure 5.6B).

To investigate whether ASC expression was correlated with amyloid deposition, renal biopsies from FMF patients were analyzed after congo red staining. Sequential sections were stained to determine the localization of ASC. In all 15 patients, there was a 100% correlation between ASC expression and the presence of amyloid deposits. That is, ASC expression was detected in every glomerulus containing amyloid deposits (Figure 5.6C-D). Generally, ASC expression was high in the region immediately surrounding the amyloid deposits (especially on the boundaries) but much lower inside of the deposits.

Discussion

In this study, we further characterize ASC speck formation in non-myeloid cells and examine ASC expression in the kidneys of FMF patients. Earlier work showed that ASC is induced by inflammatory cytokines (Masumoto et al., 1999; Shiohara et al., 2002), but the differential activation of ASC in glomeruli of FMF patients as shown here has not been previously noted. The co-existence of ASC in every glomerulus with visible amyloid was a striking finding of our study. In addition, we find the distribution of ASC expression relative to the amyloid deposits within a given glomerulus to be interesting: the highest concentrations of

ASC are found in cells bordering the amyloid deposits. There are two possible reasons for this, one trivial and one potentially instructive. First, amyloid deposits may simply mask ASC epitopes and may not allow the simultaneous visualization of ASC in the presence of concentrated amyloid. Alternatively, it is also possible that in the areas where amyloid is most prevalent, cellular ASC is no longer distributed throughout the cell, but has already been collected into specks. Though ASC specks are readily visible in flat, well-spread cells under culture conditions, it is more difficult to detect them *in situ* in a tissue section. We did, in a few instances, identify speck-like structures within the amyloid-containing regions, but it was impossible to say with certainty that these were ASC specks.

ASC expression was never detected in glomeruli from non-FMF patients, though patients with other types of autoinflammatory diseases were not examined. To rule out the possibility that glomerular cell apoptosis alone might be sufficient to promote amyloid deposition, we included five patients with a diagnosis of focal segmental glomerular sclerosis (FGS). FGS is characterized by sclerosis in glomerular tufts that is distributed in a segmental manner (Kanjanabuch et al., 2006). Glomerular cell apoptosis is believed to be an important event in the progression of this disease (Chihara et al., 2006). The fact that none of the five FGS patients exhibited glomerular amyloid deposition nor glomerular expression of ASC leads us to conclude that the process of glomerular apoptosis itself is not a trigger for glomerular amyloidosis, nor for ASC induction. This further supports our hypothesis that a more specific functional

connection may exist between ASC expression and amyloid deposition in glomeruli of FMF patients.

FMF patients are particularly prone to amyloidosis; before the advent of colchicine treatment, this complication was responsible for significant mortality in FMF patients (Gafni et al., 1968). A variant of FMF, type II FMF, has been documented in which amyloid deposition and resulting uremia precedes the more typical inflammatory attacks (van der Hilst et al., 2005). It appears, however, that the connection between mutant forms of pyrin and amyloid deposition is not a simple, direct one, since not all FMF patients develop this complication and the extent of amyloidosis can vary markedly even among members of the same family carrying the same mutation. Moreover, clear environmental effects have been documented; for example, the rate of amyloidosis is vastly different in Armenians living in the United States (almost nil) compared to those in their native country (24%) (Schwabe and Peters, 1974). Some factors that appear to predispose to amyloidosis have been identified: the M694V mutation has been linked to severe disease and increased risk of amyloidosis in some studies (Ben-Chetrit and Backenroth, 2001; Brik et al., 1999; Cazeneuve et al., 2000; Cazeneuve et al., 1999; Gershoni-Baruch et al., 2003; Langevitz et al., 1999; Mansour et al., 2001; Mimouni et al., 2000), but not others (Akar et al., 2001; Altiok et al., 2003; Yalcinkaya et al., 2000); the SAA1 gene haplotype is associated with up to seven fold increased risk for amyloidosis (Altiok et al., 2003; Cazeneuve et al., 2000; Gershoni-Baruch et al., 2003; Yilmaz et al., 2003)

and male patients have up to a four fold increased risk for amyloid deposition compared to females (Cazeneuve et al., 2000; Gershoni-Baruch et al., 2003; Saatci et al., 1997). Thus, though it is clear that amyloidosis is a common complication of FMF, it is also obvious that a multifactorial mechanism is at play.

The process of amyloid deposition has been studied in cell culture and in mouse models and kinetic models have been proposed (Murphy, 2007). These suggest a bi-phasic mechanism in which a long lag period (predicting a nucleation step) is followed by a rapid deposition phase. Stabilization of formed amyloid fibrils is also an important part of the kinetics of amyloid accumulation. The initial lag period prior to nucleation can be shortened in experimental systems by adding a poorly characterized glycerol extract of spleen from an amyloidotic animal (Axelrad et al., 1982). This material contains a poorly characterized substance known as amyloid accelerating factor or AEF; it has been proposed that AEF may act as a seed around which amyloid polymerizes (Magy et al., 2003). The key structural component of AEF is thought to be its beta sheet content (Kisilevsky et al., 1999). In this regard, it is interesting to note that both domains of ASC (the CARD and the PyD) are composed of six alpha helical bundles and there is little, if any, beta sheet in the ASC molecule (Hofmann, 1999). However, the C-terminal PRYSPRY domain of pyrin is composed of multi-stranded beta sheets, arranged in a beta sandwich (Grutter et al., 2006; Masters et al., 2006; Woo et al., 2006). This structure has been described as highly similar to that of the pea lectin (Grutter et al., 2006).

Interestingly, the structure of human serum amyloid P component (SAP), a protein that binds to all forms of amyloid and is present in all amyloid deposits, has also been likened to that of the pea lectin (Emsley et al., 1994). Indeed, the overall structure of SAP closely resembles that of pyrin's PRYSPRY domain (compare Figure 3 in (Grutter et al., 2006) to Figure 2 in (Emsley et al., 1994)). SAP is not required for amyloid deposition in vitro, but in SAP-deficient animals, amyloid deposition is slowed (Tennent et al., 1995). SAP has also been implicated in the stabilization of amyloid fibrils in vivo (Hind et al., 1984). The striking structural similarities between SAP and the pyrin PRYSPRY domain are particularly interesting given the fact over 65% of the FMF-associated pyrin mutations are found in the PRYSPRY domain (INFEVERS database). If PRYSPRY does indeed associate with amyloid similar to the way that SAP does, then pyrin mutations could have the potential to alter some aspect of amyloid formation/degradation kinetics.

But if pyrin and ASC are together responsible for amyloid deposition, which cells of the glomerulus might express both proteins? There are at least three possible answers to this question. First, infiltrating inflammatory cells (e.g., monocytes, neutrophils) are known to express both ASC and pyrin. Second, recent studies indicate that after glomerular injury, bone marrow stem cells can give rise to mesangial cells of the glomeruli; up to 12% of the glomerular cells were found to be bone marrow-derived after induction of experimental glomerulonephritis (Ito et al., 2000). Third, pyrin is expressed in a variety of

fibroblastic cells (Centola et al., 2000; Diaz et al., 2004). In the kidney, myofibroblasts, which can derive from mesangial cells or vascular cells, play a role in wound healing and disappear by apoptosis after the injury is resolved (Schmitt-Graff et al., 1994).

The data presented in this manuscript predict that in the presence of mutant forms of pyrin, ASC specks form more readily in a cell that expresses both proteins. Furthermore, we show that ASC specks can outlive the apoptotic cells and survive in the extracellular environment. We propose therefore that the presence of a large number of extracellular pyrin-coated specks could promote amyloid deposition in FMF patients. Other unknown factors may modulate ASC expression, speck formation, speck extrusion, speck stability, or aspects of amyloid deposition and stability, accounting for the well-known variability in amyloid deposition among FMF patients.

The association between ASC specks and the microtubule organizing center as well as the requirement for an intact microtubular system for efficient speck formation represent two additional important findings of our studies. Given these findings, it is interesting that data from several laboratories indicate that colchicine inhibits amyloid formation in a variety of experimental systems (Brandwein et al., 1985; Kedar et al., 1975). Though the different studies used different amyloid formation protocols, some have concluded that colchicine inhibits the nucleating step (Brandwein et al., 1985; Kedar et al., 1975) while

others suggest that the growth phase is inhibited (Kisilevsky et al., 1977; Shirahama and Cohen, 1974; Shtrasburg et al., 2001). Our work indicates that the disruption of the microtubule system by nocodazole dramatically reduces speck nucleation. These data, together with the ASC expression data above, suggest the hypothesis that ASC specks (perhaps in association with pyrin) may promote amyloidogenesis in FMF patients. However, further direct evidence using amyloid forming systems in vivo and in vitro is required to substantiate this hypothesis.

