

Functional analysis of the *RNF114* psoriasis susceptibility gene implicates innate immune responses to double-stranded RNA in disease pathogenesis

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Psoriasis is an immune-mediated skin disease, the aetiology of which remains poorly understood. In recent years, genome-wide association studies (GWAS) have helped to illuminate the molecular basis of this condition, by demonstrating the pathogenic involvement of multiple genes from the IL-23 and NF- κ B pathways. A GWAS carried out by our group also identified *RNF114*, a gene encoding a novel ubiquitin binding protein, as a determinant for psoriasis susceptibility. Although the function of *RNF114* is unknown, its paralogue *RNF125* has been shown to regulate the RIG-I/MDA5 innate antiviral response. This signalling cascade, which is activated by the presence of double-stranded RNA (dsRNA) within the cytoplasm, induces the production of type I interferon (IFN) through the activation of the IRF3 and NF- κ B transcription factors. Here, we explore the hypothesis that *RNF114* may also modulate RIG-I/MDA5 signalling. We show that *RNF114* associates with ubiquitinated proteins and that it is a soluble cytosolic protein that can be induced by interferons and synthetic dsRNA. Moreover, we demonstrate that *RNF114* over-expression enhances NF- κ B and IRF3 reporter activity and increases type I and type III IFN mRNA levels. These results indicate that *RNF114* regulates a positive feedback loop that enhances dsRNA induced production of type I IFN. Thus, our data point to a novel pathogenic pathway, where dysregulation of RIG-I/MDA5 signalling leads to the over-production of type I IFN, a key early mediator of epithelial inflammation.

INTRODUCTION

Psoriasis is a chronic, immune-mediated skin disorder, which affects 1–2% of the general population (1). The disease is characterized by the occurrence of red scaly plaques that are infiltrated by T-lymphocytes, neutrophils and dendritic cells (2). Epidemiological studies have associated the appearance of these skin lesions with a number of environmental factors, including mechanical stress and infections (1). It has also been suggested that innate immune pathways may provide a link between disease triggers and abnormal T-cell activation (3). This hypothesis is consistent with results

obtained on independent animal models and showing that: (i) stimulation of the innate TLR7/TLR8 toll-like receptors with a synthetic agonist is sufficient to induce T-cell dependent, psoriasis-like skin inflammation in mice (4) and (ii) TLR7-driven production of type I IFN is a key early event in the pathogenesis of psoriasis (3).

Familial clustering of cases being well established, psoriasis is widely regarded as a complex genetic trait, requiring the presence of inherited susceptibility alleles in multiple genes (1). On this basis, a number of genome-wide association scans (GWAS) have been carried out in recent years (5–12). These studies, which have uncovered a number of novel

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disease susceptibility genes, have provided valuable insights into the biology of psoriasis by highlighting the pathogenic involvement of the IL-23 and NF- κ B signalling pathways (6,7).

A GWAS carried by our group has also identified *RNF114* as a novel psoriasis susceptibility determinant (5), a result that was subsequently replicated in three further data set (7,10,11). Although the function of *RNF114* is unknown, bioinformatic analyses indicate that the gene encodes a protein with an N-terminal RING domain, followed by three zinc fingers and an ubiquitin interaction motif (UIM) (13). RING domains are typically found in E3 ubiquitin ligases, the enzymes which confer substrate specificity to the process of protein ubiquitination. RING motifs are essential to E3 function as they mediate the interaction with the E2 enzymes that bind activated ubiquitin molecules (14).

Homology searches identified three additional proteins sharing RNF114's distinctive domain architecture: RNF125, RNF166 and RNF138 (13). Although RNF166 has not been investigated at the functional level, both RNF125 and RNF138 have been shown to display strong ubiquitin ligase activity (13,15). In particular, RNF125 contributes to the ubiquitination of the RIG-I and MDA5 innate antiviral receptors (16). These specialized nucleic acid sensors recognize cytosolic double-stranded RNA (dsRNA) as an intermediate product of viral replication and drive the production of type I interferon (IFN) through the activation of transcription factors IRF3 and NF- κ B (17). RNF125 participates in the regulation of this signalling cascade through a negative feedback mechanism, whereby the accumulation of type I IFN up-regulates RNF125, leading to the ubiquitination and proteasomal degradation of RIG-I and MDA5 (16).

In this study, we hypothesized that RNF114 may also modulate the RIG-I/MDA5 response. Our experiments show that RNF114 is a positive regulator of this innate immune pathway and suggest that this gene may contribute to psoriasis susceptibility by up-regulating the production of type I IFN, a key early mediator of epithelial inflammation (3).

RESULTS

RNF114 is a soluble cytoplasmic protein

RIG-I and MDA5 are ubiquitously expressed cytosolic dsRNA receptors and their action is mostly regulated by soluble cytoplasmic proteins (17). On this basis, we sought to establish the sub-cellular localization of RNF114, as a first step towards an assessment of its involvement in RIG-I/MDA5 signalling.

