**STAT4 expression and activation is increased during mitosis *in vitro* and *in vivo* in skin and mucosa derived cell types: implications in neoplastic and inflammatory skin diseases**

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Running Title: STAT4 facilitates progression in mitosis

Key words: STAT4, keratinocytes proliferation, tyrosine phosphorylation, serine phosphorylation, psoriasis, squamous cell carcinoma.

Authors for correspondence: Caterina Ferreli (ferreli@unica.it) **Abstract**

Background

The signal transducer and activator of transcription-4 (STAT4/Stat4) is a transcription factor known to convey signals from interleukin-12, interleukin-23, and interferon-alpha/beta to the nucleus, resulting in activation of dendritic cells, T-helper cell differentiation and production of interferon-gamma.

Objective

To demonstrate a novel role for STAT4 in cell mitosis.

Results

Phosphoserine STAT4 (pSerSTAT4) is increased in cells undergoing mitosis, and is distributed throughout the cytoplasm during this stage of the cell cycle whilst phosphotyrosine STAT4 (pTyrSTAT4) is confined to the chromosomal compartment. This distinct pattern of pSerSTAT4 during mitosis is seen in vitro in human keratinocytes and in other cell types. This is also present in vivo in cells undergoing mitosis in normal skin, psoriasis and squamous cell carcinoma.

Inhibition of STAT4 phosphorylation by lisofylline and depletion of STAT4 by RNA interference results in a delay in progression of mitosis and leads to a reduction in cells completing cytokinesis.

Conclusion

Our data demonstrate that STAT4 plays a role in enabling the normal and timely division of cells undergoing mitosis.

**Introduction**

Signal transducer and activator of transcription (STAT/Stat) proteins are a family of transcription factors comprising seven members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) which are involved in a variety of biological processes, including regulation of immune responses, cell growth and survival 1, 2. STAT monomers live latently in the cytoplasm but, after incident signals by extracellular cytokines, hormones and growth factors which activate Janus kinases (JAKs) resulting in tyrosine phosphorylation of the STAT cytoplasmic tail, STATs homo- or heterodimerise via interaction of the tyrosine phosphorylated segment with the conserved SH2 domains and translocate into the nucleus where they initiate transcription 3,4. Some STATs, for example STAT4, contain a second phosphorylation site on serine residues within a P(M)SP motif at their C-terminus (S721 in the case of STAT4), and phosphorylation at this site seems important for complete transcriptional activity within the nucleus 3,5,6. In some situations, serine phosphorylation of STATs can occur in the absence of tyrosine phosphorylation, leading to a change in the subcellular distribution of the STAT, for example serine phosphorylation of STAT3 can result in pSerSTAT3 being exported from the nucleus into the cytoplasm 7.

STAT4 is expressed by lymphoid cells8, but has also been identified in the myeloid lineage (monocytes, macrophages and dendritic cells 9), endothelial cells 10, testis 11,12, heart 13, and skin 14. In immunocytes, including monocytes and T cells, STAT4 mediates signals from interleukin-12 (IL-12), interleukin-23 (IL-23), and type 1 interferon cytokines resulting in monocyte activation, production of interferon-gamma and differentiation of Th1 and Th17 cells 15,16. STAT4 also plays a critical role as a lineage-determining factor in Th1 development and in altering the balance between Th17 and Tregs in autoimmune diseases 17,18, as well as in the suppression of proliferation of conventional T cells secondary to interleukin-35 which is mediated by STAT4 / STAT1 heterodimers 19. Furthermore, STAT4 genetic polymorphisms have been documented as a risk factor for a variety of autoimmune disorders, including rheumatoid arthritis 20, systemic lupus erythematosus 20,21, systemic sclerosis 22,23, and primary biliary cirrhosis 24,25. However, in addition to its immunological activities, there is some evidence that STAT4 may have effects in non-immunological cells. For example, based on its presence in male germ cells which have completed meiosis in the testis, it has been hypothesised that STAT4 may have a role in onset of zygotic transcription and/or cell division in the early embryo 11. Additional evidence for a role by STAT4 in cell proliferation comes from the observation of a reduction in the growth rate of vascular smooth muscle cells following RNAi targeting of STAT4 in vitro 26.