FMF patients are particularly prone to amyloidosis, but amyloid is also seen in patients with CAPS and TRAPS, two of the other so-called autoinflammatory diseases. However, colchicine treatment is not effective for CAPS and TRAPS patients (Bustorff et al., 1995). A possible explanation for this therapeutic difference might be that the promotion of amyloid in FMF patients is a consequence of the presence of mutant pyrin on agglutinated ASC, rather than a consequence of agglutinated ASC alone. It is also possible that other aspects of the molecular composition of amyloid deposits themselves differ in FMF, CAPS and TRAPS. This remains to be studied. In the case of CAPS, it is interesting that cryopyrin shares with pyrin the ability to interact with ASC and, in fact, this interaction is the basis for the generation of at least one form of inflammasome, a multiprotein complex that contains Caspase-1 and can efficiently process IL-1β (Mariathasan, 2007). Whether cryopyrin can also promote speck formation and

whether speck-associated cryopyrin can survive in the extracellular space and play a role in the induction of amyloid remains to be studied.

In summary, the data presented here reveal a potentially important connection between mutant forms of pyrin, ASC expression and amyloidosis in FMF patients. Further functional studies are clearly required to more completely explore this tantalizing link. These findings might not only explain the high incidence of amyloidosis in untreated FMF patients, but also could suggest a mechanism at the cellular level for the remarkable efficacy of colchicine in preventing and even reversing amyloidosis specifically in these individuals.

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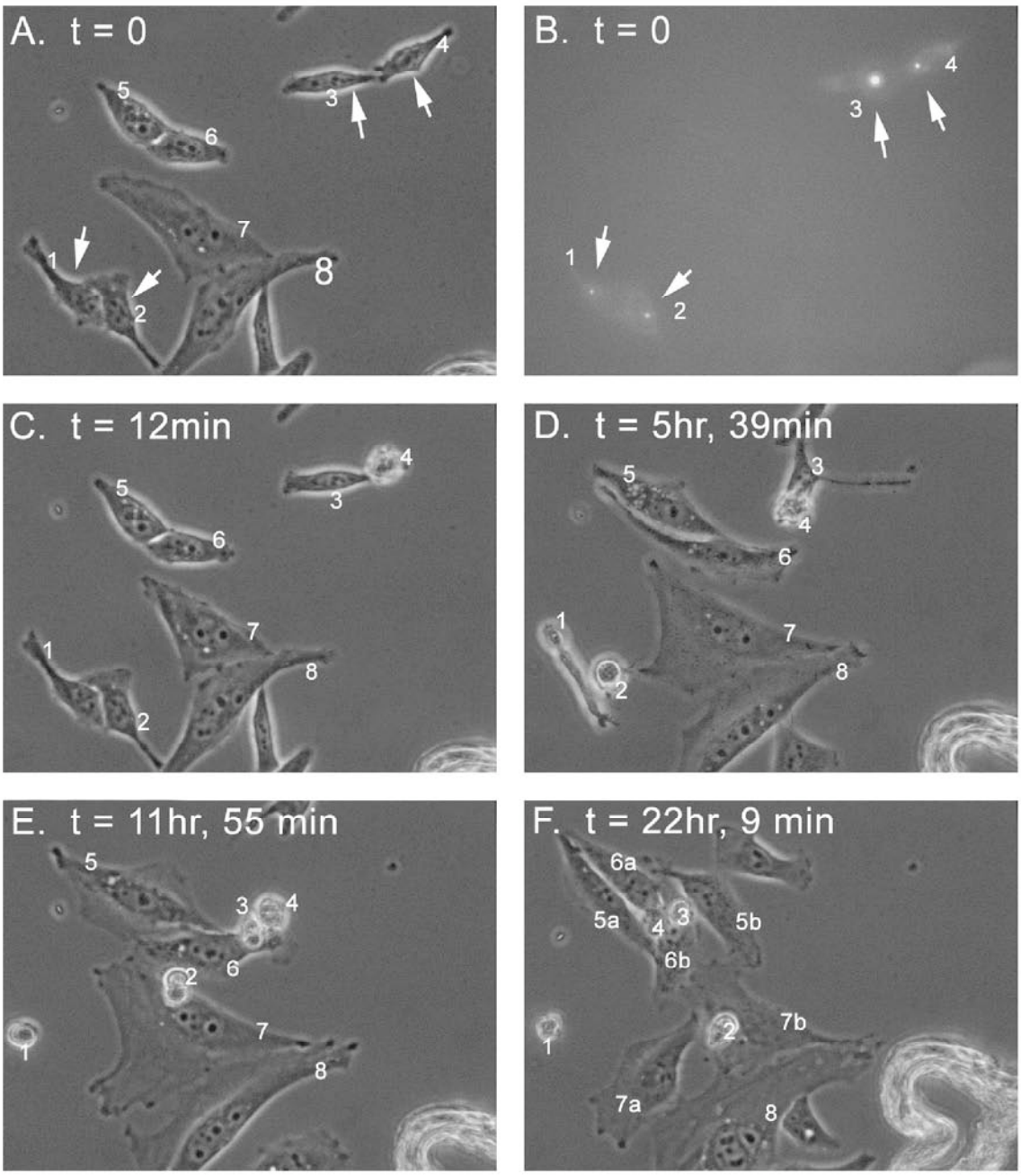


Figure 5.1: The presence of specks presages cell death. HeLa cells were transfected with ASC-YFP and imaged for 24 hours as described in the text. Each cell was given a number and the fate of the cells was followed with time. Panels A and B show phase contrast (A) and fluorescence (B) images of the starting field. Panels C through F are extracted from the movie at the times shown. All four cells with specks (#1-4) undergo cell death during the course of the 24 hours of observation. None of the non-transfected cells (#5-8) die, but three of these divide (#5-7). Timelapse carried out at 400X by Philip Schaner.

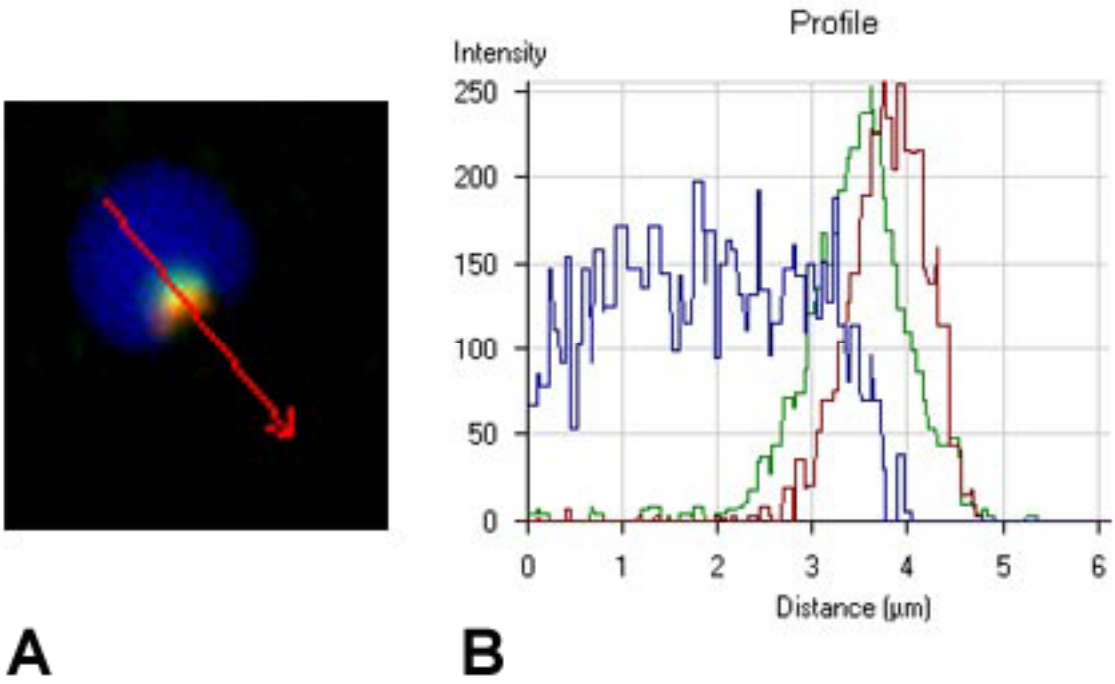


Figure 5.2: ASC specks are cytosolic. To further clarify subcellular localization of the speck, we used confocal microscopy. Figure 5.2B contains a tracing of the signal from the cell in Figure 5.2A that contains an ASC speck, and clearly shows the speck as cytosolic. The vector of the tracing is shown by the red line in Figure 5.2A. The speck is labeled with AlexaFluor568 conjugated anti-FLAG to detect FLAG-tagged ASC, while the nucleus is labeled with DAPI. Microscopy by Philip Schaner.