We initially investigated whether RNF114 localizes to the nucleus or cytoplasm, by analysing the results of cell fractionation experiments. We carried out western blots, using a monoclonal anti-RNF114 antibody, which detects a single band in a variety of primary and immortalized cell lines, and only generates a weak signal in cells transfected with RNF114 siRNAs (Supplementary Material, Figs S1 and S3A). Figure 1A shows that RNF114 is predominantly present in the cytoplasmic fraction of the HaCaT keratinocyte cell line, under conditions where incubation with anti-tubulin and anti-lamin B1 antibodies demonstrated the purity of cytosolic and nuclear extracts. The analysis of four additional cell

lines (HEK293T, HT1080, HepG2 and Jurkat) generated comparable results (data not shown). To extend these findings to primary cells, we carried out immunofluorescence experiments on skin keratinocytes obtained from healthy donors. These studies confirmed that RNF114 localizes predominantly to the cytoplasm (Fig. 1B). We next sought to determine whether RNF114 was a soluble protein or a membrane associated one, like RNF125. For this purpose, we used western blotting to analyse HeLa cell homogenates that had been loaded at the bottom of a discontinuous sucrose gradient and separated by centrifugation. As demonstrated with the transferrin receptor, membrane-associated proteins float to the 10–65% sucrose interface at the top of the gradient (Fig. 1C). RNF114, however, was absent from this fraction and only found within the pool of soluble cytoplasmic proteins (Fig. 1C).

Taken together, these experiments demonstrate that RNF114 is a cytoplasmic soluble protein, which is compatible with a function in RIG-I/MDA5 signalling.

RNF114 associates with ubiquitinated proteins and has ubiquitin ligase activity

Previous studies carried out by our group have demonstrated that a recombinant RNF114 UIM domain can bind poly-ubiquitin chains *in vitro* (5). Given that ubiquitination and de-ubiquitination processes play an important role in regulating RIG-I/MDA5 signalling (18), we investigated whether RNF114 also associates with ubiquitinated proteins in living cells. We used a recombinant protein composed of the UBA ubiquitin binding domain of Dsk2p fused to GST (GST-UBA), in order to pulldown ubiquitinated proteins from lysates of HEK293T cells that had been transfected with HA-ubiquitin and myc-tagged RNF114 constructs. In parallel, we used a mutant GST-UBA protein (GST-UBA-m) that is unable to bind ubiquitin (19) as a negative control (Fig. 2A, central panel). Finally, we carried out western blots with an anti-myc-tag antibody, which clearly demonstrated that RNF114 is associated with the pool of ubiquitinated proteins pulled down by GST-UBA (Fig. 2A, right panel, top).

We next assessed whether RNF114 has ubiquitin ligase activity like its paralogue RNF125 (13). We carried out *in vitro* ubiquitination assays, using RNF114-myc immunoprecipitated from transfected HEK293T cells. Following incubation with ubiquitin, E1 ubiquitin-activating enzyme and the E2 ubiquitin conjugating enzyme UbcH5a, we observed the accumulation of auto-ubiquitination products, indicative of RNF114 enzymatic activity (Fig. 2B). This was moderate compared with the activity of RNF125, which catalysed the conversion of all input protein to higher molecular weight ubiquitinated forms (Fig. 2B). To further explore these findings, we performed additional *in vitro* ubiquitination assays, using RNF114 GST-fusion proteins purified from *E.coli*. These experiments confirmed that RNF114 has ubiquitin ligase activity in the presence of UbcH5a (Fig. 2C), an observation that is consistent with a reported interaction between these two proteins (20). Moreover, the activity of wild-type RNF114 clearly exceeded that of a RING mutant (RINGmut, bearing two substitutions at conserved cysteines in the RING domain, Fig. 2C). Thus, the RNF114 auto-

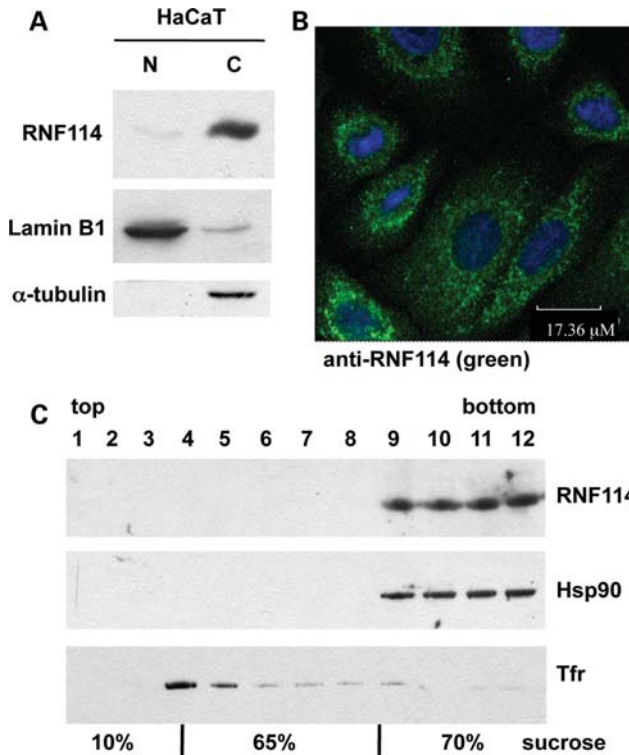


Figure 1. RNF114 localizes to the cytoplasm. (A) Nuclear (N) and cytoplasmic (C) fractions from the HaCaT keratinocyte cell line were analysed by western blot using an anti-RNF114 antibody. Antibodies detecting known nuclear (Lamin B1) and cytoplasmic proteins (α -tubulin) were used as controls. (B) Primary keratinocytes obtained from healthy donors were analysed by immunofluorescence, using an anti-RNF114 antibody (green). Nuclei were stained with DAPI (blue). (C) HeLa cells were homogenized in hypotonic buffer and fractionated on discontinuous 70/65/10% sucrose gradients. Fractions were collected from the top (fraction 1) and aliquots were analysed by western blot. Antibodies against known soluble (Hsp90) and membrane-associated proteins (transferrin receptor, Tfr) were used as controls.

ubiquitination observed in these experiments reflects the presence of a genuine enzymatic activity for this protein.

