The caspase-cleaved form of LYN mediates a psoriasis-like inflammatory syndrome in mice

Sandrine Marchetti^{1,2,3}, Parvati Gamas^{1,2,3},
Nathalie Belhacène^{1,2,3}, Sebastien
Grosso^{1,2,3}, Ludivine A Pradelli^{2,4},
Pascal Colosetti^{1,2,3}, Claus Johansen⁵,
Lars Iversen⁵, Marcel Deckert^{2,6},
Frédéric Luciano^{1,2,3}, Paul Hofman^{2,7,8},
Nicolas Ortonne⁹, Abdallah Khemis¹⁰,
Bernard Mari¹¹, Jean-Paul Ortonne¹⁰,
Jean-Ehrland Ricci^{2,4} and
Patrick Auberger^{1,2,3,*}

¹INSERM, U895, Centre Méditerranéen de Médecine Moléculaire (C3M), Team 2, Nice, France, ²Université de Nice Sophia-Antipolis, Faculté de Médecine, Signalisation et pathologies (IFR50), Nice, France, ³Equipe labellisée par la Ligue Nationale contre le Cancer, INSERM, U895, Nice, France, ⁴INSERM, U895, Centre Méditerranéen de Médecine Moléculaire (C3M), Team 3, Nice, France, ⁵Department of Dermatology, Aarhus University Hospital, Aarhus C, Denmark, ⁶INSERM, U576, Nice, France, ⁷INSERM ERI-21/EA4319, Nice, France, ⁸CHU de Nice, Laboratory of Clinical and Experimental Pathology, Nice, France, ⁹Department of pathology, AP-HP, Groupe Hospitalier Henri Mondor-Albert Chenevier, Creteil, France, ¹⁰CHU de NICE, Department of Dermatology, Nice, France and ¹¹CNRS, UMR6097, Institut de Pharmacologie Moléculaire et Cellulaire, Université de Nice-Sophia Antipolis, Valbonne, France

We showed previously that Lyn is a substrate for caspases, a family of cysteine proteases, involved in the regulation of apoptosis and inflammation. Here, we report that expression of the caspase-cleaved form of Lyn (LynΔN), in mice, mediates a chronic inflammatory syndrome resembling human psoriasis. Genetic ablation of TNF receptor 1 in a LynΔN background rescues a normal phenotype, indicating that LynΔN mice phenotype is TNF-α-dependent. The predominant role of T cells in the disease occurring in LynΔN mice was highlighted by the distinct improvement of LynΔN mice phenotype in a Rag1-deficient background. Using pan-genomic profiling, we also established that LynΔN mice show an increased expression of STAT-3 and inhibitory members of the NFκB pathway. Accordingly, LynΔN alters NFκB activity underlying a link between inhibition of NFκB and LynΔN mice phenotype. Finally, analysis of Lyn expression in human skin biopsies of psoriatic patients led to the detection of Lyn cleavage product whose expression correlates with the activation of caspase 1. Our data identify a new role for Lyn as a regulator of psoriasis through its cleavage by caspases.

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Introduction

The Src family of non-receptor protein tyrosine kinase, includes Src, Lyn, Fyn, Lck, Hck, Fgr, Blk, Yes and Yrk, and has a crucial function in cell-cycle control, adhesion, migration, proliferation, survival and differentiation of multicellular organisms (Thomas and Brugge, 1997). All Src family members share a common structural organization consisting of an N-terminal unique domain that contains acylation sites required for their localization to the plasma membrane; SH3 and SH2 (src homology) domains followed by a C-terminal catalytic tyrosine-kinase domain, and finally a region that contains conserved regulatory tyrosine phosphorylation sites (Ingley, 2008). Src kinases are activated by a wide variety of cell-surface receptors. The membrane localization of Src tyrosine kinases seems essential to trigger the intracellular signalling pathways. Indeed, removal of their acylation residues gives a completely non-functional protein in receptor signalling (Cross *et al*, 1985; Garber *et al*, 1985; Kabouridis *et al*, 1997; Lang *et al*, 1999).

The role of Src tyrosine kinase Lyn has been well established in haematopoietic cells (Xu *et al*, 2005), but several studies now indicate that Lyn also controls the behaviour of other tissues and cell types (Chen *et al*, 1996; Stettner *et al*, 2005; Zhao *et al*, 2006; Gong *et al*, 2008). We have recently identified Lyn as a new substrate for the apoptotic executioner caspases 3 and 7, observed to function as one on activation of B-cell antigen receptor and death receptors of the TNF family in immature B cells (Luciano *et al*, 2003). The cleavage of Lyn by caspases occurs in its N-terminal region (after Asp18) and leads to the relocation of the kinase from the plasma membrane to the cytosol. When overexpressed in immature B cells, this caspase-cleaved form, referred to as LynΔN, behaves as a cell death inhibitor (Luciano *et al*, 2003).

Caspases define a large family of aspartate-specific cysteine-dependent proteases with central functions during apoptosis. However, it is also clearly admitted that besides their well-characterized role in apoptosis, caspases can also have pivotal function in proliferation, differentiation and inflammation (Nadiri *et al*, 2006; Lamkanfi *et al*, 2007). Briefly, caspases are classified into two subfamilies: (i) inflammatory and (ii) apoptotic caspases. Activation of these is linked to the formation of multimeric protein complexes. Thus, during inflammation, the formation of the inflammasome leads to the activation of the inflammatory caspase-1 that is required at least for the processing and subsequent release of active pro-inflammatory cytokines, such as IL-1β and IL-18 (Martinon and Tschopp, 2007).