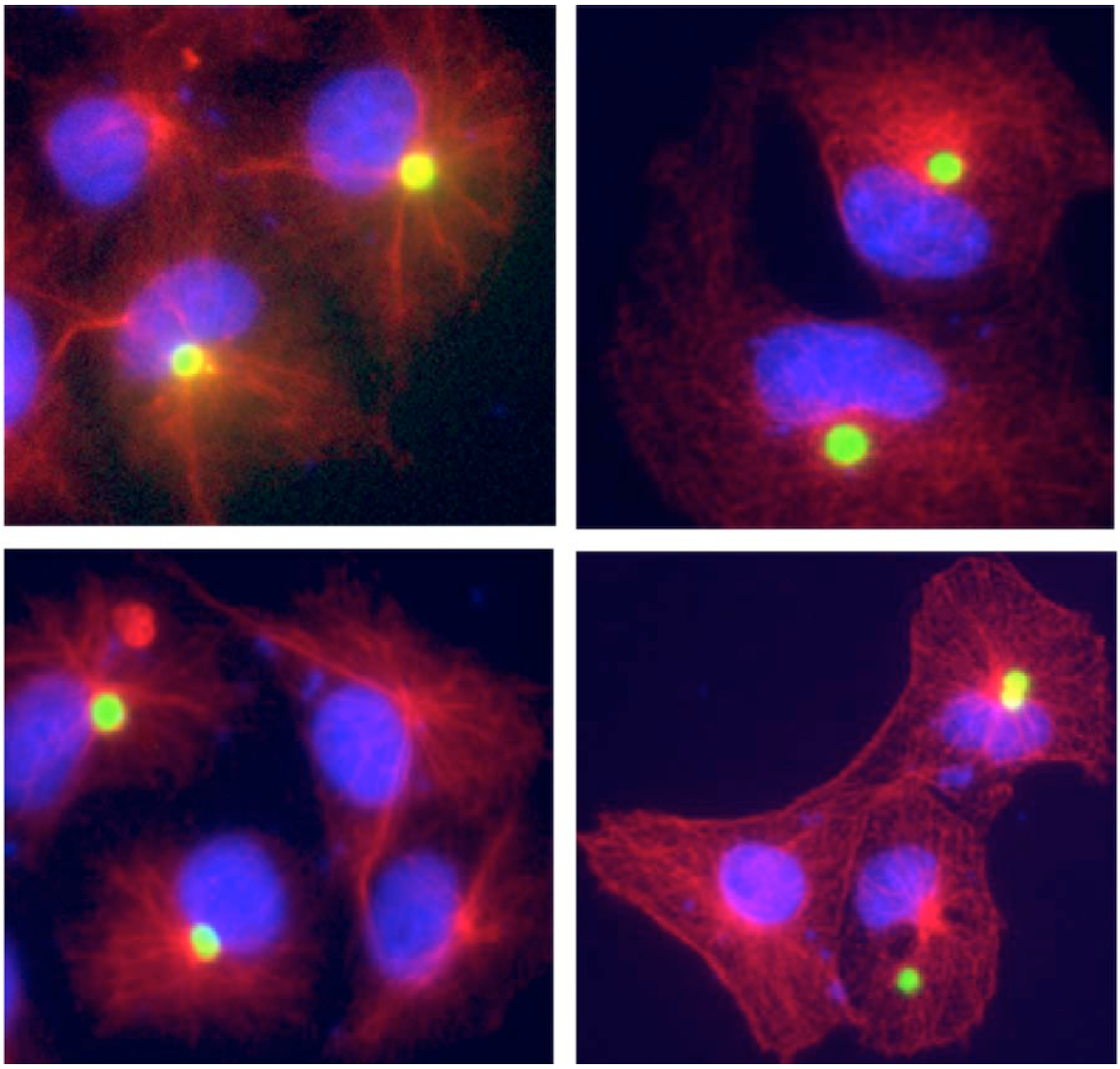
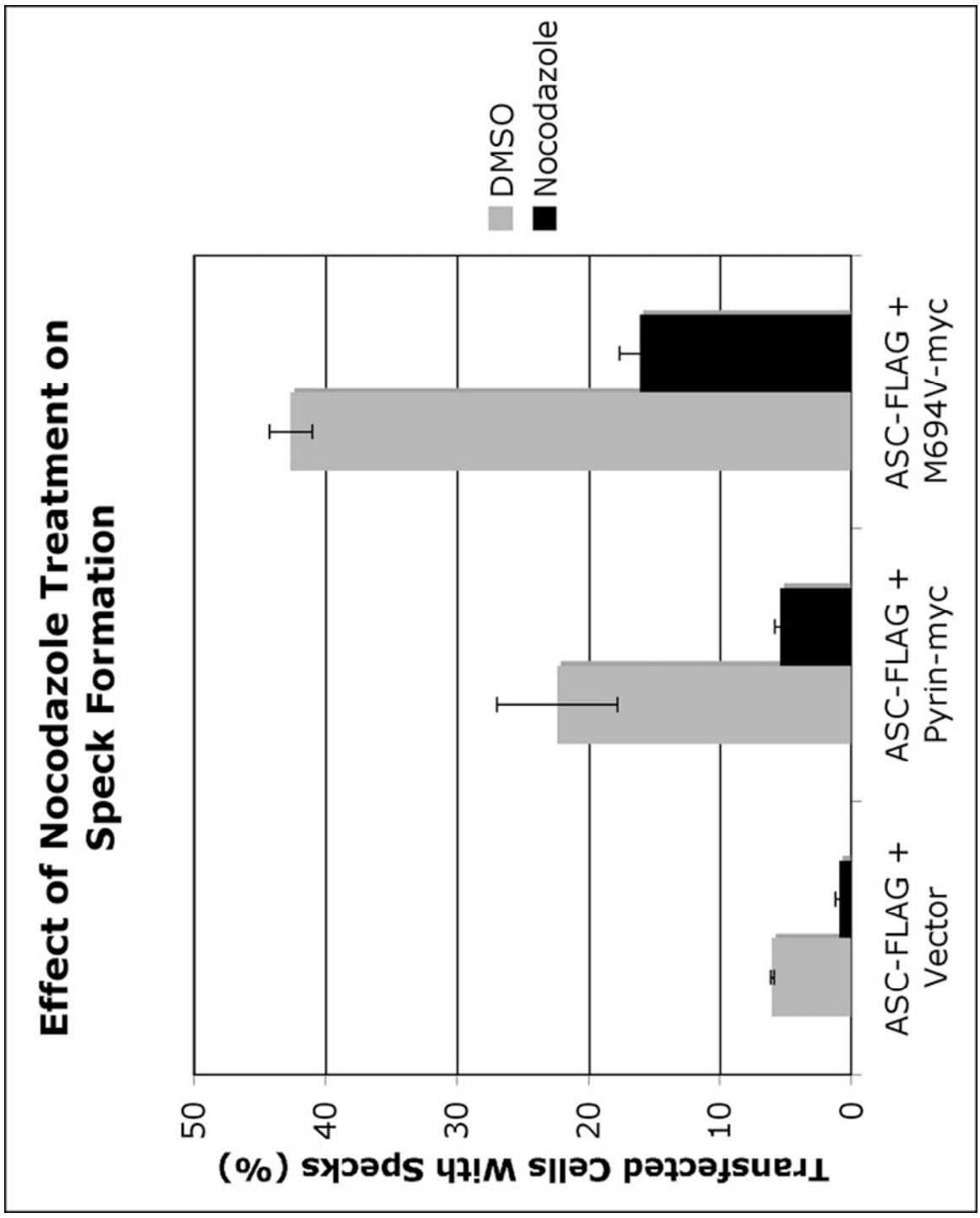


Figure 5.3: Specks co-localize with the microtubule organizing center. COS-7 cells were transfected with ASC and then stained for both ASC and tubulin. Over 90% of ASC specks were co-localized with the MTOC. Examples of four fields are shown. Photographs taken at 600X.

Figure 5.4: Nocodazole reduces the rate of speck formation. HeLa cells were transfected with ASC-FLAG, and then treated with DMSO or nocodazole. The addition of nocodazole reduced speck formation from 6% to 0.9%. While co-transfection of pyrin with ASC increased speck formation to 22%, nocodazole reduced this rate to 5%. Co-transfection of M694V pyrin further increased the rate of speck formation to 43%, and nocodazole reduced this rate to 16%.


