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SHARPIN regulates mitochondria-dependent apoptosis in keratinocytes

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Abstract

Background—The chronic proliferative dermatitis mutation (CPDM) in mice, due to *Sharpin* deficiency (*Sharpin*^{cpdm}), is a multisystem disorder characterized by peripheral blood eosinophilia and eosinophil infiltration of affected tissues including the skin, bone marrow, spleen, lung, heart, and other organs. The epidermis has numerous apoptotic keratinocytes which increase with age, coalesce, form vesicles, and rupture causing ulceration.

Objective—To clarify the molecular pathways involved in the keratinocyte apoptosis caused by loss of function of SHARPIN in mice.

Method—10-week-old *Sharpin*^{cpdm} and wildtype mice were used for experiments. Ultrastructural changes of skin were evaluated by transmission electron microscopy. Cross points of mitochondrial pathway were analyzed by *in vitro* and *in vivo* cellular and molecular assays.

Results—77.5% skin cells in *Sharpin*^{cpdm} mice were functionally apoptotic and dead cells, compared to only 18.1% unhealthy skin cells in wildtype mice, indicated by annexin-V/propidium iodide FACS analysis. Mitochondria in keratinocytes were disrupted containing prominent electron dense inclusions and membrane potential depolarization, accompanied by a shift in protein expression between the anti-apoptotic BCL2 and pro-apoptotic BAX proteins. Enzymatic activities of caspases 9 and 3, but not 8, were markedly increased in *Sharpin*^{cpdm} keratinocytes. Caspase-3 was cleaved in most cells in skin of 10-week-old mutant mice.

Conclusion—The present results indicated that keratinocyte apoptosis in *Sharpin*^{cpdm} mice was regulated by an intrinsic caspase-dependent mitochondria pathway.

Keywords

Sharpin; mice; keratinocyte; apoptosis; mitochondria

INTRODUCTION

Chronic inflammatory scaly skin diseases, e.g., psoriasis and atopic dermatitis, are common heterogeneous dermatoses in humans that result in psychosocial morbidity and a decrease in

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health-related quality of life[1,2]. The chronic proliferative dermatitis mutation (CPDM) in mice is an autosomal recessive, spontaneous, systemic inflammatory disorder. Mice develop granulocytosis affecting the liver, spleen, and lymph nodes. Their secondary lymphoid organs do not develop normally which was initially interpreted as an absence of Peyer's patches in adult mice[3–5]. The skin lesions were described as "psoriasiform dermatitis" in that the mice develop inflamed, scaly skin, somewhat reminiscent of psoriasis[3,6–8]. At the molecular level this mouse mutation does not mimic psoriasis in that there is relatively normal keratinocyte (KC) differentiation[9], a Th2 but not Th1 type inflammatory response[10], eosinophilia, and other differences. Loss of function of SHARPIN was identified as the genetic basis of CPDM (current symbol *Sharpin*^{cpdm})[11]. SHARPIN is a novel protein thought to interact with SHANK family proteins and is widely expressed in multiple tissues in both the human and mouse (http://biogps.gnf.org/)[12]. SHARPIN is predicted to be a protein of 380 amino acids with the exon 8 splice variant encoding 315 amino acids. Although the predicted protein does not show extensive homology with other known proteins, 3 previously defined motifs are present, such as the Hoil1-N domain of ubiquitin-protein ligase activity, region AA172-305 interacting with SHANK, and C terminal zinc finger domain[13]. The actual function of SHARPIN is poorly understood in mammals, although apparently it is associated with neurologic signaling[12].

The spontaneous null mutation of *Sharpin* (*Sharpin*^{cpdm}) causes mice to undergo abnormal KC death in the skin, esophagus, and forestomach[14]. It is now well established that many physiological processes require a balance between apoptosis and cell proliferation. Homeostasis in the skin is maintained by a balance between cell proliferation of basal KCs and terminal differentiation of suprabasal KCs. Differentiation involves irreversible exit of KCs from the cell cycle and transit through the suprabasal, spinous, granular, and ultimately cornified layers of the epidermis. It is essential for KCs to survive during the differentiation processes to carry out their barrier and systemic functions before ultimately dying by cornification[15]. The transition of KCs from the granular to cornified layer during differentiation resembles a special form of cell death in some aspects[16,17]. The process is genetically preprogrammed, in which KCs become enucleated, lose all organelles, shrink, and die without inducing an inflammatory response. Different from other, more common forms of apoptosis, classical apoptotic caspases (CASP 3, 8, and 9) are not activated during KC terminal differentiation[18].