To decipher the biological effect of the caspase-cleaved form of Lyn, we generated transgenic mice that expressed this transgene in all tissues. LynΔN transgenic mice develop a skin inflammatory syndrome that shows features common in psoriasis (i.e. hyperkeratosis, scaling, inflammatory-cell infiltrates and increased cytokine expression, including TNF-α and IL-23). Backcrossing LynΔN mice in a TNF receptor 1-deficient background fully rescues the normal mouse

*Corresponding author. Equipe 2, INSERM U895, Faculté de Médecine Pasteur, 28 Avenue de Valombrose, Nice, Cedex 2 06107, France.
Tel.: +33 493 3770 14; Fax: +33 493 8478 52;
E-mail: auberger@unice.fr

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Figure 1 Phenotype of Lyn Δ N transgenic mice. (A) Lyn Δ N transgenic mice developed skin defects characterized by inflexible thickened skin and scaling patches. They were runted and smaller than control mice. (B, C) The 4-week-old Lyn Δ N mice with skin defects resembling scalp psoriasis with a macroscopic view of scaly patches affecting ear (B) and hairy back skin (C). (D) The 1-week-old Lyn Δ N mouse with scaling ear and inflammatory oedema affecting the paw.

phenotype, indicating its dependence on the pro-inflammatory cytokine TNF- α . Moreover, Lyn Δ N phenotype was distinctly improved in a Rag1-deficient background underlying the role of T lymphocytes in the pathogenesis of the disease. We also establish that Lyn Δ N increases STAT-3 activation and diminishes NF κ B activation, both conditions that have been previously reported to induce a psoriasis-like syndrome in mice (Pasparakis *et al*, 2002; Sano *et al*, 2005; Rebholz *et al*, 2007). Finally, analysis of Lyn expression in skin biopsies from patients suffering from psoriasis identifies caspase-cleaved form of Lyn whose expression correlates with the activation of inflammatory procaspase 1.

Therefore, our data identify Lyn Δ N transgenic mice as a new model of psoriasis that could be suitable for pre-clinical studies aimed at understanding and curing this disease.

Results

Characterization of Lyn Δ N transgenic mice

To study the biological relevance of the caspase-cleaved form of Lyn, we generated transgenic mice expressing Lyn Δ N in all tissues. Four independent founders expressing Lyn Δ N were obtained, and all developed the same phenotype. Lyn Δ N was highly expressed in heart and muscle, moderately in spleen, thymus, lung and kidney, and to a weaker extent in skin and liver (Supplementary Figure S1A). Lyn Δ N expression and activity was maintained at the same level in transgenic mice, throughout the lifespan of mice (Supplementary Figure S1B and data not shown).

Lyn Δ N mice were born in the expected Mendelian ratio and were macroscopically indistinguishable from their control littermates until postnatal day 6 (Supplementary Figure S3A). Then, all Lyn Δ N transgenic mice developed drastic skin defects characterized by inflexible thickened skin and hyperkeratosis, with major abnormalities around day 15

(Figure 1A). Lyn Δ N mice could be classified into 2 subgroups based on the severity of their skin phenotype, either mild with small scaly patches affecting ears, tails, paws and the hairy back skin (Figure 1B and C), or acute and characterized by skin lesions covering the whole body. The majority of mice showing the acute phenotype (about 50%) died within 2 weeks. Such mice were runted and showed decreased feeding activity but the exact cause of death remained unknown. It should be noted that the severity of the skin phenotype was unrelated to the level of Lyn Δ N transgene expression (data not shown). Some Lyn Δ N mice also presented with oedema of paws (Figure 1D) with alteration of nails, suggesting an inflammatory response of the joints.

Although they consistently presented with growth retardation and loss of weight (Supplementary Figure S2A), transgenic mice were fertile and their lifespan was roughly similar to that of control mice. To confirm that Lyn Δ N mice phenotype was indeed linked to the expression of the caspase-cleaved form of Lyn, we generated transgenic mice expressing the caspase non-cleavable form of Lyn (LynD18A). LynD18A progeny failed to develop Lyn Δ N phenotype (Supplementary Figure S2B) although transgene expression and relative enzymatic activity of Lyn Δ N and LynD18A in skin and different other organs (data not shown) were comparable (Supplementary Figure S2C).

We showed previously that Fyn, another member of the Src family of tyrosine kinases, is also cleaved by caspases through a similar mechanism (Ricci *et al*, 2001). We thus generated transgenic mice expressing the caspase-cleaved form of Fyn (Fyn Δ N) in a similar manner as described above for Lyn Δ N mice. Three founders were obtained that gave progeny with no apparent phenotype (Supplementary Figure S2B) showing that skin defects in Lyn Δ N mice is intrinsic to the expression of the cleaved form of Lyn and not to the expression of any other Src kinase.

Figure 2 Inflammatory skin phenotype of LynΔN transgenic mice. (A) Histological comparison of skin sections from 2-week-old LynΔN and control mice reflecting the hallmarks of psoriasis. Haematoxylin/eosin staining revealed a marked epidermis hyperplasia, a large number of nuclei in the cornified layer (arrowheads), hyperkeratosis and inflammatory infiltrates in the epidermis and dermis of LynΔN skin (arrows). Bar scale: 100 μm. (B) Skin sections from 2-week-old LynΔN (lower panel) and control (upper panel) mice were stained with antibody against Ki67. Nuclei were counterstained with DAPI (red). Bar scale: 100 μm. (C) Skin sections from 2-week-old LynΔN (lower panels) and control (upper panels) mice were stained with antibody against Keratin 14, Keratin 10, Keratin 6, Loricrin and β1-integrin (green). Nuclei were counterstained with DAPI (blue). Bar scale: 100 μm. (D) Immunostaining of skin sections from 2-week-old LynΔN and control mice with anti-CD4 antibody to detect T cells; anti-Gr-1 antibody to detect granulocytes and F4/80 to detect macrophages (red). Laminin 1 staining (green) delimited epidermis from dermis and blue staining (DAPI) showed nuclei. Bar scale: 50 μm. (E) Immunostaining of skin sections from 2-week-old LynΔN anti-Gr1 revealed the presence of granulocytes foci into the epidermis. Bar scale: 50 μm.

Analysis of the skin phenotype of LynΔN transgenic mice

To characterize the skin lesions observed in LynΔN mice, we carried out histological and immunofluorescence analysis of skin samples isolated from transgenic and control mice (Figure 2). The LynΔN mice skin showed marked hyperplasia of the epidermis (acanthosis), loss of granular layer (hypogranulosis), thickened, cornified layer (hyperkeratosis) containing a large number of nuclei, compatible with parakeratosis (Figure 2A). The skin from transgenic mice also showed increased Ki67 expression, indicating enhanced keratinocyte proliferation (Figure 2B). Analysis of proliferation and differentiation markers showed an enhanced expression of keratin 6 in the entire epidermis and broadening of keratin 14-expressing-keratinocyte layers in the epidermal compartment of LynΔN mice, together with a reduced expression of keratin 10 and loricrin (Figure 2C). Moreover, integrin-β1 was strongly upregulated in LynΔN mice as compared with control mice (Figure 2C). It should be noted that such an upregulation of integrin-β1 in the skin of psoriatic patient has been already reported by Conrad *et al* (2007). The skin phenotype, detected as early as day 3 after birth, was optimal at 2 weeks and a reversion of the skin phenotype was

observed in all surviving mice by week 4 (Supplementary Figure S3). LynΔN expression and activity in skin of transgenic mice remained unaffected through the mice life-span, indicating that the phenotype reversion was not because of the decreased transgene expression or activity (Supplementary Figure S1B).