RNF114 expression is up-regulated by IFN α , IFN γ and synthetic dsRNA

Most of the genes that participate in RIG-I/MDA5 signalling are up-regulated by type I and type II IFNs (16,18,21). To assess whether *RNF114* shows a similar response, we used real-time PCR to measure transcript levels in HaCaT cells treated with IFN α or IFN γ . We found that both cytokines significantly up-regulate *RNF114* expression (Fig. 3A). To validate these findings, we repeated these experiments in primary keratinocytes obtained from healthy donors. We again observed an increase in *RNF114* transcript levels following IFN treatment (Fig. 3B), an effect that was also visible at the protein level (Fig. 3C). Further experiments verified that this response is both time- and dose-dependent (Fig. 3D–G). Of note, treatment of primary keratinocytes with inflammatory cytokines that are not relevant to the regulation of RIG-I signalling (TNF α , IL-17 and IL-22) failed to induce a significant change in *RNF114* transcript levels (Supplementary Material, Fig. S2).

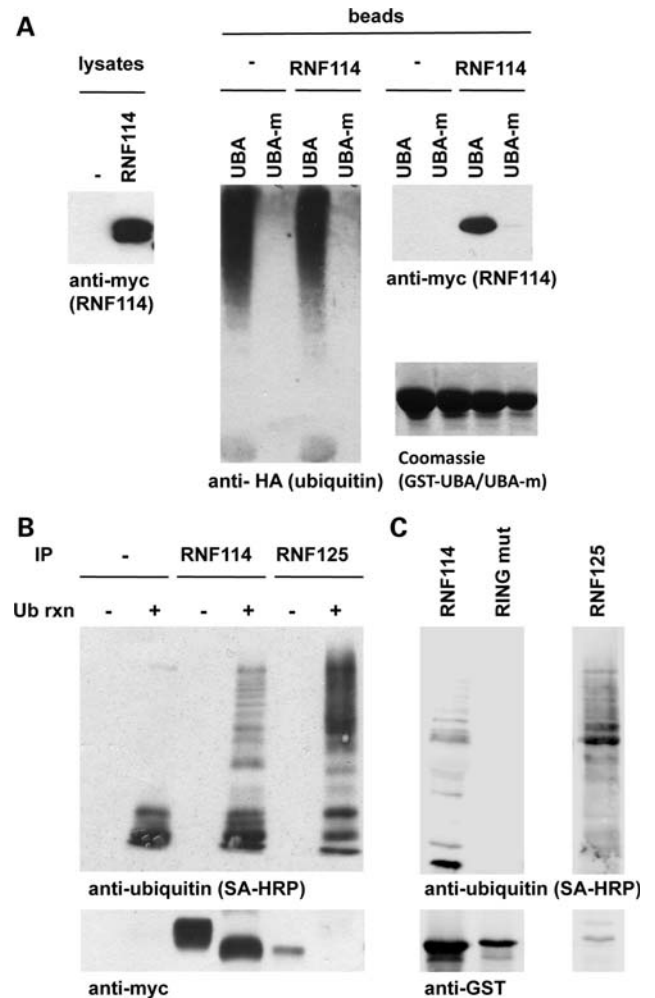


Figure 2. RNF114 binds ubiquitinated proteins and has ubiquitin ligase activity *in vitro*. (A) HEK293T cells were transfected with myc-tagged RNF114 and HA-ubiquitin. Ubiquitinated proteins were isolated from whole-cell lysates by incubation with GST–UBA beads and detected with anti-HA by western blotting (central panel). Pulldowns with a mutant form of GST–UBA (GST–UBA-m) that is unable to bind ubiquitin were performed as negative controls. Western blotting with anti-myc was performed to detect the association of RNF114 with ubiquitinated proteins (right, top panel). Pulldown samples were stained using Coomassie to demonstrate the presence of comparable amounts of GST–UBA and GST–UBAm (right, bottom panel). (B) RNF114-myc and RNF125-myc were immunoprecipitated from transfected HEK293T cells and the sample was split into two equal parts. One half was incubated for 90 min at 30°C in reaction buffer only (–), while the other half was incubated in reaction buffer supplemented with purified E1, the E2 UbcH5a, biotinylated ubiquitin, DTT and ATP (+). Ubiquitination products were revealed by western blots, using a streptavidin–HRP (SA–HRP) conjugate (top), and immunoprecipitated RNF114 and RNF125 were detected with anti-myc (bottom). The difference in migration between RNF114-myc in the (–) and (+) lane results from the presence of DTT in the (+) sample. An anti-myc immunoprecipitate from untransfected HEK293T cells was used as a negative control in this experiment. (C) Bacterially expressed RNF114 and RNF125 GST-fusion proteins were captured on glutathione–sepharose beads and used in *in vitro* ubiquitination reactions as described in (B). The RNF114 C29A,C32A mutant (RING mut) contains two cysteine-to-alanine substitutions at conserved residues in the RING domain. The reactions were analysed by western blotting using a streptavidin–HRP conjugate to detect modification of the GST fusion proteins with biotinylated ubiquitin (top panel), and with anti-GST to detect the GST-fusion proteins (bottom panel). The RNF114 and RNF125 samples were analysed on the same nitrocellulose blot (for SA–HRP or anti-GST) and the images are of the same X-ray autoradiography exposure of these blots.