Apoptosis is induced by a plethora of physiologic and pathologic stimuli that activate the apoptotic machinery in a stimulus-specific manner. Two general mechanisms of apoptosis were described for mammalian cells: the intrinsic pathway, activated in response to cellular stresses, and the extrinsic pathway, initiated at the cell surface upon activation of death receptors by binding of their cognate ligands[19,20]. The intrinsic "mitochondrial pathway" transduces cellular stress/damage signals, resulting in release of cytochrome c-1 (CYC1) and other mitochondrial proteins from the intermembrane space. CYC1 binds to adaptor molecules forming an "apoptosome"[21]. The formation of this complex leads to the activation of its effector, CASP9, which can then activate downstream effector caspases (e.g., CASP3), resulting in apoptosis. In contrast, the extrinsic pathway is induced by the binding of death ligands (TNF, FAS) to their cognate death receptors at the cell surface. Activated death receptors oligomerize, thereby inducing the formation of the death-inducing signaling complex (DISC) on their intracellular parts[22]. The DISC recruits CASP8 molecules that dimerize and activate each other forming an active tetramer, the release of which initiates the caspase cascade causing the proteolysis of hundreds of target proteins and eventually cell death[23]. The extrinsic and intrinsic pathways converge at the level of the effector caspases, and interaction can occur between the two pathways.

We show here that loss of SHARPIN function in a mouse model causes marked KC apoptosis through the mitochondrial pathway in a caspase-dependent manner.

MATERIALS AND METHODS

Mice

C57BL/KaLawRij-*Sharpin*^{cpdm}/RijSunJ (JR# 007599; The Jackson Laboratory, Bar Harbor, ME) and C57BL/KaLawRijSunJ-+/+ mice were maintained in the humidity, temperature, and light cycle (12:12) controlled vivarium under specific pathogen-free conditions. Mice were housed in double-pen polycarbonate cages (330 cm^2 floor area) at a maximum capacity of four mice per pen. Mice were allowed free access to autoclaved food (NIH 31, 6% fat; LabDiet 5K52, Purina Mills, St. Louis, MO) and acidified water (pH 2.8–3.2). This work was done with the approval of the Institutional Animal Care and Use Committee approved protocols.

Immunohistochemistry

Dorsal skin was collected and fixed by immersion in Fekete's acid alcohol formalin solution and then transferred after 12 hr into 70% ethanol until processed. Fixed tissues were embedded routinely in paraffin and serially sectioned at 6 μm. Paraffin sections were labeled at 1:100 for cleaved CASP3 (Cell Signaling, Danvers, MA). The number of positive cells were counted per linear mm of tissue section.

Transmission electron microscopy (TEM)

Strips of full-thickness skin samples (5 × 10 mm) from 2, 4, 6, 8, and 10 week old mice were immersed immediately at the time of necropsy in cold fixative consisting of 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and processed routinely. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEM-1230 transmission electron microscope (Jeol USA Inc., Peabody, MA). Quantitative analysis for damaged mitochondria was performed for 10 randomly selected fields at 20,000 × magnification and element analysis for mitochondria inclusion followed at 120,000 × magnification using an EDAX X-ray microanalsys system (Mahwah, NJ).

Apoptosis assays *in vivo* and *in vitro*

Apoptosis was evaluated on paraffin embedded sections of skin tissues by Tdt-mediated dUTP nick-end labeling (TUNEL) using *In Situ* Cell Death Detection Kit TMR red (Roche) per the manufacturer instructions. Rehydrated tissues were treated in 0.1 M citrate buffer, pH 6.0 with 350W microwave irradiation for 5 min. Slides were then incubated with 50 μl of TdT incubation buffer at 37 °C for 60 minutes inside a dark humid chamber. The reaction was terminated by immersing the slides in 2X standard saline citrate for 15 min. The tissue orientation was determined by final staining with 100 ng/ml DAPI solution. Apoptotic cells were visualized immediately with a confocal microscope, resulting in localized red fluorescence within the nucleus of the apoptotic cells.

Epidermal KCs were prepared from adult mice essentially as described previously[24]. For flow cytometric analysis, suspensions of KCs (1×10^6) from 10 week old mice were fixed in 70% EtOH at 4 °C. After fixation cells were permeabilized with 0.01% Triton X-100 and centrifuged. The pellets were resuspended in PBS and treated with PE conjugated Annexin-V and propidium iodide. Cells were counted using flow cytometry (BD Biosience). Approximately 25,000 events were acquired for analysis using Cell Quest 3.3 Software (BD Biosience). A histogram plot of FITC fluorescence (x axis) versus counts (y axis) was generated in logarithmic fluorescence intensity.

Evaluation of mitochondrial membrane potential

Fresh KCs isolated from 3 *Sharpin*^{cpdm} and 3 +/+ female mice at 10 weeks of age were stained with a bivariate mitochondrial membrane potential cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolycarbocyanine iodide (JC1) following the manufacturer's protocol (Cell Technology Inc, Mountain View, CA). JC1 is a potentiometric dye that exhibits a membrane potential – dependent loss as polarized mitochondria transition depolarized mitochondria; the loss of membrane potential is indicated by the fluorescence emission shift from red to green.