One of the main features of psoriasis is the occurrence of inflammatory cell infiltrates into the epidermis and dermis. The skin sections from LynΔN mice showed inflammatory infiltrates into the epidermis and dermis (Figure 2A). An immunofluorescence analysis confirmed the presence of CD4+ T cells and an increased number of granulocytes and macrophages in the skin of LynΔN transgenic mice (Figure 2D). In certain instances, granulocyte foci were detected in the epidermis of transgenic mice (Figure 2E). This finding indicates that skin hyperproliferation is accompanied by a haematopoietic cell infiltration, suggesting that an inflammatory response is probably involved in the LynΔN transgenic mice phenotype. Accordingly, analysis of inflammatory cytokine mRNA expression in the skin of 2-week-old mice showed an increase in TNF-α, TNF-β, IL-1β, IL-17, IL-18 and IL-23 levels (Figure 3A and B). Furthermore, we identified the upregulation of both the chemotactic proteins,

Figure 3 Increased expression of pro-inflammatory cytokines in LynΔN mice. **(A)** Real-time quantitative PCR of indicated pro-inflammatory cytokines using RNA isolated from 2-week-old LynΔN and control mice skin. Data presented are representative of three independent experiments. **(B)** Semi-quantitative RT-PCR analysis of IL-1β, TNF-α, IL-23, S100A8, S100A9 and actin using RNA isolated from 2-week-old LynΔN and control mice skin.

S100A8 and S100A9, known to be involved in psoriasis pathogenesis (Figure 3B; Zenz *et al*, 2005; Chan *et al*, 2006). The increased level of pro-inflammatory cytokines was closely related to the severity of LynΔN mice phenotype. By contrast, all surviving mice showed normalized levels of pro-inflammatory cytokines (data not shown).

LynΔN transgenic mice phenotype is strictly dependent on TNF signalling

As TNF-α expression was strikingly increased in LynΔN transgenic mice and was largely reported to be involved in skin inflammation, we next investigated whether blocking the TNF signalling can rescue the LynΔN mice disease. To this end, we crossed LynΔN mice with TNFR1-deficient mice (Pfeffer *et al*, 1993). Remarkably, LynΔN/TNFR1^{-/-} mice were exempt of skin disease, with normal skin architecture compared with LynΔN/TNFR1^{+/-} mice (Figure 4A and B). Although ~50% of LynΔN/TNFR1^{+/-} mice died before day 15 after birth, all LynΔN/TNFR1^{-/-} reached adulthood (data not shown), indicating that the inflammatory syndrome was the main cause of death in LynΔN mice. Accordingly, IL-1β, S100A8 and IL-23 expression returned to control level in LynΔN/TNFR1^{-/-} mice as compared with LynΔN/TNFR1^{+/-} (Figure 4C). To validate the clinical relevance of LynΔN mice as a new model for psoriasis, we treated them with etanercept, a leading agent used for treating patients with psoriasis (Nickoloff and Nestle, 2004). Etanercept was administered at dose of 50 μg/day to 2-day-old LynΔN mice for 5 days. The Figure 4D shows that etanercept treatment leads to a distinct improvement of skin lesions in all injected mice as compared with untreated controls.

Next, we addressed the role of T cells in the pathogenesis of the LynΔN mice phenotype. To do so, we backcrossed LynΔN mice with Rag1-deficient mice (Mombaerts *et al*, 1992). Conversely, compared with LynΔN/Rag1^{+/-} mice, which developed a psoriasis-like syndrome indistinguishable

from that of LynΔN mice, the skin inflammatory disease was considerably improved in LynΔN/Rag1^{-/-} (Figure 4E), clearly indicating that T lymphocytes have a significant function in LynΔN mice phenotype.

Comparative analysis of gene expression in the skin of WT and LynΔN mice

To gain insights into the molecular mechanism of LynΔN effect, we compared the pan-genomic profile of several 5-day-old control and LynΔN skin mice before the initiation of the inflammatory syndrome. We decided to carry out the experiment at this time point to identify genes whose regulation is directly linked to the expression of LynΔN and not a consequence of the inflammatory process. The expression of more than 300 genes was found to be altered in the skin of LynΔN transgenic animals (for the complete list of regulated genes please refer to Materials and methods section). The Supplementary Table S1 recapitulates the most relevant genes in psoriasis-like skin inflammatory syndrome. Our pan-genomic approach confirmed the upregulation of keratin 14 and downregulation of keratin 10 and loricrin, and also revealed modulation of other skin differentiation markers, known to be altered during psoriasis, including upregulation of keratin 16 (Supplementary Table S1). Moreover, as mentioned earlier we confirmed the upregulation of many cytokines, such as S100A8, S100A9, TNF-α and IL-1β (Supplementary Table S1 and Figure 3A and B).

Interestingly, the pan-genomic approach used herein allowed us to focus on two signalling pathways already reported to be involved in psoriasis pathogenesis in both mouse and human. Indeed, we observed the upregulation of STAT-3 mRNA, but of no other STAT mRNA (Supplementary Table S1). This was confirmed at the protein level (Figure 5A) in LynΔN, but not in LynD18A or FynΔN mice (Figure 5B). Upregulation of STAT-3 was accompanied with an increase in its tyrosine phosphorylation status and consequently by an increase of its activity.

Moreover, another very interesting finding of this pan-genomic analysis was the upregulation of inhibitory molecules of the NFκB signalling pathway, including IκBα, IκBζ and Bcl-3 in

LynΔN mice as compared with WT mice (Figure 5C). An increased IκBα expression was also confirmed at the protein level in the skin of LynΔN mice (Figure 5D).