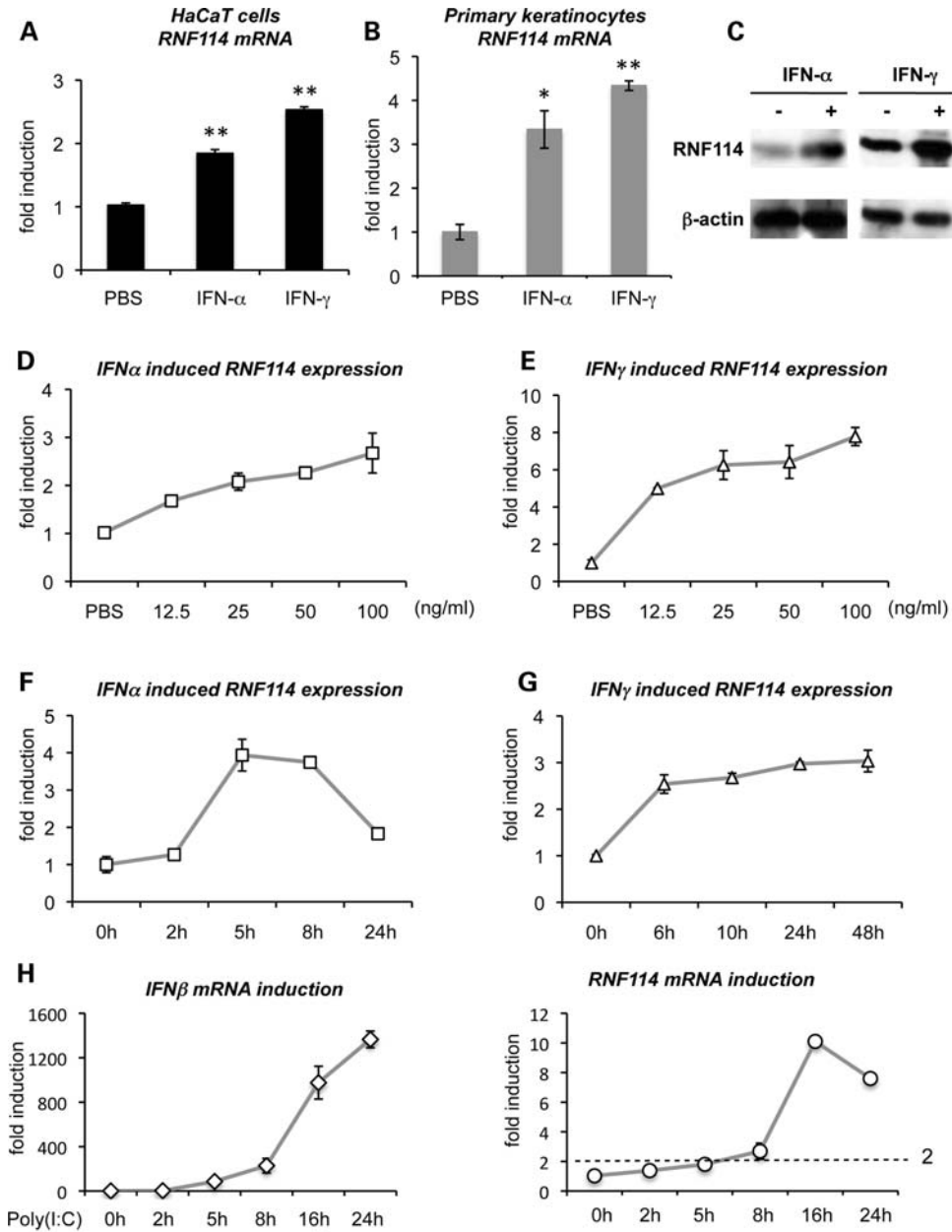


Figure 3. RNF114 expression is up-regulated by IFN α , IFN γ and synthetic dsRNA. (A) HaCaT cells were treated with 100 ng/ml IFN α (5 h) or 50 ng/ml IFN γ (24 h). *RNF114* induction was measured by real-time PCR, using *PPIA* as an internal control; ** $P < 0.005$. (B) Primary keratinocytes obtained from healthy donors ($n = 2$) were treated and analysed as in (A); * $P < 0.05$, ** $P < 0.005$. (C) Primary keratinocytes were treated with 100 ng/ml IFN α (24 h) or 50 ng/ml IFN γ (48 h) and *RNF114* induction was measured by western blot, using a monoclonal anti-RNF114 antibody. (D and E) Primary keratinocytes were treated in duplicate with the indicated concentrations of cytokine for 5 (IFN α) or 24 h (IFN γ). *RNF114* induction was measured by real-time PCR, using *PPIA* as an internal control. (F and G) Primary keratinocytes were treated in duplicate with 100 ng/ml IFN α or 50 ng/ml IFN γ . Cells were harvested at the indicated time points and *RNF114* induction was measured by real-time PCR, using *PPIA* as an internal control. (H) HEK293T cells were transfected with 2 μ g/ml poly(I:C) for the indicated times. *IFNB1* (left) and *RNF114* (right) induction was measured by real-time PCR, using *PPIA* as internal control.

To further explore the relationship between RNF114 and cellular responses to cytosolic dsRNA, we measured gene expression levels in HEK293T cells that had been transfected with the synthetic dsRNA analogue polyinosinic:polycytidylic acid [poly(I:C)]. We found that this stimulation, which mimics the presence of viral RNA within the cytoplasm, results in a time-dependent increase in RNF114 transcript levels (Fig. 3H). In particular, we observed that the up-regulation of RNF114 lags a few hours behind the induction of IFN β

(Fig. 3H), suggesting that the effect of poly(I:C) on RNF114 expression is mediated by type I IFNs produced following activation of the RIG-I/MDA5 pathway.