Assessment of CASP3, CASP8, and CASP9 enzyme activities

CASP3, CASP8, and CASP9 activity in KCs isolated from 3 *Sharpin*^{cpdm} and 3 +/+ female mice at 10 weeks of age was measured using the Caspase-Glo assay kit (Promega, Madison, WI) as directed by the manufacturer, with modifications. Briefly, fresh KCs (5×10^3 cells/well) were plated in a black-walled 96-well luminometer plate and incubated with Caspase-Glo 3, 8, or 9 buffer with luciferase and proluminescent substrates (provided by the manufacturer), containing one of the following amino acid sequences: DEVD, LETD, or LEHD, which are cleaved by CASP3, CASP8, and CASP9, respectively. An equal volume (100 μl) of the buffer–substrate mixture was added to each test well of the plate which was gently mixed at 37 °C with shaking at 500 rpm for 1 min and then incubated at RT for 2 hours. The luminescence of each well was measured in a plate-reading luminometer (PerkinElmer, Waltham, MA). The control for background luminescence was tissue culture media alone. Three independent experiments were performed in triplicate.

Immunoblotting of proteins involved in apoptotic pathways

Fresh KCs were harvested from *Sharpin*^{cpdm} and +/+ female mice at age of 10 weeks. The mitochondria fraction was isolated from KCs using a mitochondria isolation kit (Thermo Scientific, Rockford, IL) per the manufacturer's instructions and analyzed by Western blot as described below. Ten ng of proteins and 5 ul of multicolor ladder (Bio-Rad, Hercules, CA) were fractionated using Criterion XT Bis-Tris gel (Bio-Rad) and electrophoretically transferred and blotted onto immune-blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Nonspecific binding was blocked by overnight incubation of the membranes with blocking buffer (5% nonfat dry milk, 0.05% Tween-20 in TBS). The blots were then incubated 1 h with primary antibodies (BCL2, BAX) at 1:1000 in blocking buffer. After 3 washes, the membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Thermo Scientific, Rockford, IL) at 1:2000 in blocking buffer 1 h. The blots were developed using enhanced ECL substrate detection kit (PerkinElmer), and then scanned and quantified using LAS-1000 plus (Fujifilm AG, Dielsdorf, Switzerland). Densitometry analysis was performed using software Image J.

RESULTS

Keratinocyte apoptosis in *Sharpin*^{cpdm}/*Sharpin*^{cpdm} mice via mitochondria pathway in a caspase-dependent manner

In opposition to the intensive proliferation observed in the basal epithelium, increased numbers of apoptotic cells were found in epithelial tissues of *Sharpin*^{cpdm}/*Sharpin*^{cpdm} (hereafter referred as *Sharpin*^{cpdm}) mutant mice, including the epidermis of the trunk and face, esophagus, and stomach. A molecular hallmark of apoptosis is DNA degradation to high molecular weight or oligonucleosome-length fragments[25]. DNA fragmentation was evaluated in KCs using the TUNEL assay. Cells with nuclear damage were TUNEL-positive, whereas cells with a nucleus still morphologically intact were negative. As shown

in figure 1, TUNEL positive KCs increased in skin of *Sharpin*^{cpdm} mutant mice compared to those in wildtype (hereafter referred as +/+) mice at 10 weeks of age.

Annexin-V is an appropriate marker of any type of cell death—apoptosis, autophagy, or necrosis—because cells undergoing either apoptosis or autophagy have been shown to externalize phosphatidylserines and because corpses generated by any mechanism ultimately lose their plasma membrane integrity[26]. Propidium iodide staining similarly reveals cells lacking membrane integrity. Flow cytometry was used to quantify Annexin-V/propidium-positive cells in the whole population as the number of population doublings increased. Both Annexin-V and propidium-negative cells ranged from 81.9% in the controls to 22.5% in the *Sharpin*^{cpdm} mice at 10 weeks of age (Figure 1), indicating increasing dead KCs in the skin of *Sharpin*^{cpdm} mice. CASP3 was cleaved in *Sharpin*^{cpdm} KCs and increased along with disease progression from 14 cells/mm of skin at the age of 2 weeks to 261 cells/mm at 10-wk age (Figure 2). Cleaveage of CASP3 initially and most prominently occurred in follicular KCs but was also evident in interfollicular epidermis of *Sharpin*^{cpdm} mutant mice as disease developed (Figure 2).

Loss of mitochondria membrane potential (MMP) induces mitochondria disruption in *Sharpin*^{cpdm} keratinocytes

To more specifically document that KCs die by apoptosis, the ultrastructure of KCs was evaluated in the dorsal skin. KCs of the healthy controls displayed a normal organization with a typical nucleus, several dispersed organelles, some rare vesicles, and a keratin intermediate filament network. Normal mitochondria were numerous and had two sets of membranes, a smooth continuous outer coat and an inner folded membrane that formed the cristae. By contrast, *Sharpin*^{cpdm} mitochondria had round, dark, electron dense inclusions surrounded by the inner membrane of the cristae, corresponding to changes of mitochondrial membrane potential[27]. These inclusions were present in KCs from the stratum basale to the stratum granulosum in *Sharpin*^{cpdm} mutant mice at 4, 6, 8, and 10 weeks of age and increased along with disease development (Figure 2H-K), but not in mitochondria in KCs in the skin of +/+ mice (Figure 2L) and 2 week old *Sharpin*^{cpdm} mutants (Figure 2G). Element analysis, specifically looking at calcium levels, did not reveal differences between damaged mitochontria in mutant mice compared to normal mitochondria in controls (data not shown).