Effect of Nocodazole Treatment on Speck Formation
Transfected Cells With Specks (%)
0
10
20
30
40
50
DMSO
Nocodazole
ASC-FLAG + Vector
ASC-FLAG + Pyrin-myc
ASC-FLAG + M694V-myc

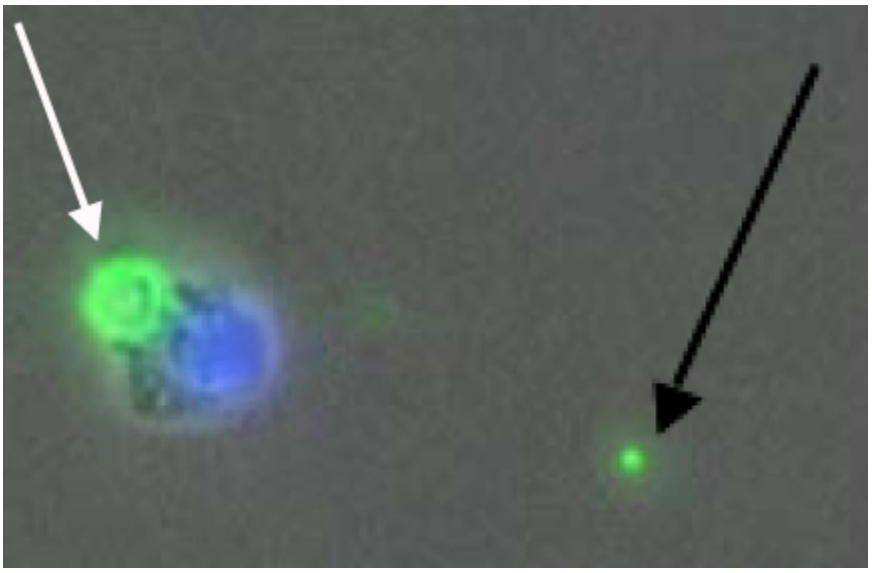


Figure 5.5: Specks remain stable in extracellular space. Cultures containing COS-7 cells that overexpress ASC contained stable extracellular specks (black arrow). Some dying cells appeared to be expelling the specks into extracellular space (white arrow). Photographs taken at 600X by Neil Richards.

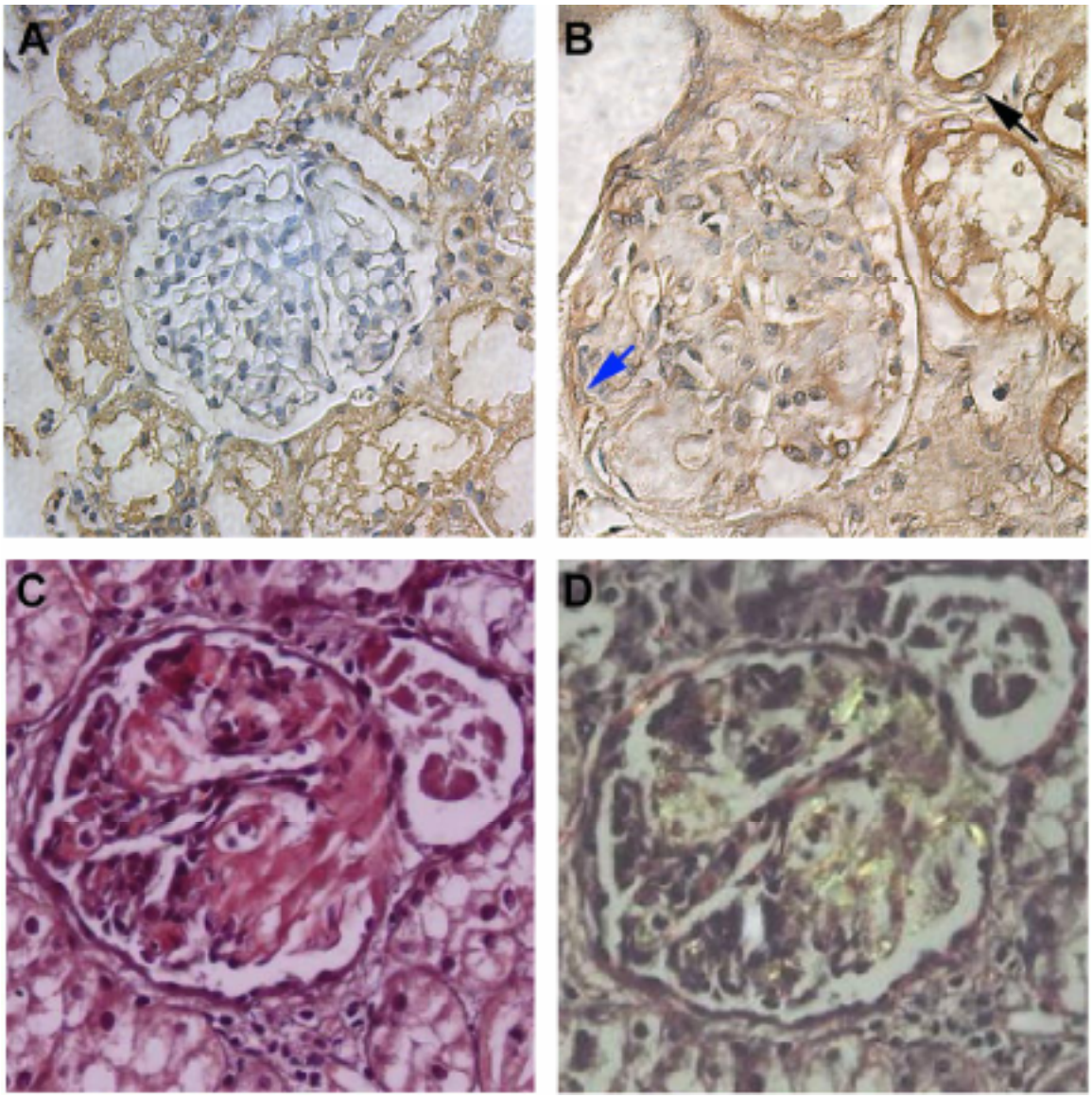


Figure 5.6: ASC is expressed in the glomeruli of FMF patients with amyloidosis. Immunostaining of a control kidney showing that ASC is expressed in renal tubules only (A). In FMF patients with amyloid, ASC is expressed in tubules (black arrow; B) and in glomeruli (blue arrow; B). Birefringence of congo red staining under polarized light shows clear amyloid deposition in the FMF kidney (C-D). ASC appears to be expressed at the boundaries of amyloid deposition. Photographs taken at 40X by Banu Balci-Peynircioglu.

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Chapter 6

Conclusion

Introduction

The work in this thesis has explored the interaction of pyrin with several proteins that our laboratory identified in a yeast two hybrid screen (Richards et al., 2001): ASC, PSTPIP1 and Siva. The cDNA for the interaction screen was generated from neutrophils harvested from the knee of a patient with gout, but the interactions described have since been verified in several additional cell types in which pyrin is expressed (monocyte/macrophages, synoviocytes). The work presented in this thesis, combined with work from other laboratories, indicates that these interactions are crucial to pyrin's function in several cellular pathways, including cell death, inflammation, cytoskeletal signaling, and the building of multiprotein complexes.

When the *MEFV* gene was initially cloned in 1997, analysis of the sequence revealed very little about the function of the pyrin protein. In fact, the guess, based on homologies to related proteins and the presence of apparent nuclear localization domains (1997a; 1997b), was that pyrin might be a transcription factor; this idea has thus far not been substantiated. Rather, much

of pyrin's function seems to take place in the cytoplasm. Indeed, all three proteins that we have studied can be found in the cytoplasm, though ASC and especially Siva, also apparently reside in the nucleus. Interestingly, there are still unpublished data from the Kastner laboratory suggesting that pyrin is cleaved by Caspase-1 and that a portion of the cleaved pyrin molecule can enter the nucleus and may function to modulate NF-κB signaling (Chae et al., unpublished observations). Thus, a clear nuclear function for pyrin may still emerge.

The work presented in this thesis represents significant progress in the study of pyrin, and some of the new discoveries extend into other syndromes in the field of autoinflammatory disease. However, new questions have arisen as a result of our work and some older questions remain unanswered. Some of the most interesting and important questions for future research are addressed in this chapter, including: a) What is the nature of the selective pressure that has colored the evolution of pyrin? b) How do FMF mutations alter the function of pyrin? c) What triggers FMF attacks? d) Why do FMF patients so often present with amyloidosis? e) Is there a functional connection between pyrin and cell migration? f) How does pyrin modulate the formation and function of ASC specks?