RNF114 is a positive regulator of dsRNA-induced type I IFN production

Having established that RNF114 is up-regulated by RIG-I/MDA5 signalling, we investigated whether it also plays a

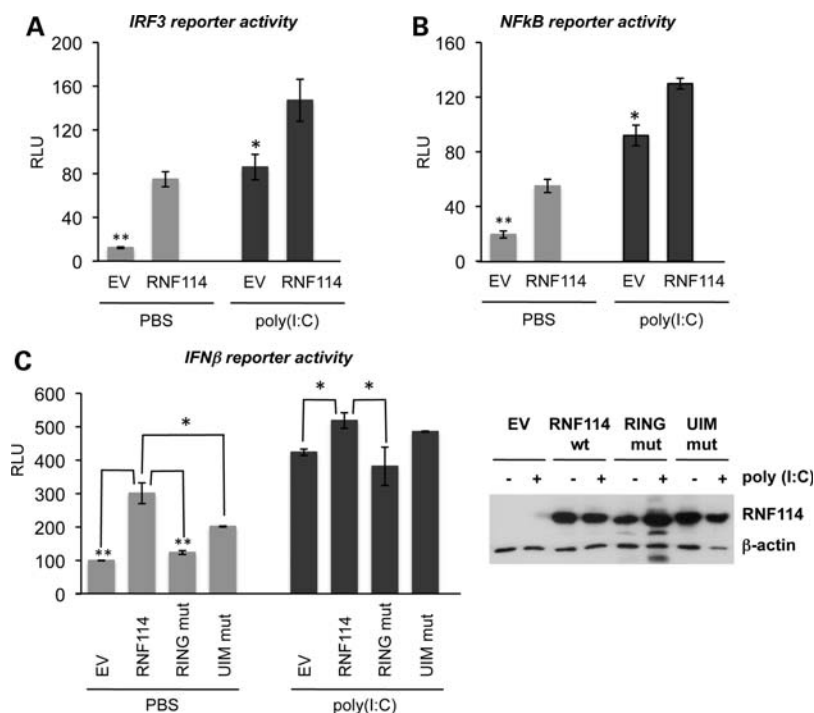


Figure 4. RNF114 positively regulates poly(I:C) induced IFN β production. (A and B) HEK293T cells were transfected with pcDNA3.1-myc/His plasmids containing either RNF114 cDNA (RNF114) or no insert (EV: empty vector) together with pJ7lacZ and a luciferase reporter plasmid for IRF3 (pISG54-luc in A) or NF κ B (pPRDII-luc in B). After 30 h, cells were transfected with 1 μ g/ml poly(I:C) or mock transfected with PBS. Beta-galactosidase and luciferase activities were measured after a further 18 h, using a dual-light reporter assay. The plots refer to normalized luciferase ratios, measured in one of three independent experiments, each carried out in triplicate; * $P < 0.01$, ** $P < 0.001$. (C) HEK293T cells were transfected with pJ7lacZ and the indicated plasmids, together with the IFN β reporter IFN β -luc. After 30 h, cells were transfected with either PBS or 1 μ g/ml poly(I:C). Beta-galactosidase and luciferase activities were measured after a further 18 h, using a dual-light reporter assay. The plots refer to normalized luciferase ratios, measured in one of three independent experiments, each carried out in triplicate (left). RING mut: mutated RNF114 construct, bearing two Ala substitutions at Cys residues 29 and 32 in the RING domain; UIM mut: RNF114 construct, bearing a Pro substitution at the conserved Ser 224 in the UIM domain; * $P < 0.05$, ** $P < 0.001$. The expression of all RNF114 constructs was verified by western blot (right).

role in this pathway. First, we assessed cellular responses to cytoplasmic dsRNA in two independent RNF114 knockdown cell lines, generated through the lentiviral transduction of short hairpin RNAs (sh-RNAs) into HEK293T cells. Following poly(I:C) transfection, we detected a significant type I IFN induction (measured as increased transcription of the *IFNB1* gene) in control cells, where we also observed a marked increase in the expression of other inflammatory mediators, known to be induced by the stimulus. At the same time, we observed a heterogeneous response in the two knockdown cell lines, which displayed clear differences in the cytokine levels induced by poly(I:C) stimulation (Supplementary Material, Fig. S3A–C). Owing to the difficult interpretation of these findings, we adopted a different approach and sought to investigate the effects of RNF114 over-expression on transcription factors NF- κ B and IRF3, both of which are activated downstream of RIG-I and MDA5 and required for type I IFN expression. Using luciferase reporter constructs, we found that the transfection of RNF114 cDNA into HEK293T cells resulted in an increase of basal and poly(I:C)-induced activity, for both reporters (Fig. 4A and B).

In order to dissect the functional role of RNF114 RING and UIM domains in dsRNA-induced type I IFN transcription, we generated two mutant constructs and assessed their ability to

up-regulate the activity of an IFNB1 reporter. These experiments showed that the presence of an intact RING domain is essential to RNF114 function, as over-expression of a RING mutant construct failed to up-regulate basal and poly-IC induced reporter activity (Fig. 4C).

Although type I IFN is the main product generated through the activation of RIG-I/MDA5 signalling, it is well known that the stimulation of this pathway results in the production of other chemokines and cytokines (22). Thus, we sought to establish whether RNF114 over-expression influences the induction of any of these molecules. We transfected HEK293T cells with RNF114 cDNA and found that the gene over-expression was sufficient to induce the transcription of genes encoding type I (*IFNB1*) and type III (*IL-29*) IFNs as well as the CCL5 and CXCL10 chemokines (Fig. 5A–D). As the IFN β released by infected cells causes the production of a second type I IFN wave (23), we investigated whether cell-culture medium derived from HEK293T cells transfected with RNF114 cDNA could induce further IFN β expression. Indeed, we detected enhanced *IFNB1* mRNA levels in THP1 monocytes cultured in medium obtained from RNF114 transfected cells (Fig. 5E). This provided additional evidence for the induction of type I IFN by over-expression of RNF114.