Figure 4 LynΔN transgenic mice phenotype is both dependent on TNF signalling and functional lymphocytes. (A) The 10-day-old LynΔN/TNFR1^{+/-} and LynΔN/TNFR1^{-/-} mice. Note that the invalidation of TNFR1 in LynΔN mice prevents the development of skin lesions. (B) Skin sections from 10-day-old LynΔN (b,c,e,f) and control (a,d) mice in TNFR1^{+/-} (a-c) or TNFR1^{-/-} (d-f) background were stained with haematoxylin/eosin (a,b,d,e) or an antibody directed against Keratin 14 (c,f) (nuclei are stained in blue). Bar scale: 50 μm. (C) Semi-quantitative RT-PCR analysis of S100A8, S100A9, IL-1β, IL-23 and actin using RNA isolated from 2-week-old LynΔN and control mice skin. (D) Treatment of 2-day-old LynΔN mice with 50 μg of etanercept (daily subcutaneous injection) for 5 days leads to a distinct improvement of skin lesions as compared with untreated mice. (E) The 10-day-old LynΔN/Rag1^{+/-} and LynΔN/Rag1^{-/-} mice. Note that in the absence of mature T cells (Rag1^{-/-}) LynΔN mice do not develop a skin disease.

Figure 5 LynΔN expression in mice skin increases STAT-3 levels and inhibitory molecules of the NFκB signalling pathway. (A) Whole-cell protein extracts from LynΔN and control mice skin were separated by SDS-PAGE and immunoblotted with the indicated antibodies. ERK2 was used as loading control. (B) Comparison of STAT-3 expression and respective tyrosine phosphorylation level on whole-cell protein extracts from LynΔN, LynD/A, FynΔN and control mice skin. ERK2 was used as loading control. (C) Semi-quantitative RT-PCR analysis of IκBα, Bcl-3, IκBζ and actin using RNA isolated from LynΔN and control mice skin. (D) Immunoblotting analysis of IκBα expression into LynΔN and control mice skin. Hsp60 was used as loading control.

Figure 6 Lyn Δ N expression impairs NF κ B signalling pathway. (A) Freshly isolated thymocytes from control or Lyn Δ N mice were left untreated or incubated with either TNF- α (20 ng/ml) or an anti-CD3 mAb (10 μ g/ml) for the times indicated. NF κ B activation was assayed by EMSA analysis of nuclear extracts. (B) Freshly isolated thymocytes from control or Lyn Δ N mice were treated as in (A). Degradation of I κ B α was assayed by western blot analysis of total cell extracts. Actin was used as a loading control. (C) Keratinocyte cultures established from control or Lyn Δ N mice were treated for the indicated times with or without TNF- α (20 ng/ml). NF κ B activation was measured as described in (A). (D) Keratinocytes from control or Lyn Δ N mice were treated as in (B). Degradation of I κ B α was assayed using western blot analysis of total cell extracts. Hsp60 was used as a loading control. (E) Keratinocytes from control of Lyn Δ N mice were treated for 1 h with or without TNF- α . p65/RelA nuclear relocation was monitored using fluorescent microscopy. Bar scale: 100 μ m.

The caspase-cleaved form of Lyn inhibits the NF κ B signalling pathway

The NF κ B signalling pathway has a crucial function in the regulation of the transcriptional responses of inflammation (Gerondakis *et al*, 2006), and mice deficient for some members of this signalling pathway developed skin disorder that resembles human psoriatic lesions (Pasparakis *et al*, 2002; Rebholz *et al*, 2007). On the basis of these reports and the results of our pan-genomic approach showing an increased expression of inhibitory regulators of NF κ B activity (i.e. I κ B α , I κ B ζ and Bcl-3), we investigated whether Lyn Δ N might alter the NF κ B signalling pathway. Briefly, NF κ B is sequestered into the cytoplasm through its interaction with the I κ B α repressor. The TNF- α stimulation triggers the activation of a serine/threonine kinase complex composed of IKK1/IKK2/NEMO, leading to I κ B α phosphorylation and its further degradation by the proteasome pathway. Following this NF κ B is free to translocate to the nucleus and to induce the transcription of a wide range of genes, more specially involved in the inflammatory response (Hayden and Ghosh, 2008). To investigate the potential role of NF κ B, we carried out experiments in both freshly isolated thymocytes and primary keratinocytes derived from control and Lyn Δ N mice, and checked the critical steps of the activation pathway described above. The NF κ B activation was analysed in thymocytes from control and Lyn Δ N mice on TNF- α or anti-CD3 mAb stimulation. As shown in Figure 6A, TNF- α and anti-CD3 mAb induced binding of NF κ B to DNA in freshly isolated thymocytes from control mice, but not in those prepared from Lyn Δ N transgenic mice. Importantly, I κ B α degradation occurred in thymocytes isolated from control or Lyn Δ N mice as well (Figure 6B). Decreased binding of NF κ B to DNA was also seen in primary keratinocytes isolated from Lyn Δ N mice with no modification of I κ B α degradation (Figure 6C and D). Accordingly, p65/RelA translocation to the nucleus was not altered in keratinocytes expressing Lyn Δ N (Figure 6E). This is consistent with the increased

expression of I κ B ζ and Bcl-3, two members of the I κ B family, previously described to directly regulate NF κ B activity in the nucleus (Richard *et al*, 1999; Motoyama *et al*, 2005).

Similar results were obtained using the Ramos B cell line overexpressing Lyn Δ N, previously used to identify Lyn cleavage and function *in vitro* (Luciano *et al*, 2003), or mouse embryonic fibroblasts derived from transgenic mice (Supplementary Figure S4). Finally, as previously mentioned, caspase cleavage of Lyn leads to the relocation of the kinase from plasma membrane to cytosol (Luciano *et al*, 2003). The transfection of Lyn Δ N fused to GFP confirmed that Lyn Δ N is mainly localized in the cytosol and also showed that leptomycin B, an inhibitor of nuclear export, induced the accumulation of Lyn Δ N in the nucleus (Supplementary Figure S4E). Thus, Lyn Δ N modulates NF κ B activity directly or indirectly in the nucleus.