The loss of MMP is caspase dependent in keratinocytes and thus occurs after caspase activation. To evaluate MMP in affected KCs in *Sharpin*^{cpdm} mice, we assayed MMP during apoptosis in freshly isolated *Sharpin*^{cpdm} and wildtype KCs. High fluorescence at 585 nm corresponds to the aggregated form of the dye and is proportional to an intact MMP, whereas loss of MMP leads to a loss of 585 nm fluorescence and an increase in fluorescence at 510 nm. Wildtype (+/+) KCs exhibit high 585 nm fluorescence, indicating normal MMP (Figure 3A). *Sharpin*^{cpdm} KCs exhibit in loss of 585 nm fluorescence and an increase in fluorescence at 510 nm, implicating a loss of MMP (Figure 3B).

Protein expression shift of BCL2/BAX with activation of CASP3 and CASP9 in *Sharpin*^{cpdm} keratinocytes

To identify the apoptotic pathway in which SHARPIN may have an essential role, protein levels of BCL2 and BAX in KCs of 10 week old *Sharpin*^{cpdm} mutant and +/+ mice were evaluated. Western blot analysis revealed downregulated expression of BCL2 but upregulated expression of BAX in the mitochondrial fraction of *Sharpin*^{cpdm} KCs compared to controls at 10 weeks of age when clinical disease is prominent (Figure 4). The expression ratio of BCL2/BAX was 0.86±0.11 in the mutant mitochondria, compared to that of 1.8±0.2 in the wildtype mitochondria.

Mitochondrial damage can be caused by the activation of either the extrinsic or intrinsic apoptosis pathways. To determine if KC apoptosis in *Sharpin*^{cpdm} mice is associated with one or both of these two apoptosis pathways, activities of CASP3, CASP8, and CASP9 were evaluated in freshly isolated KCs. The activities of cell death executioner CASP3 and the intrinsic marker CASP9 were significantly increased ($p<0.05$) (Figure 5). The extrinsic pathway enzyme CASP8 was mildly increased, but not significantly in *Sharpin*^{cpdm} KCs compared to control cells, implicating the intrinsic apoptosis pathway was causing apoptosis in *Sharpin*^{cpdm} mice.

DISCUSSION

The results described here provide important insights into the role of *Sharpin* in KC apoptosis. Decreased expression of SHARPIN increased susceptibility of KCs to apoptosis due to loss of MMP, protein expression shifts of BCL2/BAX, CASP9, and CASP3 activation which resulted in the morphological changes consistent with apoptosis.

Diseases associated with increased KC apoptosis tend to be acute, those associated with decreased apoptosis tend to be chronic[28]. Most skin diseases or cutaneous lesions characterized by epidermal hyperplasia or hyperkeratosis likely involve decreased KC apoptosis. As described previously, *Sharpin*^{cpdm} mice present with a “psoriasiform dermatitis” characterized by hyperproliferation of the epidermis associated with inflammation in the dermis. Although earlier work categorized CPDM as a spontaneous mouse model of human psoriasis [6], human genome wide association studies showed no significant linkage to *SHARPIN* in the psoriasis cohort[29,30].

In the mutant mice, apoptosis was also observed in all cornified squamous epithelia including epidermis, esophagus, and forestomach. Apoptotic KCs were distributed among all layers of epidermis and dramatically increased as the disease progressed. As mice age, the thick, scaling, inflamed skin becomes pruritic and mice are euthanized when scratches and ulcers result at 10 weeks of age following IACUC standards.

In a previous study describing the ultrastructure of epidermis in mice with chronic proliferative dermatitis, disrupted mitochondria were reported in epidermal KCs[14]. Both extrinsic (receptor-mediated) and intrinsic (mitochondria-mediated) apoptosis pathways result in these morphological changes in mitochondria and it remained obscure if mitochondrial damage is a primary intrinsic event regulated by *Sharpin* or a secondary, extrinsic event of due to the severe dermal inflammation.

Through a death domain mediated binding of the adaptor proteins TRADD (TNFRSF1A) and FADD (all death receptors), death receptors recruit and activate the apoptosis-initiating proteases CASP8 and/or CASP10 that induce apoptosis either by direct activation of effector caspases or via a BAX/ BCL2-antagonist/killer 1 (BAK1)-dependent MMP triggered by CASP8-mediated cleavage of BH3 interacting domain death agonist (BID). Alternatively, CYC1 released from disrupted mitochondria induces cleavage of CASP9 with formation of the “apoptosome”, which activates effector caspases. Abnormal mitochondria were confirmed untrastucturally in KCs of mutant skin increasing with age, consistent with increasing cleaved CASP3 positive cells. Apoptotic cells share a number of common features such as cell shrinkage, membrane blebbing, chromatin cleavage, nuclear condensation, and formation of pyknotic bodies of condensed chromatin[31]. These apoptosis features were not seen in the skin of 2 week old *Sharpin*^{cpdm} mutants which already have detectable cleaved CASP3 positive cells, implicating mitochondria damage and CASP3 cleavage were the cause instead of consequence of apoptosis in this case. FACS analysis using JC1 identified loss of MMP in KCs of *Sharpin*^{cpdm} mice, connecting mitochondrial