FMF mutations – A possible heterozygote advantage?

The high FMF carrier frequency in certain populations (Aksentijevich et al.,

1999) and the fact that disease-causing mutations in humans recapitulate wild type sequences in primates (Schaner et al., 2001) are two findings that suggest that the *MEFV* locus is under significant selection pressure and that mutations in pyrin alter the functional nature of the protein rather than destroy its function. At least two possible sources of selective advantage have been postulated and both involve increased resistance to intracellular pathogens endemic to the Mediterranean basin. One such proposed pathogen causes the disease brucellosis (Ross, 2007). Interestingly, the disease now known as "brucellosis" was originally called "Mediterranean Fever" (Wilkinson, 1993). Caused by bacteria from the genus *Brucella*, particularly *Brucella abortus*, brucellosis is marked by periods of fever accompanied by sweating, weakness, anemia, arthralgia, and myalgia. The symptoms of the disease in fact have a slight resemblance to FMF, though brucellosis is chronic rather than recurrent and of course has a bacterial basis, while FMF attacks are considered "sterile" inflammation. The bacteria usually are transmitted either through direct contact with infected animals (particularly sheep or goats) or by consumption of raw milk or soft cheeses made from raw milk of infected animals. Soft cheeses made from raw goat and sheep milk are still prevalent in Middle Eastern and Mediterranean diets, and may account for the high incidence of brucellosis in these populations (Pappas et al., 2005). Left untreated, brucellosis can lead to death from systemic infections (Al Dahouk et al., 2006; Park et al., 2007), although the use of modern antibiotics has made death due to brucellosis rare. Even with modern antibiotics the bacteria that cause brucellosis are highly

resistant to treatment, and the disease requires a nine-month course of antibiotics to prevent later re-emergence. In fact, this resistance to treatment made *Brucella* the bacteria of choice for use in biological weapons in the 1970s (Pappas et al., 2006). Host defense against the bacteria is dependent upon high levels of IFN-γ (Pappas et al., 2005). Since carriers of FMF mutations appear to exist normally in a "hyper-inflammatory" state that includes higher basal levels of IFN-γ, it has been proposed that FMF heterozygotes may be naturally protected against brucellosis (Ross, 2007). Interestingly, folklore surrounding FMF includes the idea that attacks are precipitated by drinking the milk of goats. It is possible that this is indeed that case, as the immune system of FMF patients may be already tipped toward a pro-inflammatory state and infections, even mild ones, might promote attacks, with further exacerbation of inflammation. Such inflammatory attacks, while painful, would further ramp up the pro-inflammatory state and therefore might greatly facilitate the clearance of the pathogen.

A possible connection to brucellosis is interesting in another regard. Recently, He et al (He et al., 2006) assayed the transcriptional response of the macrophage host cell to infection with a virulent strain of *Brucella melitensis* and noted a robust up-regulation of Siva. The fact that Siva is a pro-apoptotic pyrin-interacting protein places pyrin in a position to modulate cell death pathways in the infected macrophage host, a control point that has the potential to limit the infection.

Another postulated advantage for carriers of FMF mutations is an increased resistance to tuberculosis (Cattan et al., 2007). The city of Tunis in the north African Mediterranean nation of Tunisia offers a unique look into this possibility due to both its multi-ethnic population makeup and its public health records. From 1881 to 1955, Tunis was under the protectorate of the French government, which conducted population censuses every five years (except during the two world wars). These surveys collected information on mortality due to infectious diseases among the varied ethnic groups of the city. During this time, Tunis had a large and relatively wealthy European population (mostly of Italian and French descent), who enjoyed a high standard of living and access to medical care. In addition, Tunis had a well-established Muslim population, and a Jewish population that been present since Phoenician times (Cattan et al., 2007). Muslim and Jewish communities were mostly poverty-stricken, and were relegated to areas of the city where infectious disease was rampant and access to medical care was poor. This led to a higher mortality rate in these populations due to infectious diseases such as malaria, typhoid, smallpox, typhus, influenza, and measles (Cattan et al., 2007). Importantly however, while tuberculosis consistently resulted in high mortality among the Muslim population of Tunis (an average of 593 deaths per 100,000 people yearly from 1919 – 1939), the Jews of Tunis reported deaths from tuberculosis at a level much lower than even the rate reported in the European populations (193 deaths per 100,000 people yearly from 1919 – 1939 among the French population; 81 deaths per 100,000 people yearly from 1919 – 1939 among the Jewish population (Cattan et al., 2007)).

This lower death rate was not due to lower infection rates in the Jewish populations, as records from the time indicate that the incidence of infection was similar in all ethnic groups. A potential explanation for the apparent protection of the Jews from death caused by tuberculosis is that they had a particularly high FMF carrier rate and that these carriers were more likely to survive a tuberculosis infection due to their consistently heightened pro-inflammatory state. Indeed, descendents of this population have a carrier frequency today of 20-30% for the M694V mutation alone (heterozygotes and homozygotes included (Onen, 2006)). It has also been noted that this community is traditionally very isolated and marriage outside of the ethnic group is highly discouraged (Cattan et al., 2007), leading to concentration of this FMF allele.

While the Cattan study is unique in its extensiveness, Other smaller studies comparing tuberculosis death rates among various populations in cities throughout Europe and the United States have supported the finding that populations of Sephardic Jews tend to have low rates of mortality due to tuberculosis (Cattan 2007 et al., see Table 6). This makes the hypothesis that FMF mutations, and M694V in particular, may play a role in defense against tuberculosis an important area for future study.

How do FMF mutations alter the function of pyrin?

Despite significant advances in the field of pyrin research, it is still unclear

how FMF mutations alter the function of pyrin in such a way as to cause FMF attacks. Our work suggests at least two conceivable scenarios that might be responsible for the robust inflammatory response seen in this disease. One hypothesis is that FMF is a sensing problem. Expression of mutant pyrin in resident macrophages or dendritic cells in tissues may alter the response of these important sensors of inflammatory stimuli in such a way that minor triggers (which should normally be ignored) lead to over-stimulation of the cell, and result in increased inflammatory signaling and consequently, a massive influx of neutrophils. The fact that pyrin interacts with ASC, a major component of the inflammasome, suggests the potential that mutant forms of pyrin might alter inflammasome signaling, though this remains to be directly demonstrated.

Another possibility is that FMF is a reaction problem. Our findings of pyrin on the leading edge of migrating cells and documentation of pyrin's interaction with the actin-associated protein, PSTPIP1, suggest a possible perturbation in migration. If minor chemotactic stimuli lead to a robust migratory response, perhaps the neutrophils are the source of the problem.

In either scenario, it is possible that modulation of apoptosis plays a role, especially since pyrin interacts with two apoptotic signaling proteins, ASC and Siva. In the case of monocytes, a delay in cell death would lead to prolonged inflammatory signaling, while in the case of neutrophils, this would lead to prolonged accumulation of cells in the inflamed tissue.

Though any of these speculations are plausible, no clear answers are yet available. FMF mutations are predicted to alter the binding affinity of pyrin for its partners (see Chapter 1). This hypothesis is based on the structure of the RFP domain where the majority of FMF mutations are clustered. Indeed, mutations that correlate with more severe phenotypes are found on protein loops that are predicted to be critical for protein binding (James et al., 2007). One recent study demonstrated a lowered affinity of the M694V pyrin mutant for Caspase-1 (Chae et al., 2006), lending credence to this hypothesis, since the pyrin-Caspase-1 interaction seems to require the RFP domain of pyrin. However, the purported functional outcome of this altered binding is Caspase-1-mediated pyrin cleavage; yet a consistent association between degree of cleavage and expression of wild type or mutant pyrin could not be demonstrated. In studies of other pyrin binding partners, no difference in interaction affinity has been seen with FMF mutations.

Another intriguing idea is that the mutations affect how pyrin is folded. Recent studies have provided evidence that pyrin exists as an autoinhibited homotrimer, in which the B box in exon 3 is bound to and masks the PyD encoded by exon 1 (Yu et al., 2007). This study suggests that binding to PSTPIP1 via exon 3 activates pyrin, opening the PyD to interact with ASC and precipitate in inflammatory signaling. If this model is correct, increased binding of PSTPIP1 to pyrin would lead to increased activation of pyrin and increased inflammatory signaling through ASC (Yu et al., 2007). This could nicely explain

PAPA syndrome, an autoinflammatory disease caused by mutations in PSTPIP1. PAPA mutations increase PSTPIP1's affinity for pyrin (see Chapter 4). But the model does not fit with our data, nor does it satisfactorily explain the effects of FMF mutations. Our data in Chapter 4 indicate that when pyrin, ASC and PSTPIP1 are together in the cell, pyrin prefers to interact with ASC. In such cases, PSTPIP1 filaments can often be seen, but pyrin is seen in association with ASC specks, rather than decorating the filaments. Only when mutant forms of PSTPIP1 (with higher affinity for pyrin) are present does this picture change. In this case, PSTPIP1 is recruited to ASC specks along with pyrin. Thus, pyrin does not require PSTPIP1 to "activate" its binding to ASC, as suggested by the Yu et al. model (Yu et al., 2007).