Expression of Lyn Δ N in human psoriatic skin biopsies

Transgenic mice expressing the caspase-cleaved form of Lyn developed an inflammatory skin disease that shares many, if not all, features of human psoriasis. Recently, Johansen *et al* (2007) have shown that caspase 1 activity was increased in lesional psoriatic epidermis. An analysis of caspase 1 expression in whole-cell extracts from biopsies, obtained from non-lesional and lesional psoriatic skin, carried out on six patients confirmed that caspase 1 activity is increased in lesional psoriatic epidermis (Figure 7A). Moreover, we also checked for the activation status of the apoptotic executioner caspase 7 recently reported to be activated in a caspase 1-dependent manner during inflammatory conditions (Lamkanfi *et al*, 2008). Caspase 7 is indeed activated in lesional skin of three patients suffering from psoriasis (Figure 7B), concomitantly with caspase 1 activation (data not shown). Accordingly, it is noteworthy that Lyn is efficiently cleaved by recombinant caspase 7 *in vitro* (Figure 7C). On the basis of these observations, we next investigated Lyn expression and cleavage in both non-lesional and lesional psoriatic skin from the

Figure 7 Expression of Lyn in human psoriatic skin biopsies. Whole-cell extracts were prepared from biopsies isolated from lesional and non-lesional psoriatic skin and analysed by western blot. SDS-PAGE separated proteins were probed with antibody recognizing procaspase 1 and intermediate form of caspase 1 (A, upper panels) and procaspase 7 and active form (B, upper panel). ERK2 (A and B, lower panels) was used as loading control, (C) Full-length Lyn and LynD18A cDNAs were transcribed and translated *in vitro* with ^{35} S-methionine and incubated with purified recombinant caspases 3, 6, and 7 (25 ng) for 15 h at 37°C. The reaction products were then analysed using SDS-PAGE and autoradiography. (D) Whole-cell extracts were prepared from biopsies isolated from lesional and non-lesional psoriatic skin and analysed for Lyn expression.

six above-mentioned patients. A western blot analysis with two different anti-Lyn antibodies showed that the caspase-cleaved form of Lyn is detected at significantly higher levels in lesional skin in psoriatic patients (Figure 7D and data not shown), underlying the correlation between the presence of the cleaved form of Lyn and activation of caspases 1 and 7.

Discussion

The data presented herein show that ubiquitous expression of the caspase-cleaved form of the Src tyrosine kinase, Lyn, induces a skin inflammatory syndrome that recapitulates the main, if not all, features of human psoriasis (please refer to Table I for histological and clinical comparison of human psoriasis and Lyn Δ N mice phenotype). Immediately after birth, Lyn Δ N transgenic mice develop a massive skin inflammation associated with epidermal hyperplasia, hyperkeratosis, inflammatory cell infiltration, including that of T lymphocytes, granulocytes and macrophages, and an increased expression of cytokines, such as IL-1 β , TNF- α , IL-17, IL-18 and IL-23. Moreover, a large number of Lyn Δ N mice died within 2 weeks after birth. Importantly, genetic ablation of TNFR1 completely rescued the skin phenotype showing that TNF signalling is critical for the establishment of the inflammatory syndrome. Interestingly, all Lyn Δ N/TNFR1 $^{-/-}$ mice reached adulthood, indicating that TNF-dependent inflammation is the main cause of death in Lyn Δ N mice, as previously reported for K14-IKK2 $^{-/-}$ mice (Pasparakis *et al*, 2002). Previous studies have already pointed out the importance of TNF and also of IL-23 in psoriasis pathogenesis (Pasparakis *et al*, 2002; Chan *et al*,

2006) and therapies using neutralizing antibody directed against these molecules are in process (Lowes *et al*, 2007).

As psoriasis is a T cell-mediated autoimmune disease, it was of importance to determine whether lymphocytes were involved in the pathogenesis of the psoriasis-like syndrome occurring in Lyn Δ N mice. The predominant role of T cells in the inflammatory skin disease occurring in Lyn Δ N mice was highlighted by the distinct improvement of Lyn Δ N mice phenotype in a Rag1 $^{-/-}$ -deficient background. Importantly, the persistence of a weak residual disease in Lyn Δ N/Rag1 $^{-/-}$ mice probably suggests a non-negligible role of granulocytes and macrophages in the phenotype of Lyn Δ N mice, as it is also the case in K14-IKK2 $^{-/-}$ mice (Stratis *et al*, 2006).

In addition, the importance of T cells was further substantiated by the inhibition of NF κ B activation by TNF- α and anti-CD3 triggering in thymocytes isolated from Lyn Δ N mice. Interestingly, this defect in NF κ B activation was detected in thymocytes and keratinocytes isolated from Lyn Δ N mice, indicating that both T cells and keratinocytes participated in the mice phenotype.

We have previously shown that on caspase cleavage beyond aspartate 18 Lyn is relocated from the plasma membrane to the cytosol but its SH2/SH3 domains and tyrosine kinase activity remain intact (Luciano *et al*, 2001). Mice deficient for Lyn suffer a deficit of mature B cells (Nishizumi *et al*, 1995; Chan *et al*, 1997) and an autoimmune disease (Harder *et al*, 2001) but no skin defects have been reported in these mice, strongly suggesting that Lyn Δ N mice phenotype is not a consequence of a dominant-negative effect of Lyn Δ N toward native Lyn signalling.

Table 1 Comparison of clinical and histological features of human psoriasis and LynΔN mice phenotype

	Human psoriasis	LynN transgenic mouse
<i>Clinical</i>		
Erythema	Yes	Yes
Infiltration (thickening)	Yes	Yes
White silvery scaling	Yes	Yes
<i>Histological</i>		
Hyperkeratosis	Yes	Yes
Parakeratosis	Yes	(Yes/No)?
Granular layer	No	Diminished
Acanthosis	Yes	Yes
Epidermal spongiosis	Yes	Yes
Inflammatory oedema	Yes	Yes
<i>Recruitment to epidermis of</i>		
T lymphocytes	Yes	Yes
Neutrophils	Yes	Yes
<i>Recruitment to dermis of</i>		
Inflammatory infiltrate	Yes	Yes
<i>Differentiation markers</i>		
Upregulation: Krt14, Krt6, Krt16	Yes	Yes
Downregulation: Krt10, Krt15, loricrin	Yes	Yes
<i>Cytokines</i>		
TNF, IL-1β, S100A8, S100A9, IL-23	Yes	Yes

The role of the Src tyrosine kinase Lyn has been well established in haematopoietic cells (Xu *et al*, 2005), but several studies also indicate that Lyn controls the behaviour of other tissues and cell types (Chen *et al*, 1996; Stettner *et al*, 2005; Zhao *et al*, 2006; Gong *et al*, 2008). The expression of Lyn in mouse keratinocytes has been previously reported (Calautti *et al*, 1995; Joseloff *et al*, 2002). In this line, we analysed Lyn expression in skin sections from control and LynΔN transgenic mice (Supplementary Figure S5). Importantly, Lyn was expressed almost essentially in the basal layer of the epidermis (Supplementary Figure S5A). In LynΔN transgenic mice, Lyn was expressed all along the epidermis (Supplementary Figure S5B). Interestingly, Lyn was also present in some cells of the dermis in both control and LynΔN mice (Supplementary Figure S5A and B). Moreover, expression of Lyn was confirmed by western blotting in primary mouse keratinocytes (Supplementary Figure S5C) and in primary human keratinocytes (data not shown).