Taken together, these results indicate that RNF114 enhances the production of dsRNA-induced inflammatory molecules.

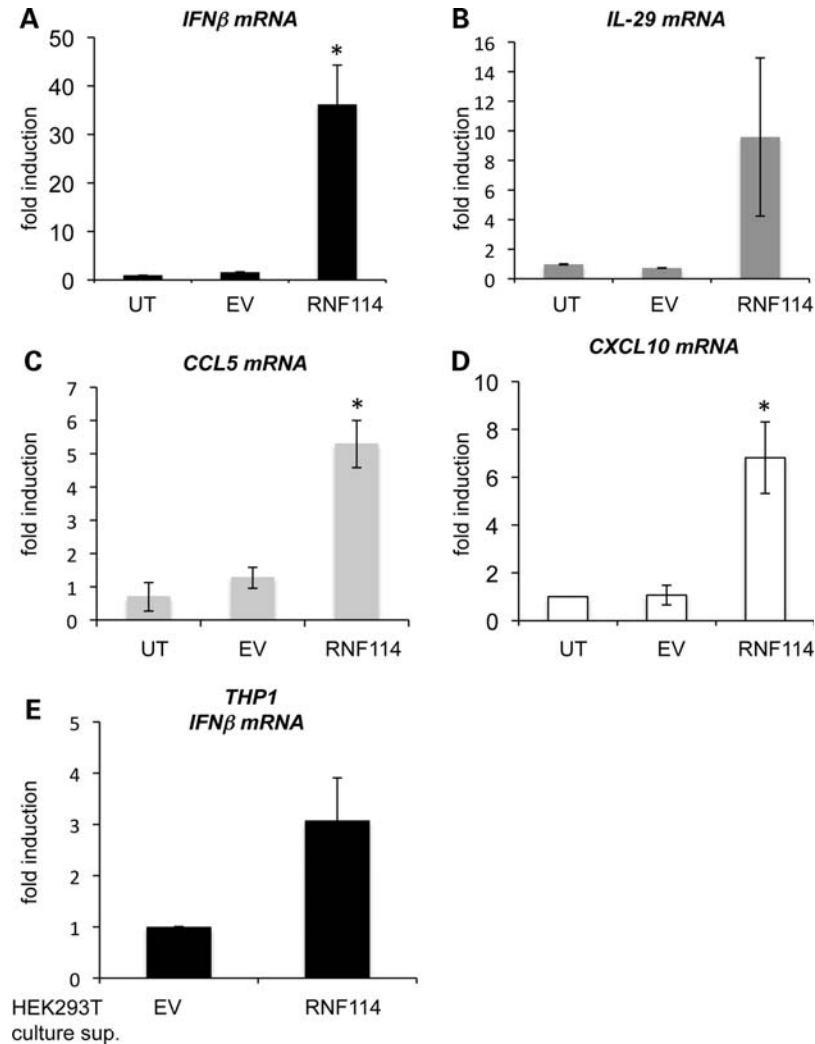


Figure 5. RNF114 over-expression results in the induction of IFN β , IL-29, CCL5 and CXCL10. (A–D) HEK293T cells were transfected with pcDNA3.1-myc/His plasmids containing either RNF114 cDNA (RNF114) or no insert (EV: empty vector) or were left untransfected (UT). Forty-eight hours post-transfection, cells were harvested and the expression of *IFNB1* (A), *IL-29* (B), *CCL5* (C) and *CXCL10* (D) was measured by real-time PCR, using *PPIA* as internal control. * $P < 0.005$ relative to the EV control. (E) THP1 cells were cultured for 24 h in cell-culture supernatants of HEK293T cells that had been transfected for 48 h with either an empty pcDNA3.1-myc/His vector (EV) or one containing RNF114 cDNA (RNF114). THP1 cells were harvested and *IFNB1* mRNA induction was measured by real-time PCR.

Since HEK293T cells do not express the TLR3 receptor for extracellular dsRNA (24) and do not respond to extracellular poly(I:C) stimulation (Supplementary Material, Fig. S4), our findings demonstrate that RNF114 specifically regulates the innate antiviral response to intracellular dsRNA.

DISCUSSION

The advent of GWAS has demonstrated that highly powered, hypothesis-free genetic studies have unique potential to uncover novel disease pathways for common and complex diseases. Here, we have integrated the results of a GWAS, with a range of functional studies informed by the results of bio-informatic analyses. This combined approach has allowed us to identify *RNF114* as a regulator of RIG-I/MDA5 signalling, thus pointing to the disruption of innate antiviral responses as a pathogenic mechanism in psoriasis.

Our work was initiated by the observation that *RNF114* is a paralogue of *RNF125* (13). The latter encodes a RING domain E3 ligase, which suppresses dsRNA-induced interferon production by promoting the proteasomal degradation of RIG-I and MDA5 (16). Our experiments demonstrated that RNF114 is a cytoplasmic protein, which can be up-regulated by IFNs and dsRNA, similar to RNF125 and to an equivalent extent. We also showed that RNF114 can bind ubiquitinated proteins, as well as free poly-ubiquitin molecules. This is of considerable interest, as emerging data point to a critical role of ubiquitin binding proteins [e.g. the DUBA and TNFAIP3/A20 de-ubiquitinating enzymes (25,26)] and unanchored ubiquitin chains (27,28) in the regulation of RIG-I signalling.