This model also seems at odds with the distribution of pyrin mutations, most of which are found in pyrin's RFP domain, encoded by exon 10, with a smaller cluster in exon 2. It is easy to imagine how mutations in the PyD encoded by exon 1 or the B box encoded by exon 3 could potentially interfere with this predicted autoinhibition, leaving pyrin free to interact constitutively with ASC and resulting in constitutive inflammatory signaling. But mutations in exons 2 and 10 would not be predicted to interfere with autoinhibition according to the model of Yu et al.

Interestingly, a different version of the Yu et al. autoinhibition model was suggested earlier to explain another interesting aspect of pyrin biology: the fact

that the mouse pyrin protein lacks an RFP domain, the very region that seems to be so highly important in human disease, as judged by the number of mutations that it harbors (Schaner and Gumucio, 2005). Though no data exist to support this speculation, Schaner posited that the RFP domain might interact with exon 2 and that this interaction might inhibit a basal activity of the protein. It was further proposed that the binding of a ligand or other protein to the RFP domain might cause a conformational shift that could open pyrin and allow it to function. In this case, the mouse protein, lacking an RFP domain, could be a constitutively active form of pyrin, perhaps permitting it to function more robustly in the dirty murine environment. Mutations in pyrin could then be thought of as sensitizing changes that lowered its threshold for “activation”.

The fact that over a decade has passed since the cloning of pyrin, yet the function of the wild type molecule and the pathoetiology of the mutations have not yet been elucidated, makes for an interesting, if frustrating, story. Progress in this field might be accelerated if a mouse model were available (though given the differences between mouse and human pyrin, this is not a certainty). Perhaps the further analysis of additional pyrin-interacting proteins, found in our laboratory (Lark, Galectin, mitochondrial kinase; Neil Richards, personal communication) will hold the keys to this question.

FMF attacks – what is the trigger?

Once again, there is no clear answer to this question, but clues might be found in exploring the few anecdotal triggers of FMF attacks. For example, some patients report experiencing attacks near the time of menstruation, or after the consumption of a fatty meal. There may be a physiological basis for these observations. Menstruation is initiated by the release of a family of lipid hormones known as the prostaglandins (Vijayakumar and Walters, 1981). In some systems, prostaglandins are upregulated by NF-κB and mediate inflammatory signaling (Paradowska et al., 2007). This process may be important in the pathogenesis of rheumatoid arthritis (Paradowska et al., 2007). Recently prostaglandin E2 has been specifically linked to p53 activation in synovial cells (Faour et al., 2006). While it has not been directly established whether this results in downstream upregulation of Siva, the fact that Siva is a direct p53 target gene makes this pathway an interesting area for investigation.

Eating a fatty meal results in the production of low-density lipoproteins (LDL). When LDL are taken up by endocytosis in macrophages, the cell becomes a type of inflammatory macrophage known as a foam cell. These foam cells then release inflammatory cytokines such as IL-6, IL-10, IL-1β, and TNFα (Elabbassi and Al-Nooryani, 2006; Ma et al., 2007; Yudkin et al., 2000). Since high levels of these cytokines have been reported during FMF attacks, this may

represent a situation in which a delicate inflammatory balance has been slightly perturbed, and massive inflammation results.

In several cases, reported FMF attack triggers are pharmacological agents that were found to induce FMF attacks in patients being treated for other disorders. One such agent is isotretinoin, a retinoid used in the treatment of severe acne and marketed under the brand name Accutane (Roche). While the precise mechanism of action of isotretinoin in treating acne is unknown, it is known to induce apoptosis in B lymphocytes (Nelson et al., 2008; Sundelin et al., 2007), although these cells do not express pyrin. Isotretinoin is also used (although rarely) to treat acute promyelocytic leukemia due to its ability to induce the production of the inflammatory cytokine IL-6, which results in differentiation of cancerous cells (Xie et al., 2000). This effect was first noticed in HL-60 cells, a promyelocytic cell line that also expresses ASC (Masumoto et al., 1999). It would be of interest to specifically search in cell culture assays for a cellular link between isotrentinoin and ASC expression, speck formation, and/or inflammasome function. Isotrentinoin has also been documented to induce pyoderma gangrenosum. This is of interest since PAPA syndrome (Pyogenic sterile Arthritis, Pyoderma gangrenosum and Acne) is caused by mutations in PSTPIP1 (a pyrin interacting protein examined in Chapter 4) and it has been speculated that pyrin and PSTPIP1 are part of the same inflammatory pathway.

Another documented FMF attack trigger is the chemotherapeutic agent

cisplatin. This link was discovered when an FMF patient receiving cisplatin as treatment for adenocarcinoma of the lung was observed to suffer multiple severe FMF attacks (Toubi et al., 2003). Cisplatin works by crosslinking DNA, which interferes with mitosis and activates DNA repair mechanisms. Where DNA repair is not possible, apoptosis is induced. Patients that are undergoing treatment with cisplatin exhibit high levels of several inflammatory cytokines, including IL-6, IL-1, IL-8, and TNFα (Toubi et al., 2003). It has been proposed that overproduction of one or more of these cytokines after cisplatin treatment may be responsible for causing these FMF attacks, although the mechanism behind its effects is unknown. Of course, as discussed in Chapter 3, cell death after cisplatin treatment has been shown to involve Siva (Balci-Peynircioglu et al., 2008; Chu et al., 2005), and this could also be an important connection to investigate. Cell culture studies on monocyte/macrophages could be used to examine whether pyrin can modulate cytokine expression or Siva-induced death after cisplatin treatment.

An additional factor that may be important in identifying the cause behind pharmacological induction of FMF attacks is the fact that both isotrentinoin and cisplatin result in an increase in soluble Fas in the culture medium of some cell lines (Sundelin et al., 2007). PSTPIP1 has been implicated in the control of reverse Fas signaling, as expression of PSTPIP1 has been shown to increase intracellular localization of Fas and thus to regulate its cytotoxic activity (Baum et al., 2005). Additionally, Fas has been shown to transiently upregulate expression

of ASC in neutrophils (Shiohara et al., 2002). This upregulation is linked to inflammation and apoptosis downstream, as it can be blocked with a pan-caspase inhibitor (Shiohara et al., 2002). ASC expression in neutrophils is also increased by proinflammatory cytokines such as IL-1β, IFNα, IFNγ, and TNFα (Shiohara et al., 2002). Since the expression of many of these factors is also induced by isotrentinoin and cisplatin, one area of future study many be to block caspase activity in neutrophils exposed to these chemicals in an attempt to block the upregulation of ASC and subsequent cytokine production.

Another important area to consider while searching for an FMF attack trigger is that a few pharmacological agents have been found to prevent FMF attacks. The first and most familiar agent is of course colchicine, which has been found to provide improvement or complete remission of FMF symptoms in the vast majority of patients, although the mechanism behind colchicine's efficacy in treating FMF is unknown. Colchicine binds to microtubules in cells to prevent their polymerization. We have shown that a drug with a similar mode of action, nocodazole, destroys the tubular filaments formed by PSTPIP1 (see Chapter 4) and inhibits ASC speck formation (see Chapter 5). Colchicine may therefore modulate cytoskeletal and inflammatory signaling. Additionally, colchicine has been shown to inhibit neutrophil motility and therefore may inhibit neutrophil influx after inflammatory stimulation (Griswold et al., 1989).

In FMF patients where colchicine treatment proves ineffective, a few

alternative treatments have been described (reviewed in (Bhat et al., 2007)). One such treatment is prazosin, a drug that suppresses the catecholamine response (Kataoka et al., 1998). In one FMF patient that had previously been experiencing 12 to 24 attacks each year, administration of prazosin completely prevented attacks for over a year. Furthermore, subsequent withdrawl of the prazosin resulted in the recurrence of the attacks (Kataoka et al., 1998). Catecholamines have been shown to induce the expression of inflammatory mediators in monocytes (Seematter et al., 2004), which may provide clues to the connection between this pathway and FMF attacks. Another agent that has proven effective in treating FMF in some cases is the IL-1 receptor antagonist anakinra, a drug used for the treatment of rheumatoid arthritis and marketed under the brand name Kineret (Amgen) (Belkhir et al., 2007; Calligaris et al., 2007; Gattringer et al., 2007; Kuijk et al., 2007). Not only did patients treated with anakinra show resolution of symptoms, circulating levels of SAA and CRP were also reduced to very near the normal range (Gattringer et al., 2007). Since IL-1 levels are elevated in FMF patients, it is hypothesized that blocking its action may work to prevent FMF attacks.