Thus, it seems that the main difference between LynΔN and native Lyn is the subcellular localization of the protein. We hypothesized that once relocated LynΔN might have access to new substrates in the cytoplasm and/or the nucleus that could participate in the pathogenesis of the skin disease showed by LynΔN transgenic mice. By contrast, mice expressing the caspase-cleaved form of Fyn failed to develop any apparent phenotypic abnormalities, indicating that LynΔN and FynΔN show distinct intrinsic properties probably linked to the set of substrates they specifically control. Another point of particular interest of this study comes from the observation that transgenic mice overexpressing an unclea-

vable form of Lyn, in which aspartate 18 has been mutated to an alanine, failed to develop the skin inflammatory syndrome observed in LynΔN mice. This mouse model reinforces the idea that the localization, rather than the amount of Lyn, is critical for the establishment of LynΔN transgenic mice phenotype. These findings are in agreement with several lines of evidence suggesting that the subcellular localization of different Src kinase members is at least as important as the regulation of their kinase activity (Garber *et al*, 1985; Kabouridis *et al*, 1997; Lang *et al*, 1999; Ricci *et al*, 2001; Luciano *et al*, 2003). Thus, conversely to native membrane-anchored Lyn, the relocated caspase-cleaved form of Lyn most probably controls specific targets involved in inflammatory responses.

A histological analysis of the heart and muscle, tissues in which LynΔN expression is the highest, failed to reveal any defects. By contrast, the skin, in which transgene expression is modest, showed major hallmarks of inflammation. However, although expression level and relative kinase activity of LynΔN are constant throughout the mouse lifespan, spontaneous inflammatory syndrome showed by LynΔN mice, those survive, is improved by week 5. The disappearance of inflammatory skin disease has already been reported for transgenic mice expressing constitutive active STAT-3 specifically in epidermis (Sano *et al*, 2005) and reversion of skin lesion is one of the remarkable features of psoriasis (Nickoloff *et al*, 2007). Importantly, STAT-3 transgenic mice developed a spontaneous psoriasis-like disorder very early after birth, whereas in aged mice psoriasis this was observed only on wounding. Interestingly, a comparative pan-genomic analysis of control and LynΔN mice skin revealed a specific upregulation of STAT-3 with no modification of expression of any other STAT proteins. Moreover, STAT-3 was constitutively activated in the skin of LynΔN mice, strongly suggesting a relationship between STAT-3 and LynΔN expression. Taking into account the fact that tyrosine phosphorylation of STAT transcription factor by Src kinase, such as Lyn has been recently described (Wang *et al*, 2007), it is tempting to speculate that transcriptional regulation and activation of STAT-3 by LynΔN may be responsible for the transgenic mice phenotype.

As previously specified, the skin disease of LynΔN mice also shares some features with the one occurring in IKK2^{-/-}, NEMO^{-/-} or IκBα^{-/-} mice (Pasparakis *et al*, 2002; Nenci *et al*, 2006; Rebholz *et al*, 2007), characterized by an early onset of hyperkeratosis and immune cell infiltration after birth. These observations, together with the fact that skin of LynΔN mice expressed high level of IL-1β and TNF-α prompted us to investigate the potential role of NFκB signalling pathway in LynΔN phenotype. We found a marked decrease in NFκB binding activity, in both freshly isolated thymocytes and primary keratinocytes, without significant alteration of the upstream NFκB activation pathway on TNF-α stimulation. Moreover, a net decrease in the trans-activation of κB-Luciferase reporter gene expression was detected in Ramos B cells overexpressing LynΔN (Supplementary Figure S5D). Therefore, alteration of NFκB activation by LynΔN probably occurs at the level of target gene regulation in agreement with the observed localization of LynΔN in the nucleus. Interestingly, Greten *et al* (2007) recently showed that NFκB is a negative regulator of IL-1β secretion. Thus, the increased expression of IL-1β observed in

LynΔN mice is consistent with the potential inhibition of NFκB signalling pathway by LynΔN. Attempts to detect a direct interaction between p65 and p50 NFκB subunits and LynΔN were unsuccessful. This, however, does not rule out the possibility of an indirect effect of LynΔN on these NFκB subunits. Another point of particular interest of this study is the transcriptional regulation by LynΔN of several known inhibitors of the NFκB pathway, including increased expression of IκBα, IκBζ and Bcl-3. Among them the latter two are known to be localized in the nucleus (Zhang *et al*, 1994; Motoyama *et al*, 2005). The exact mechanism by which LynΔN alters the NFκB pathway warrants a detailed further study. Nevertheless, we could imagine a model in which LynΔN-mediated inhibition of NFκB activation is mediated through transcriptional induction of Bcl-3 by STAT-3 (Brocke-Heidrich *et al*, 2006). In such model, LynΔN would activate STAT-3, which in turn would increase Bcl-3 expression, leading to the inhibition of NFκB activation. This model is in line with the observation that transgenic mice expressing constitutively activated STAT-3 in the epidermis develop a psoriasis-like syndrome and show an increased IκBα expression (Sano *et al*, 2005).

Psoriasis is a T cell-mediated autoimmune disease of unknown etiology, which is characterized by acanthosis, parakeratosis (i.e the presence of nuclei in the corneum substratum) and immune cell infiltration (Lowes *et al*, 2007). Accordingly, the psoriasis-like disease showed by LynΔN mice was improved distinctly in LynΔN/Rag1^{-/-} mice. As LynΔN mice recapitulate the main features of human psoriasis (Table I), we sought to analyse whether Lyn expression and cleavage were modulated in human psoriatic skin lesions. An analysis of Lyn expression in human psoriatic skin biopsies showed the presence of a cleaved form of Lyn, which correlates with caspase 1 and caspase 7 activation. The cleavage of caspase 1 has been already reported in human psoriasis biopsies (Johansen *et al*, 2007), but to our knowledge this is the first description of caspase 7 activation in this disease. Accordingly, Lamkanfi *et al* (2008) very recently established that apoptotic executioner caspase 7 is activated directly by caspase 1 during an inflammatory process, reinforcing the concept of a crosstalk between these two classes of caspases. In addition, Lyn cleavage was not detected in non-lesional skin biopsies, in which caspases 1 and 7 activation was absent, strongly suggesting a causal link between these two events. It should be noted that Ayli *et al* (2008) recently found an activation of Src tyrosine kinases in hyperproliferative epidermal disorders, including psoriasis. In this study Src kinase activity was associated with both membrane and cytoplasmic localization.