damage with apoptosis. Activities of critical caspases were detected in both extrinsic and intrinsic apoptosis pathways. Although severe inflammation exists in *Sharpin*^{cpdm} skin, activity of CASP8 was not significantly increased, suggesting that extrinsic apoptosis is not the key executioner. In contrast, activity of CASP9, activated by release of mitochondrial contents, together with the activity of executor CASP3, was dramatically increased. These results support KC apoptosis in *Sharpin*^{cpdm} mice as an intrinsic problem.

In summary, several lines of evidence are presented indicating SHARPIN as a novel anti-apoptosis protein that protects against intrinsic mitochondrial apoptosis in mouse KCs.

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Abbreviations

BCL2	B-cell leukemia/lymphoma 2
BAK1	BCL2-antagonist/killer 1
BAX	pro-apoptotic BCL2-associated X protein
BID	BH3 interacting domain death agonist
CASP	caspase
CPDM	chronic proliferative dermatitis mutation
CYC1	cytochrome c-1
DISC	death-inducing signal complex
DIABLO	diablo homolog
FAS	TNF receptor superfamily member 6
FACS	fluorescence activated cell sorting
FADD	Fas-associated via death domain
KC	keratinocyte
JC1	tetraethylbenzimidazolycarbocyanine iodide
MMP	mitochondrial membrane potential
Sharpin	SHANK-associated RH domain interacting protein
TEM	transmission electron microscopy
TRADD	TNFRSF1A-associated via death domain
TUNEL	Tdt-mediated dUTP nick-end labeling
TNF	tumor necrosis factor

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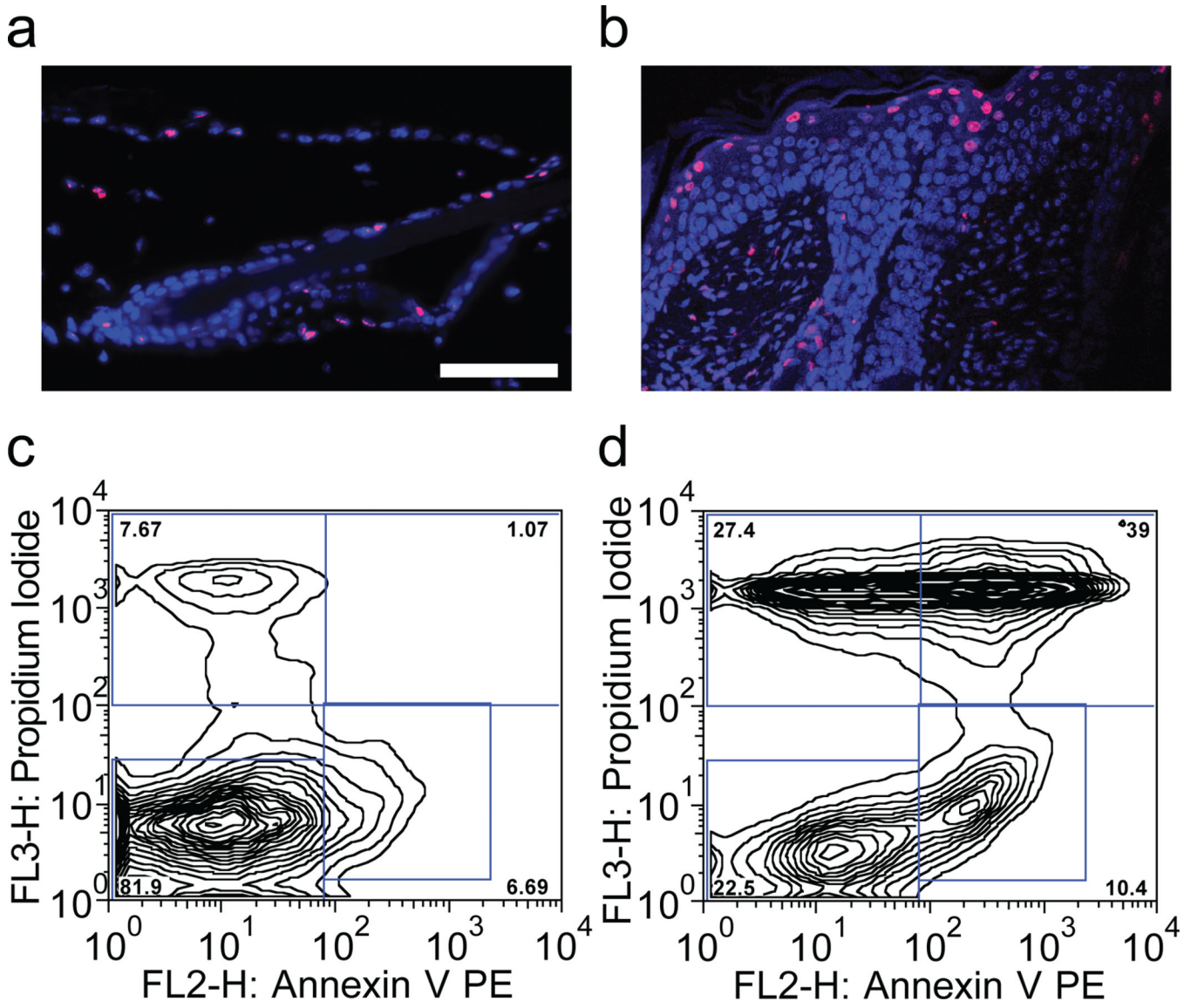