Two other pharmacological agents that have been shown to prevent FMF attacks are the TNFα antagonists etanercept, marketed under the brand name Enbrel (Immunex), and thalidomide. These treatments have been used alone (Mor et al., 2007; Sakallioglu et al., 2006; Seyahi et al., 2002) or in combination (Seyahi et al., 2006) with success. Since both of these TNFα antagonists blunt

the acute phase response, it is thought that the mechanism by which they prevent FMF attacks works through this pathway. Additionally, derivatives of thalidomide have been shown to reduce NF-κB signaling by preventing its translocation to the nucleus (de-Blanco et al., 2007). This factor may be important in determining how thalidomide works to prevent FMF attacks. Further dissection of the pathways activated and attenuated by these drugs may provide additional insights into the trigger for FMF attacks

Why do FMF patients so often present with amyloidosis?

Another aspect of FMF that presents an intriguing area of study is the propensity of FMF patients to develop amyloidosis. While patients of other autoinflammatory diseases do occasionally develop amyloidosis, the startlingly high rate of amyloidosis in FMF patients before the advent of colchicine therapy leads to the hypothesis that there is an important link between the molecular pathways involved in FMF and those responsible for amyloidosis. One potential explanation may come from data observed in our lab regarding the nature of ASC specks (see Chapter 5 for a detailed explanation of this hypothesis). We observed that ASC specks are remarkably stable, and can been seen in extracellular space long after the death of the cell in which the speck first formed. Although there is no evidence to date that ASC specks are emitted from live cells (we have only observed speck extrusion from dying cells), this idea should not be ignored in future study. In cell culture experiments, pyrin expression increases

the number of cells that contain specks (Richards et al., 2001), and this effect is even further observed with mutant pyrin. An increase in the number of specks overall would presumably lead eventually to an increase in the number of extracellular specks. Since the rate-limiting step in the formation of amyloid plaques is nucleation, we proposed that free ASC specks (perhaps in association with pyrin) might act as a nucleation platform. This hypothesis can be tested using a cell culture model of amyloid formation that has been previously described (van der Hilst et al., 2008). Briefly, cells are cultured in a medium containing both SAA and a poorly characterized factor known as amyloid enhancing factor (AEF). After 3 days in these conditions, amyloid plaques form in the culture and can be identified by Congo red staining. Cells expressing ASC alone, or in combination with wildtype or mutant pyrin, could be tested in this system for their ability to enhance amyloid formation. It would also be important to determine the effect of nocodazole and/or colchicine on amyloid formation in this system. We observed that nocodazole, a microtubule toxin with a mode of action similar to that of colchicine, reduces the number of ASC-expressing cells with specks (see Chapter 5). If colchicine treatment also reduces the nucleation of amyloid in this cell culture model, this could provide the first molecular explanation as to why colchicine therapy is so effective in the treatment and prevention of amyloidosis in FMF patients.

It is also noteworthy that the Alnemri Laboratory has reported the isolation of formed ASC specks (Fernandes-Alnemri et al., 2007). Thus, it may be

possible to test the ability of these isolated specks, with and without wild type and mutant forms of pyrin, to nucleate amyloid in a cell-free experimental setup, in which SAA (potentially even without AEF) is present. In such an assay, it would be possible to determine whether ASC alone or the ASC-pyrin combination, constitutes an amyloid accelerator. Our prediction, based on the known beta-sheet-rich structure of pyrin's RFP is that pyrin itself is involved (particularly mutant pyrin); such beta sheet structures are known amyloid accelerators. This could explain the predilection for amyoidosis in FMF patients.

Is there a functional connection between pyrin and cell migration?

Another area of pyrin research that remains to be explored is the nature of pyrin's interaction with the leading edge of migrating cells. Cell migration is critical to the development, growth, and maintenance of tissues, and depends largely upon actin reorganization and polymerization. As previously discussed, it was observed in our lab that both ASC and pyrin localize to the actin-rich leading edge of migrating cells, and to *Listeria* rocket tails (see Chapter 2), although the functional nature of this connection remains to be elucidated. A huge burst of cell migration is observed during FMF attacks, which invites the speculation that FMF is a disease of overly robust cell migration. However, it is important to remember that the principal cell involved in this influx is the neutrophil, a cell in which pyrin is nuclear. This would indicate that pyrin likely does not localize to the leading edge in these cells, although this has not been directly tested.

Therefore, if pyrin alters migration in these cells the effects may be due to actions apart from the leading edge. One clue may lie in the connection between pyrin-associated pathways and ARG kinase. Work in other laboratories has shown that an ARG-mediated molecular coupling switch between p130Cas and Crk uses phosphorylation to regulate the decision for a cell to migrate and survive or remain static and die (Bouton et al., 2001). Siva is an ARG substrate, and PSTPIP1 binds to ARG, indicating that the phosphorylation status of these proteins, and thus their action within the cell, may be functionally connected.

How might pyrin modulate ASC speck formation and function?

Several observations about pyrin and pyrin-expressing cells have been made in our laboratory, and it is not yet clear how these findings fit into the FMF picture. For example, human neutrophils and monocytes express both ASC and pyrin, as shown by immunofluorescence studies in our lab. In neutrophils, pyrin is nuclear, but ASC specks are cytoplasmic and pyrin does not co-localize with them. ASC as been shown to be able to shuttle between the nuclear and cytoplasmic compartments in other cell types (Balci-Peynircioglu et al., 2008), leaving open the possibility that ASC may enter the nucleus and interact with pyrin, although not in speck form. If this occurs, how does the functional nature of the ASC/pyrin interaction differ in this cell type versus others? It will interesting to investigate the response of neutrophils from pyrin -/- mice to inflammatory stimuli to determine whether ASC upregulation is pyrin-dependent.

Studies in multiple cell types have shown that expression of ASC leads to speck formation in the absence of other factors, yet monocytes in culture do not form specks at an appreciable rate. Provocatively however, when monocytes are co-cultured with lymphocytes (which do not express pyrin or ASC) speck formation is efficient, even without additional stimulation (Philip Schaner, unpublished observations). This indicates that some lymphocyte factor may be required for speck formation in monocytes. Several factors must be investigated if the basis for this observation is to be uncovered. First, in this experiment, monocytes and lymphocytes were separated from whole blood, but lymphocytes were not further fractionated. What fraction of lymphocytes produced the rise in speck formation in the co-cultured monocytes? Several lymphocyte subtypes, including NK cells (innate immune effectors), CD4+ cells, IL-4R+ cells (Th2), and IFNγ+ cells (Th1), need to be explored. Additionally, monocytes were cultured in direct contact with lymphocytes. Is this cell:cell contact required, or do lymphocytes release a diffusible factor that can induce speck formation in monocytes? This question can be answered by separating monocytes from lymphocytes by a filter that will allow secreted factors to diffuse through it, but will prevent contact between the two cell types. If a diffusible factor is found to be responsible, it can be further identified with commercial cytokine preparation. If, however, cell:cell contact is required, this would suggest that an integral membrane protein is responsible.

If a membrane fraction is necessary for speck formation in monocytes, one particular candidate protein immediately comes to mind: the $P2X_7$ receptor ($P2X_7R$). The $P2X_7R$ is a non-specific ATP-gated ion channel that is expressed on monocytes and macrophages. When stimulated with millimolar concentrations of ATP, it forms large non-selective plasma membrane pores that result in a complete collapse of ionic gradients (Kahlenberg and Dubyak, 2004). As previously mentioned, recent work has shown that potassium efflux from THP-1 cells results in speck formation and nearly immediate cell death by pyroptosis (Fernandes-Alnemri et al., 2007). Additionally, it has been shown that potassium efflux through $P2X_7R$ macropores results in the activation of Caspase-1 and subsequent processing of IL-1β in macrophages (Kahlenberg and Dubyak, 2004; Perregaux and Gabel, 1994). The steps linking potassium efflux through $P2X_7R$ to caspase-1 activation have yet to be clarified; however, the addition of ASC to cell lysates potentiates $P2X_7R$-mediated activation of Caspase-1 (Kahlenberg and Dubyak, 2004).