In summary, we report a new and original mechanism by which the caspase-cleaved form of Lyn triggered a psoriasis-like syndrome in mice. Many animal models for psoriasis have been developed (Gudjonsson *et al*, 2007; Schon, 2008). However, although it is important to consider that none of these animal models shows all the features of human psoriasis, each of them has proven to be useful for the better understanding of the pathogenesis of this disease. Nevertheless, the LynΔN transgenic mice phenotype described herein, which recapitulates the main characteristics of human psoriasis (Table I) can be improved by etanercept (Figure 4D). As such, it may be particularly valuable as an animal model to validate new therapeutic drugs against psoriasis.

Materials and methods

Generation of transgenic mice

To drive an ubiquitous expression of the caspase-cleaved form of Lyn (LynΔN), its uncleavable form (LynD18A) or the caspase-cleaved form of Fyn (FynΔN), we used the pCAGGS vector (a generous gift from H. Niwa), in which the cytomegalovirus (CMV) enhancer and the chicken β-actin promoter are located upstream of the MCS region. In addition, a rabbit β-globin polyadenylic acid sequence is located downstream of the MCS region (Niwa *et al*, 1991).

LynΔN, LynD18A and FynΔN were PCR amplified from the corresponding pcDNA3 plasmids (Ricci *et al*, 1999; Luciano *et al*, 2001) using the following primers: LynΔN forward 5'-GGAATTCGCGAGAAATATGTCTGAAGAC-3' and reverse 5'-GGAATTCCTACGGTTCGCTGCTGATACTGCCC-3' (each primer contains an *EcoRI* site); LynD18A forward 5'-GGGATCCAGCGAGAAATATGGGATGTATTAAATC-3' (a *Bam*H1 site) and reverse 5'-TGCTCGAGCTACGGTTGCTGATACTGCCCTTC-3' (an *Xho*I site); FynΔN forward 5'-AGATTTCGACCATGGGCGAGCCTGAACCAG-3' and reverse 5'-GGAATTCACAGGTTTTCACCGGGCTG-3' (each primer contains an *EcoRI* site). The pCAGGS-LynΔN and -FynΔN vectors were obtained by subcloning the *EcoRI*-digested PCR fragment into *EcoRI*-digested pCAGGS vector. The pCAGGS-LynD18A vector was obtained by subcloning the *Bam*H1 (blunt-ended)-*Xho*I digested PCR fragment into *Xba*I (blunt-ended)-*Xho*I digested pCAGGS vector. The vector sequences were removed by *Sal*I-*Pst*I digestion and the resulting linearized constructs were microinjected into pronuclei obtained from B6D2 mice (Service d'Experimentation Animale et de Transgénèse, SEAT, CNRS, Villejuif, France).

Transgenic founders were crossed with C57BL/6 (Janvier) mice to generate lines. F1 transgenic progeny were crossed with C57BL/6 mice to maintain lines. The phenotype of LynΔN mice described was maintained through at least 10 generations and showed total penetrance.

The expression of LynΔN and LynD18A proteins was confirmed by western blot analysis of various tissues with anti-Lyn antibody. In some experiments, LynΔN mice were backcrossed with TNFR1- or Rag1-deficient mice (Jackson Laboratory).

For genotyping, DNA was isolated from tail snips and subjected to PCR with the following primers for pCAGGS-LynΔN or pCAGGS-LynD18A: sense 5'-CCTTCTCTTTTCTCTACAGC-3' and antisense 5'-GCTCTGCACTGTTCCCTGG-3'; for pCAGGS-FynΔN: sense 5'-CCTTCTCTTTTCTCTACAGC-3' and antisense 5'-CGGGCTTCCCA CCAATCTC-3'; for TNFR1 and Rag1 deficient mice the genotyping protocol is available at the Jackson laboratory website.

The animal studies were approved by the Institutional Animal Care and Use Committee of the Centre Méditerranéen de Médecine Moléculaire (INSERM U895).

Antibodies and reagents

Rabbit anti-Lyn (sc-15), mouse anti-Lyn (sc-7274), mouse anti-Fyn (sc-434), goat anti-hsp60 (sc-1722), goat anti-actin (sc-1616), rabbit anti-caspase-1 (sc-622), rabbit anti-IκBα (sc-203), mouse anti-ERK2 (sc-1647), rabbit anti-Oct2 (sc-233), rabbit anti-p65 (sc-372) and goat anti-KI67 (sc-7846) were purchased from Santa Cruz Biotechnology. Rabbit anti-phospho src (pY-418) was purchased from Biosource. Mouse anti-caspase 8 was purchased from MBL. Anti-phospho IκBα anti-stat 3, anti-phospho stat 3 (Tyr 705), anti-stat 1, anti-caspase 7, anti-mouse HRP and anti-rabbit HRP were purchased from Cell signalling Technology. Anti-mouse HRP, anti-goat HRP, anti-mouse IgG-FITC, streptavidin-FITC and streptavidin-PE were purchased from Dako Cytomation.

Goat anti-NEMO, rat anti-CD4 and biotin anti-rat were purchased from Pharmingen (BD Bioscience, San Diego, CA). Rat anti-F4/80 was purchased from Serotec.

Rabbit anti-laminine 1 and mouse anti-keratin 14 were purchased from Sigma and rat anti-Gr 1 from R&D system. Rabbit anti-keratin 6, anti-keratin 10 and loricrin were purchased from Covance. Rat anti-β1 integrin was purchased from Chemicon international. Anti-rabbit, anti-rat and anti-mouse Alexa488 or Alexa594 were purchased from Molecular Probes (Invitrogen). Mouse and human TNF-α were from PeProtech. Mouse anti-CD3 mAb was purified in the laboratory (hybridoma 2C11).