Figure 1. Apoptosis assays confirmed increased dead keratinocytes in *Sharpin*^{cpdm} skin compared to +/+ controls

Representative data for 3 female +/+ and *Sharpin*^{cpdm} mice at 10 weeks of age. Immunofluorescence revealed few TUNEL-positive cells (red nuclei) in skin of control mice (a) compared to large numbers in *Sharpin*^{cpdm} mice (b). Annexin-V and propidium iodide are markers that differentiate dead from live cells. Annexin-V and propidium iodide positive keratinocytes (dead cells, upper two quandrants) increased in *Sharpin*^{cpdm} skin (d) compared to age- and gender-matched +/+ controls (c). Scale bar = 100 um.

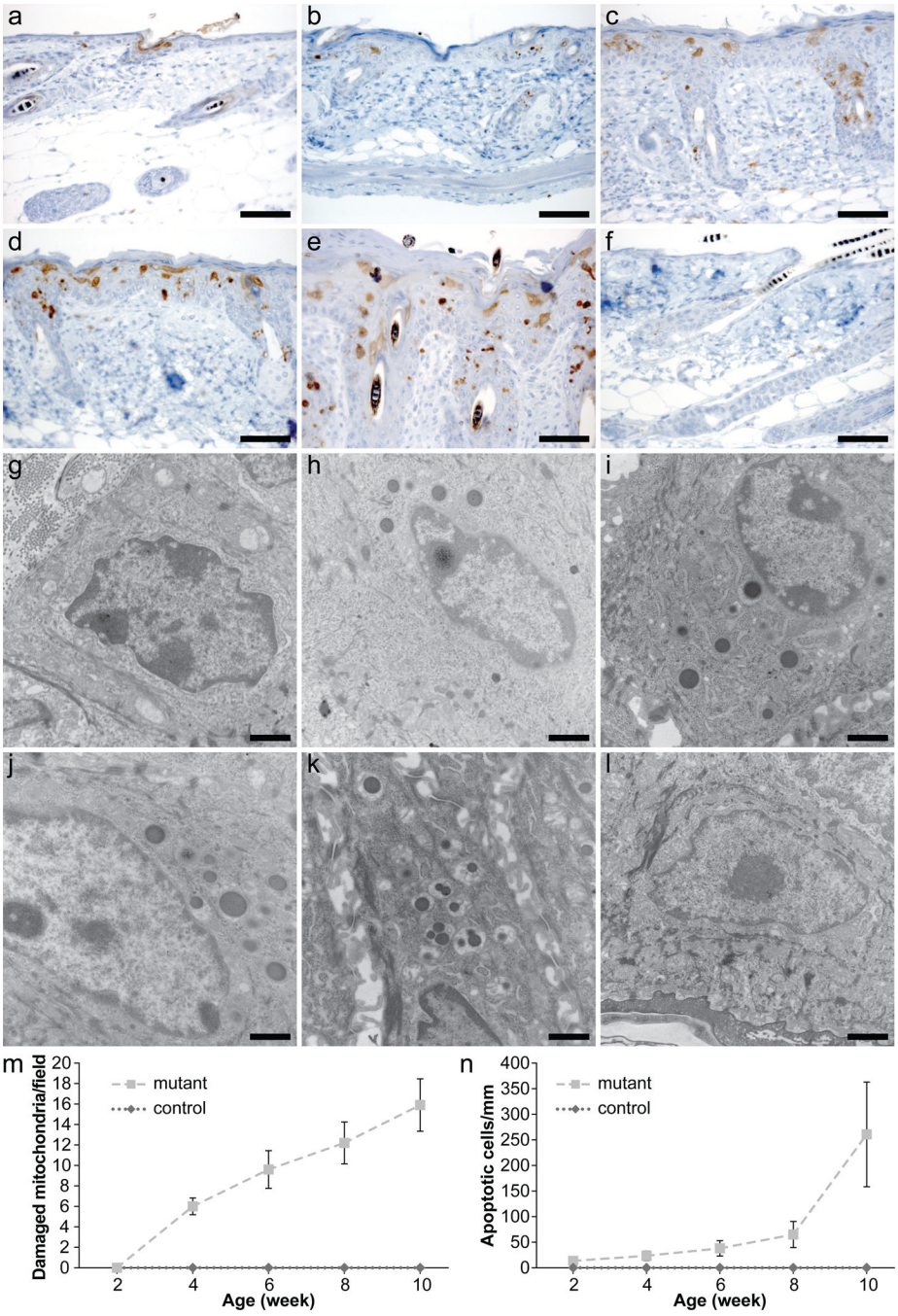

Figure 2. Caspase 3 cleavage and mitochondria damage starts before apoptosis in *Sharpin*^{cpdm} kerationocytes