Our experiments also confirmed that RNF114 is an ubiquitin ligase. Although the enzymatic activity of RNF114 appears weaker than that of RNF125, it is worth remembering that our

in vitro assays could only measure the accumulation of RNF114 auto-ubiquitination products. Thus, the identification of RNF114 physiological substrates will be ultimately required to design the experiments that are most suited for the investigation of enzymatic activity in eukaryotic cells.

To further investigate the role of RNF114 in RIG-I/MDA5 signalling, we investigated the response to dsRNA upon gene silencing and gene over-expression. The analysis of *RNF114* knockdown cells yielded a set of heterogeneous results, possibly owing to the presence of different populations within our polyclonal cell lines. At the same time, we cannot exclude the possibility that these results reflect a partially redundant role for RNF114, whereby the gene knockdown has a limited effect on cytokine production. Conversely, the results of our over-expression experiments were unambiguous, with real-time PCR experiments and three independent reporter assays consistently showing that RNF114 is a positive regulator of dsRNA-induced type I IFN production. Importantly, the effect of RNF114 over-expression on reporter activity and cytokine levels was comparable to that observed for REUL, another positive regulator of the RIG-I/MDA5 innate immune response (29). Of note, a recent large-scale screen of interferon-stimulated genes has demonstrated that RNF114 has moderate antiviral activity against HCV and that the magnitude of this effect is comparable to that observed for OAS1, a well known effector of innate antiviral responses (30).

We have repeatedly observed a marked increase in MDA5 and RIG-I expression in cells that had been co-transfected with RNF114 (Supplementary Material, Fig. S5A–C and data not shown), suggesting the interesting possibility that RNF114 might act by stabilizing RIG-I and MDA5 protein levels. Although it is tempting to speculate that this may reflect competition with negative regulators such as RNF125, co-immunoprecipitation experiments did not provide any evidence for a direct interaction between RNF114 and RIG-I or MDA5 (data not shown). Thus, further experiments will be needed to dissect the molecular mechanisms underlying our observations and to establish whether RNF114 may contribute to the up-regulation of RIG-I and MDA5, which has been observed in psoriatic skin (31).

Taken together, our data identify a feedback mechanism whereby the type I IFN produced upon viral infection up-regulates the expression of RNF114, which in turn leads to the production of more type I IFN through the RIG-I/MDA5 pathway. In this context, it is worth pointing out that type I IFN plays a key role in psoriasis, as demonstrated by observations of constitutively active IFN α signalling in patient skin lesions (32,33) and reports of psoriasis exacerbation, following IFN α treatment of hepatitis (34,35). Interestingly, our data show that RNF114 also affects the production of IL-29, suggesting that this type III IFN molecule may also be involved in the pathogenesis of psoriasis. This notion is consistent with the results of a GWAS recently carried out by our group, which detected a significant association signal in proximity of *IL28RA*, the gene encoding the IL-29 receptor (10). The same study also identified a susceptibility interval on chromosome 2q24, which encompasses *IFIH1* (the gene encoding MDA5) as well as four other genes (10).

Thus, our results integrate the findings of genetic studies into a pathogenic model, whereby the disruption of innate antiviral responses contributes to the accumulation of the cytokines that mediate epithelial inflammation. Future investigations into the regulatory role of RNF114 are expected to further elucidate the molecular mechanisms underlying this novel disease pathway.

MATERIALS AND METHODS

Cell culture and transfection

Following approval from Guy's and St Thomas Hospital Local Research Ethics Committee, primary human keratinocyte cultures were established from breast reduction tissue of consenting donors ($n = 4$), using the procedure described by Mee *et al.* (36). Cells were grown for 2–4 passages in EpiLife medium (Gibco) containing 1X Human Keratinocyte Growth Supplement (Gibco). Prior to treatment with the relevant cytokines (IFN γ , IFN α , TNF α , IL-17 and IL-22, all purchased from R&D Systems), cells were quiesced for 24 h in supplement-free medium.

HEK293T, HeLa and HaCaT cells were grown in DMEM medium (Gibco) supplemented with 10% FBS and THP1 cells were grown in RPMI medium (Gibco) supplemented with 10% FBS. Plasmids and poly(I:C) (Invivogen) were transfected into 80% confluent cultures using FuGENE 6 (Roche).

RNF114 knockdown

Stable RNF114 knockdown was achieved through transfection of HEK293T cells with lentiviral-based pAPMshRNA vectors (a gift of Jeremy Luban, Columbia University, NY, USA) containing microRNA short hairpin RNAs (shRNA) for silencing of RNF114 (or control shRNA for luciferase), as well an expression cassette to confer puromycin resistance. Forty-eight hours after transfection, cells were grown in culture medium supplemented with 2 μ g/ml puromycin, until stably transfected cells were selected.