It has been noted that ASC speck formation and subsequent death in THP-1 cells occurs in the absence of ATP stimulation, and that a naturally-occurring extracellular environment with millimolar concentrations of ATP is unlikely (Fernandes-Alnemri et al., 2007). However, such conditions do exist in certain microenvironments such as synapses between cells (Labasi et al., 2002; Verderio and Matteoli, 2001). Note that the immunological synapse is known to contain PSTPIP1, a pyrin-interacting protein discussed in Chapter 4 (Badour et

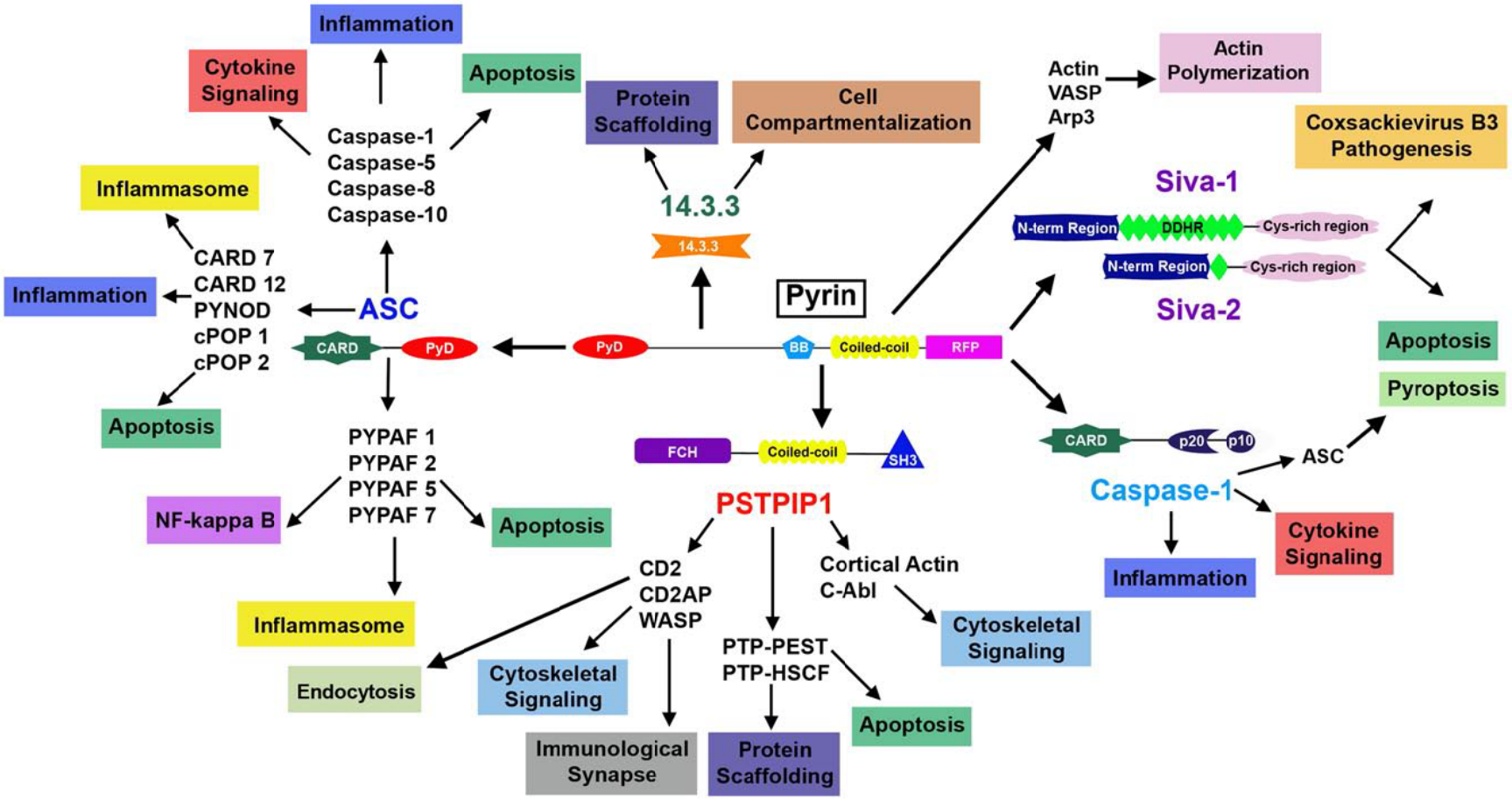
al., 2003). If monocyte:lymphocyte contact is indeed required to induce speck formation in monocytes, it may be that an immunological synapse forms between the two cells, creating the necessary microenvironment for $P2X_7R$ stimulation and potassium efflux.

Such a powerful cell death stimulus would have to be tightly controlled, especially given the rapidity of Caspase-1 activation and death following speck formation (Fernandes-Alnemri et al., 2007). This system contains such control, and would preclude widespread cell death due to a diffusible factor. Additionally, this system provides a possible mechanism for FMF attacks. It has previously been shown that cells expressing both ASC and pyrin form specks more efficiently than cells expressing ASC alone, but that these cells are delayed in their death (Richards et al., 2001). Upregulation of pyrin in response to some inflammatory stimulus could enhance the ability of a monocyte to form specks after $P2X_7R$ stimulation and may delay cell death, thereby prolonging the inflammatory signaling through Caspase-1 that is seen after speck formation. Substituting mutant pyrin further exacerbates the effect seen with wildtype pyrin, causing even more robust speck formation (Richards et al., 2001). This implies that in the presence of mutant pyrin, speck-initiated Caspase-1 signaling may be enhanced even further, leading to the robust inflammatory response seen in FMF attacks.

Conclusion

While the pyrin protein with its many binding partners and associated pathways presents a challenging and fascinating area to study, some may question the wisdom behind devoting time and energy to studying a disease that affects so few people worldwide. However, the advances in understanding innate immunity and inflammatory processes that have come about as a result of studying Familial Mediterranean Fever and other autoinflammatory diseases have been remarkable (for a summary, see Figure 6.1). We have identified critical, central inflammatory signaling molecules such as ASC and cryopyrin, and several factors that may exact tight control over their function according to the specific circumstances. We have discovered macromolecular complexes that are crucial signaling hubs in the cell. We have identified previously unknown connections between various cellular pathways, and discovered what may be general mechanisms of pathway regulation. While significant advancements have been made in recent years, much work remains to be done and will undoubtedly lead to compelling new insights in the field of innate immunity.

Figure 6.1: Pyrin-interacting proteins and associated pathways. Through connections with its binding partners, pyrin is linked to the modulation of several cellular pathways. Through its N-terminal PyD, pyrin binds to ASC and is connected to apoptosis, inflammation (both involving the inflammasome and separately from it), NF-κB, and cytokine signaling. Pyrin's subcellular localization is controlled in part through its connection to 14.3.3 proteins, which also link pyrin to the process of protein scaffilding. Through its Bbox/Coiled-coil domain, pyrin interacts with actin, VASP, and Arp3 (through which it is connected to actin polymetization), and to PSTPIP1. PSTPIP1 links pyrin to the processes of cytoskeletal signaling, formation and function of the immunological synapse, endocytosis, protein scaffolding, and apoptosis. Pyrin's RFP domain binds to Siva-1 and Siva-2, which are implicated in apoptosis and in the pathogenesis of Coxsackievirus B3. Finally, through its RFP domain pyrin binds to Caspase-1, which links it to inflammation, cytoskeletal signaling, and a form of inflammatory cell death known as pyroptosis.


Inflammation
Cytokine Signaling
Apoptosis
Caspase-1
Caspase-5
Caspase-8
Caspase-10
Protein Scaffolding
Cell Compartmentalization
14.3.3
14.3.3
Actin
VASP
Arp3
Actin Polymerization
Coxsackievirus B3 Pathogenesis
Siva-1
N-term Region
DDHR
Cys-rich region
N-term Region
Cys-rich region
Siva-2
Inflammasome
CARD 7
CARD 12
PYNOD
cPOP 1
cPOP 2
Inflammation
ASC
CARD
PyD
PyD
Pyrin
BB
Coiled-coil
RFP
Apoptosis
Pyroptosis
Apoptosis
PYPAF 1
PYPAF 2
PYPAF 5
PYPAF 7
FCH
Coiled-coil
SH3
CARD
p20
p10
ASC
Caspase-1
NF-kappa B
Apoptosis
PSTPIP1
Cytokine Signaling
Inflammation
CD2
CD2AP
WASP
Cortical Actin
C-Abl
Inflammasome
Cytoskeletal Signaling
PTP-PEST
PTP-HSCF
Endocytosis
Cytoskeletal Signaling
Apoptosis
Immunological Synapse
Protein Scaffolding

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