Some keratinocytes were positive for cleaved caspase 3 as early as 2 weeks of age for *Sharpin*^{cpdm} mice (a) and increased with disease progression at 4, 6, 8, and 10 weeks of age (b-e). No cleaved caspase-3 positive keratinocytes were found in +/+ mice at any time point (f). Mitochondria contained prominent electron dense vacuoles within cristae in KCs of 4 week old *Sharpin*^{cpdm} mutants that increased in size and number as disease progressed (h-k). By contrast, no mitochondrial changes were detected in KCs of 2 week old mutants (g) or +/+ controls at any age (l). Damaged mitochondria (m, mean +/− SD) or cleaved caspase 3 positive cells (n, mean +/− SD) were quantified by counting abnormal mitochondria or

immunohistochemically positive cells, respectively, in 10 fields per sample. Scale bar = 5 um (a-f), 500 nm (g-i).

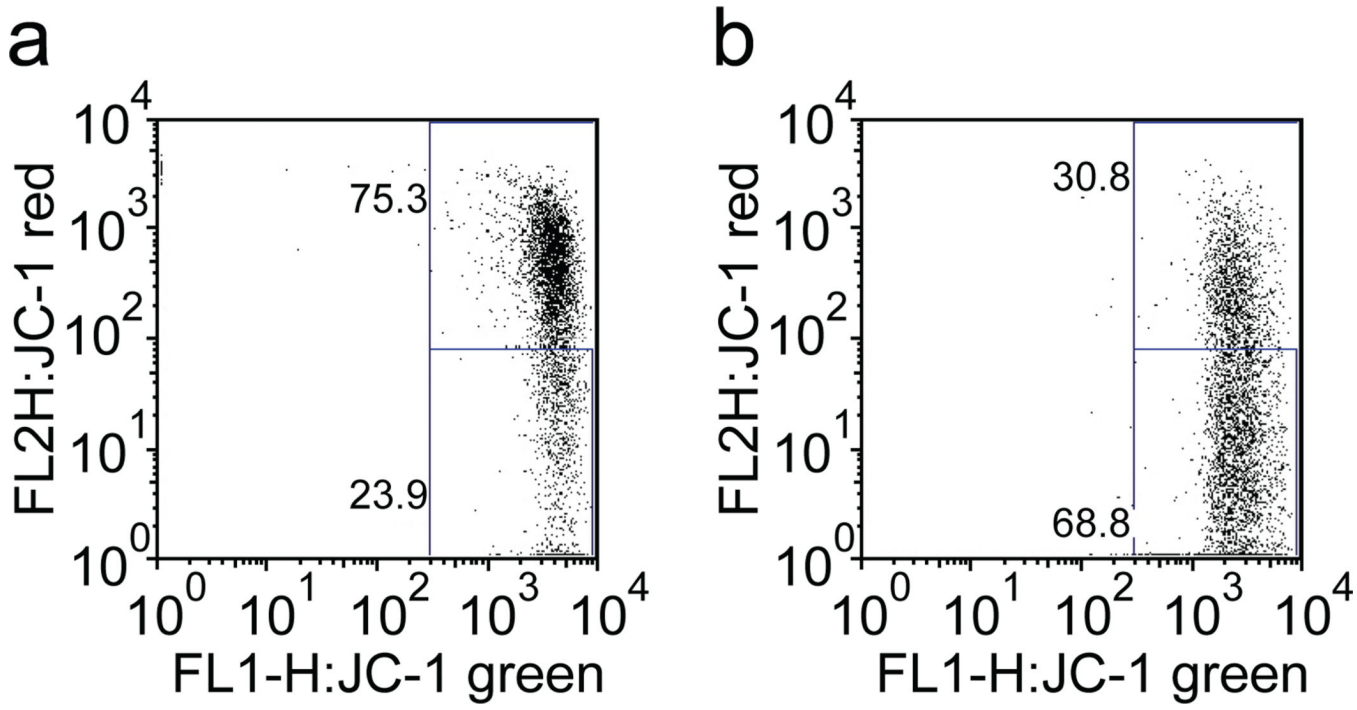

Figure 3. Loss of mitochondria membrane potential of keratinocytes from *Sharpin*^{cpdm} mice

JC-1 is a potentiometric dye that exhibits a membrane potential–dependent loss as mitochondria transition from polarized to depolarized. The loss of membrane potential is indicated by the fluorescence emission shift from red to green. In normal, non-apoptotic cells from 10 week old wildtype mice (a) JC-1 exists as a monomer in the cytosol (green, lower right quadrant) but it also accumulates as aggregates within the mitochondria (red, upper right quadrant; a, 23.9% green, 75.3% red). In mutant mice (b) that have apoptotic and necrotic cells, JC-1 exists primarily as the monomeric form (green) within the cytoplasm (68.8% green, 30.8% red) indicating that the mitochondria are damaged.