Primary mouse keratinocyte culture and thymocyte isolation

Primary keratinocyte were isolated from adult mice (Romero *et al*, 1999). Control and LynΔN mice (6–12 weeks) were killed, shaved and washed with 70% ethanol. The skin was removed, rinsed in 70% ethanol and in PBS containing 250 µg/ml penicillin/streptomycin, and all the subcutaneous tissue was scraped off. The skin was then incubated in 0.25% trypsin overnight at 4°C. The epidermis separated from the dermis was minced and placed in keratinocyte medium (William's E medium containing 2 mM glutamine, 10% chelated FCS, 5 µg/ml insulin, 10 µM cholera toxin, 2 µM triiodothyronine, 10 ng/ml epidermal growth factor, 0.4 µg/ml hydrocortisone and 18 µM adenine). Cell suspension was filtered through a 70 µm Teflon mesh, centrifuged and resuspended in complete keratinocyte medium. Cells were then seeded onto collagen I-coated dishes (25 µg/ml) in the presence of a feeder layer of lethally irradiated fibroblasts. Cells were incubated at 32°C under 8% CO₂ and the medium was changed thrice a week.

For the preparation of cells from the thymus, the organ was removed from individual mouse and passed through a nylon membrane (70 µm porosity) to obtain single-cell suspensions in RPMI 1640 medium (with 10% FCS, 50 µM β-mercaptoethanol, 100 µg/ml penicillin/streptomycin, 2 mM glutamine).

Immunoblotting

Mouse tissues homogenized with a polytron or cells were lysed in buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM Na₃VO₄, 1 mM PMSF, 1 mM leupeptin, 20 µg/ml aprotinin and 1% Triton X-100. Proteins were separated using SDS-PAGE and transferred onto PVDF membrane (Immobilon-P, Millipore). After blocking nonspecific binding sites, the membranes were incubated with specific antibodies. The membranes were washed and incubated further with horseradish peroxidase-conjugated antibody. Immunoblots were revealed by autoradiography using the enhanced chemiluminescence detection kit (Pierce).

RT-PCR and Real time quantitative-PCR analysis

Tissues were harvested and incubated in RNeasy lysis buffer (Qiagen) before RNA extraction. Total RNA was isolated using RNeasy Reagent (Qiagen) after tissues homogenization with polytron. The supernatant was cleared by centrifugation, precipitated with isopropanol and resuspended in RNase and DNase-free water.

For RT-PCR, total RNA (2 µg) was reverse transcribed using the SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions in a 40 µl final volume. cDNAs (2 µl) were amplified using 1 U of Taq polymerase (New England Biolabs, Ipswich, MA, USA) in a final volume of 25 µl buffer containing 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphate (dNTP) and 0.5 µM of the primers (for primer sequences, please referred to Supplementary data).

For RQ-PCR analysis, 1 µg of RNA, previously treated with DNaseI, was reverse transcribed using random priming and MultiScribe reverse transcriptase (Applied Biosystems). RT-PCR was carried out in an ABI PRISM 5700 Sequence Detector System (Applied Biosystems) using the SYBR Green detection protocol as outlined by the manufacturer. Gene-specific primers were designed using the Primer Express software (Applied Biosystems). The relative expression level for target genes was normalized for RNA concentrations with four different housekeeping genes (GAPDH, β-actin, HPRT and ubiquitin). The mRNA values are expressed as arbitrary units and represent the mean ± s.d. of duplicates and are representative of three independent experiments.

Preparation and immunostaining of tissue

The tissues were collected from mouse and were either preserved in Formol for histological analysis (haematoxylin/eosin staining) or were frozen at –80°C for subsequent immunostaining experiments. The tissue sections (6 µm) were fixed in ice-cold acetone (10 min at –20°C) and blocked in 2% BSA/PBS for 1 h. Then, tissue sections were incubated with the appropriate specific primary antibody for 1 h at room temperature. After three washes with PBS, tissue sections were incubated with the appropriate fluorescent-conjugated secondary antibody or streptavidin conjugates. The preparations were mounted in fluoromount (Southern Biotech) and images were acquired using Leica microscope and LAF6000 software.

Human psoriatic skin biopsies

A total of five paired non-lesional and lesional psoriatic skin biopsies were obtained from Archet Hospital of Nice (Dermatology department; Head of Department Pr Ortonne) with the consent of the patients, according to France regulation. In addition, whole-cell protein extracts from four paired non-lesional and lesional psoriatic skin biopsies were obtained from Dr Iversen (Denmark). The local ethical committee of Aarhus, Denmark approved all studies and informed consent was obtained from each patient.

Microarray experiments

Total RNA was extracted using the RNeasy kit Mini (Qiagen). The RNA was quantified using nanodrop spectrophotometry. RNA quality was evaluated using the Agilent Bioanalyzer 2100 and Lab-on-Chip Nano 6000 chip (ratio of the 28S:18S RNA ≥ 1.5).

Pan-genomic microarrays were printed using mouse RNG/MRC oligonucleotide collection as previously described by Le Brigand *et al* (2006). The list containing 25011 probes spotted on the microarray is available at <http://www.microarray.fr> (follow the link to 'mouse national set'). The RNA was labelled and hybridized as described by Moreilhon *et al* (2005). We compared in pairs three LynΔN and three control mice, and for each comparison Cyanine dyes were inverted to reduce the impact of dye bias. The experimental data and associated microarray designs have been deposited in the NCBI gene expression omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under series GSE12722 and platform record GPL1476.

Statistical analysis

The data were normalized using the print tip Lowess method (within-array normalization method) and by quantile (between-array normalization method) using the Limma package in the software package R Bioconductor (Wettenhall and Smyth, 2004). Differentially expressed genes were selected using a Benjamini-Hochberg correction of the *P*-value for multiple tests, based on a *P*-value < 0.05. All normalized data sets were registered in the GEO database under the accession number GSE12722.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: SM conceived and carried out the experiments, analysed data, and wrote the manuscript. PG, NB, LP and PC also carried out the experiments. SG and BM carried out the microarray analysis. CJ and LI provided skin biopsy extracts. MD supervised RQ-PCR experiments. PH carried out histological analysis on mice organs. NO analysed mice skin sections. AK and JPO did skin biopsy and carried out a critical analysis of data. FL and JER provided helpful discussion about the data and commented on the paper. PA conceived and directed the work, and revised the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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