Plasmid generation

The following plasmids were generous gifts from collaborators: pJ7lacZ (Talat Nasim, King's College London); myc-SMAD8 (Rajiv Machado, King's College London); pISG54-luc, pPRDII-luc, pIFN β -luc (Steve Goodbourn, St George's University of London); HA-RIG-I, HA-MDA5 (Andreas Pichlmair, CeMM, Vienna). GST-UBA, GST-UBAm (Nia Bryant, University of Glasgow). The GST-RNF125 was previously described by Giannini *et al.* (13). Plasmids bearing the RNF114 coding sequence were obtained by amplifying the gene cDNA from a pCMVSPORT6-RNF114 clone (imaGENES) and sub-cloning it into pcDNA3.1(–) myc/His (Invitrogen) between *EcoRI* and *BamHI* sites (myc-RNF114) and into pGEX5x3 (Amersham) between *EcoRI* and *NotI* sites (GST-RNF114). The RNF114 RINGmut (C29A/C32A) and RNF114 UIMmut (S224P) constructs were obtained by using the Quik-Change Site-Directed Mutagenesis kit (Stratagene). The accuracy of

all cloning and mutagenesis procedures was verified by direct sequencing of constructs.

Cellular fractionation

Nuclear and cytoplasmic fractions were separated using the NE-PER Extraction Reagents (Pierce) according to the manufacturer's protocol. Membrane-associated and soluble proteins were separated using membrane floatation on sucrose gradients. In brief, cells were washed in PBS, resuspended in 20 mM Tris pH 7.8 supplemented with 1 mM PMSF and CLAP (5 µg/ml each of chymostatin, pepstatin A, antipain hydrochloride and 10 µg/ml leupeptin hemisulphate) and sonicated. Nuclei and unbroken cells were removed by centrifugation at 1000g and the post-nuclear supernatant was adjusted to 70% sucrose (w/v) in 2.5 ml, overlaid with 6 ml 65% (w/v) sucrose/NTE (150 mM NaCl, 20 mM Tris pH 7.8, 1 mM EDTA) and 2.5 ml 10% (w/v) sucrose/NTE. Gradients were centrifuged at 120 000g for 18 h. One millilitre fractions were collected from the top and subsequently adjusted to 1% NP-40. Aliquots of these fractions were analysed by SDS-PAGE and western blotting.

Cell lysis, immunoprecipitation and western blotting

Cells were lysed in NP-40 buffer (1% NonidetP-40, 20 mM Tris pH 7.8, 150 mM NaCl, 2 mM MgCl₂), supplemented with PMSF and CLAP. To detect ubiquitinated proteins, cell lysates were supplemented with SDS (0.1%) and *N*-ethylmaleimide (5 mM).

The following antibodies were used in western blot experiments: mAb anti-RNF114 (Abnova), mAb anti-tubulin (Invitrogen), goat anti-laminB1 S-20 (Santa Cruz Biotechnology), rabbit anti-Hsp90 α/β H-114 (Santa Cruz Biotechnology), mAb anti-TfRec H68.4 (Zymed), mAb anti-myc 9E10 (Sigma), mAb anti-HA HA.11 (Covance), goat anti-GST (Amersham Pharmacia Biotech), mAb anti-β-actin (Sigma Aldrich).

In vitro ubiquitination assay and pull-down of ubiquitinated proteins

The *E. Coli* strain BL21DE3(pLysS) (Novagen) was transformed with the relevant pGEX constructs and GST fusion proteins were purified on glutathione-sepharose beads (Amersham), according to the manufacturer's instructions. For the ubiquitination assays, 10 µl of beads bearing wild-type or mutagenized GST fusion proteins (~2 µg of protein) were incubated for 90 min at 30°C, in 50 µl of reaction buffer (50 mM Tris, pH 7.4; 2.5 mM MgCl₂; 0.5 mM DTT; 10 mM ATP) together with 50 ng E1 ubiquitin activating enzyme, 2 µg biotin-conjugated ubiquitin and 1 µg of UbcH5a ubiquitin conjugating enzyme (all purchased from Biomol). Auto-ubiquitination products were revealed by western blots, using a streptavidin-HRP conjugate. To pull-down ubiquitinated proteins, NP-40 lysates of HEK293T cells transfected with myc-RNF114 and HA-ubiquitin (one-third of a 10 cm dish per recombinant protein) were incubated overnight at 4°C with 20 µl glutathione-sepharose beads bearing GST-UBA or GST-UBA-m (purified from BL21DE3

E. Coli). GST-UBA represents a fusion protein of GST and the UBA domain of Dsk2p (amino acids 328–373), whereas GST-UBAm contains two mutations at Met342 and Phe244 in the UBA, rendering it unable to bind ubiquitin (19). Pull-down products were visualized by western blot using anti-ubiquitin and anti-RNF114 antibodies.

Immunofluorescence

Cells were cultured on 13 mm diameter glass coverslips for 24 h, then fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton TX-100 (Perbio Science)/PBS. Primary and secondary antibodies were diluted in 0.2% w/v gelatin (Sigma)/PBS and incubations were carried out for 1 h at room temperature. Coverslips were mounted in Mowiol (Calbiochem-Novachem) supplemented with 4',6-diamidino-2-phenylindole (DAPI) and images were acquired on a Leica TCP SP2 AOBSTM confocal microscope.

RNA extraction and real-time PCR

Total RNA was extracted from primary keratinocytes and HaCaT cells, using the Trizol reagent (Invitrogen). Reverse transcription was carried with the Reverse-it cDNA first-strand synthesis kit (Thermo Scientific) and PCR reactions were set up using TaqMan Gene Expression assays (Applied Biosystems). Transcript levels were quantified by the ΔΔCt method (37), using *PPIA* as an endogenous control. All reactions were carried out in duplicate.

Reporter assays

HEK293T cells were transfected for 48 h with the relevant constructs, in the presence or absence of poly(I:C). Lysates were prepared with 1X Reporter Lysis Buffer (Promega) and reporter activities were measured using the Dual-Light Luciferase and β-Galactosidase Reporter Gene Assay System (Applied Biosystems).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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