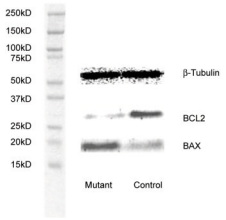

Figure 4. BCL2/BAX shift

Protein expression of BCL2 (anti-apoptotic) and BAX (pro-apoptotic) shifted in the mitochondrial fraction of keratinocytes from 10 week old *Sharpin*^{cpdm} mutant mice (left) associated with marked increase in keratinocyte apoptosis compared to +/+ control mice (right).

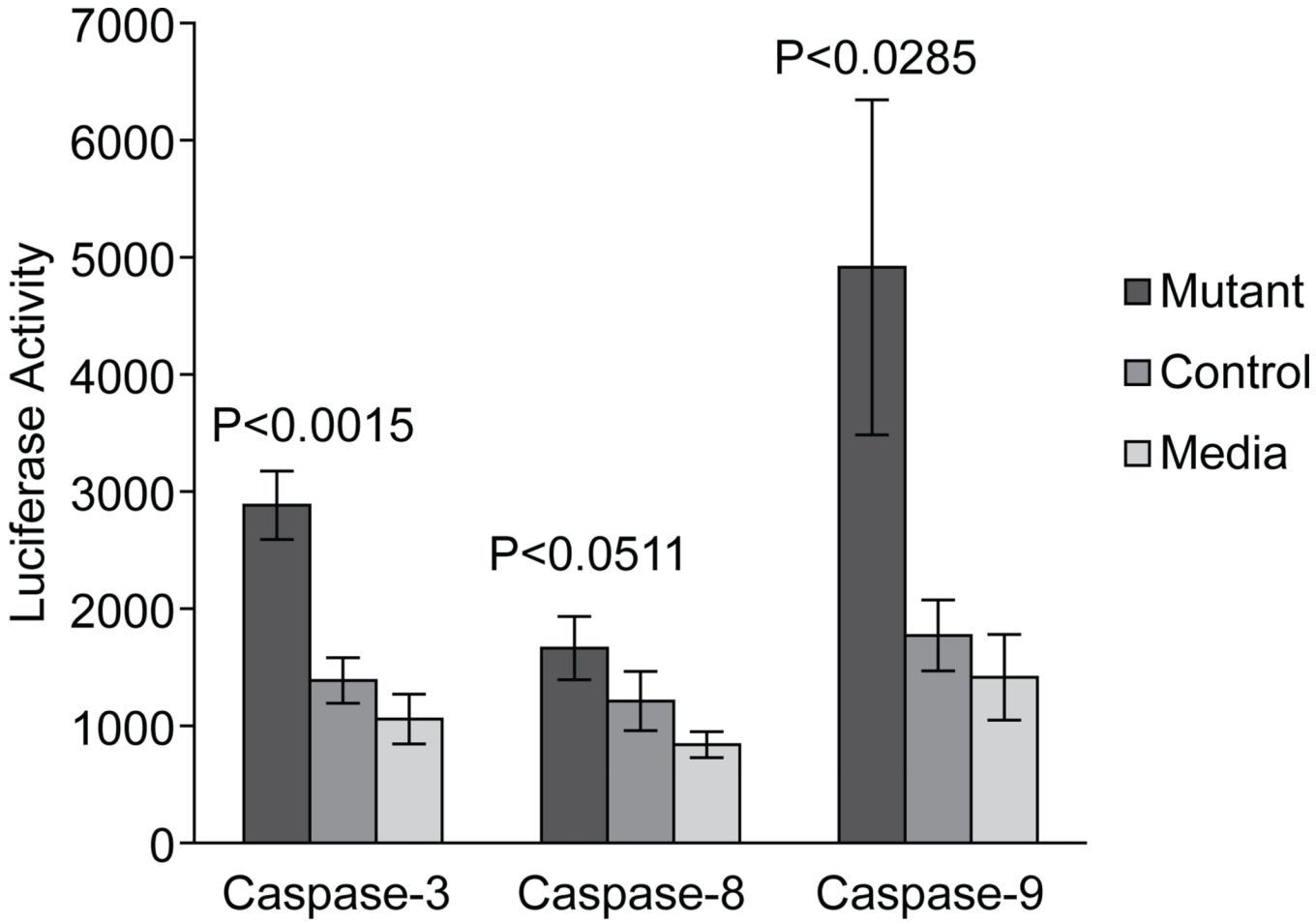

Figure 5. Caspase assays confirmed the involvement of the intrinsic apoptosis pathway

Significant activation of caspase 9 and caspase 3 ($p<0.05$), but not caspase 8, in keratinocytes from 3 female *Sharpin*^{cpdm} and +/+ mice at 10 weeks of age confirmed the role of the intrinsic mitochondrial apoptosis pathway in chronic proliferative dermatitis mice.