In the present work, based on a serendipitous observation of phosphoserine STAT4 (pSerSTAT4)-bright cells in keratinocyte cell cultures, we investigated for a role for STAT4 in cell division. Here, we report that pSerSTAT4 is increased in a variety of cell types undergoing mitosis in vitro and in vivo, and that STAT4 functions to facilitate timely completion of telophase and progression through cytokinesis during this process.

**Materials and Methods**

**Ethics Statement**

This study was approved by the ethics committees overseeing the research at the institutions where samples were ascertained and analyzed. For the human tissue and cells the approvals were obtained from the Southampton and South West Hampshire Research Ethics Committee (reference number: 07/Q1704/59) and from the ethics committee of the “ Azienda Ospedaliero Universitaria” of Cagliari (reference number: np/6428 ) . Written informed consent from the donor or the next of kin was obtained for the use of the samples in research.

All work involving the use of tissues derived from mice which were euthanized by cervical dislocation (Schedule 1) was carried out under the authority of a Project Licence held under the Animals (Scientific Procedures) Act, 1986 which had been given approval by the University of Southampton Local Ethical Review Process.

**Cell culture and tissue samples**

Human skin primary keratinocytes and dermal fibroblasts were isolated from healthy breast skin. For keratinocyte cultures, skin was incubated overnight with 2 U/ml dispase (Invitrogen, Paisley, Renfrewshire, UK) in PBS at 37°C and epidermis separated from dermis with fine forceps and dissociated in 0.05% trypsin/0.02% EDTA solution (Invitrogen, Paisley, Renfrewshire, UK) for 15 minutes. The resulting cells were collected by centrifugation and cultured in Defined Keratinocyte-SFM containing growth supplement (Invitrogen, Paisley, Renfrewshire, UK), 50 μg/ml penicillin and 50 μg/ml streptomycin, and were used within the first or second passage for the investigations outlined in this study. Dermal fibroblasts were grown in Petri dishes and expanded in T21/75-cm2 flasks containing Dulbecco’s Modified Eagle's Medium (Gibco, Paisley, Renfrewshire, UK) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Fibroblasts were then passaged weekly using trypsin-EDTA and used for assays at second or third passages. HeLa , HaCaT, and RPMI7951cells (purchased from ATCC) were cultured at 37°C with 5% CO2 in Dulbecco’s Modified Eagle's Medium (Gibco, Paisley, Renfrewshire, UK) containing 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 50 μg/ml penicillin and 50 μg/ml streptomycin. Interleukin 12 (human rDNA derived) was obtained from the National Institute for Biological Standards and Control (Potters Bar, Hertfordshire, UK) and used at 5 ng/ml concentration.

Formalin fixed human samples were used after diagnostic procedures had been completed. For the animal samples mice were sacrificed and the appropriate organs harvested and fixed in 2% buffered paraformaldehyde.

**Antibodies**

Anti-pSerSTAT4 rabbit polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Baden-Wurttemberg, Germany) was used at 1Description: [ratio]150 dilution; anti-pTyrSTAT4 rabbit polyclonal antibody (Zymed Laboratories Inc, Cambridge, Cambridgeshire, UK) at 1Description: [ratio]50; anti-STAT4 (C20) rabbit polyclonal antibody (Zymed Laboratories Inc, Cambridge, Cambridgeshire, UK) at 1Description: [ratio]100; anti-pSerSTAT1, anti-pSerSTAT3, anti-pTyrSTAT1 and anti-pTyrSTAT3 rabbit polyclonal antibodies (Cell Signaling Technology, Hitchin, Hertfordshire, UK) were each diluted at 1:50. Anti-alpha-tubulin mouse monoclonal (Santa Cruz Biotechnology, Heidelberg, Baden-Wurttemberg, Germany) was employed at 1:750 dilution. Secondary antibodies included Alexa-Fluor-546 goat anti-rabbit IgG (Invitrogen, Paisley, Renfrewshire, UK) utilised at 1:400; FITC goat anti-mouse IgG (Jackson ImmunoResearch, Newmarket, Suffolk, UK) at 1:50, Alexa-Fluor-488 goat anti-rabbit IgG (Molecular Probes, Paisley, Renfrewshire, UK) at 1:200. The chromosomal/nuclear dye TO-PRO-3 iodide (Molecular Probes, Paisley, Renfrewshire, UK) was used at 1:1000 dilution. For immunohistochemistry, a biotinylated swine anti-rabbit secondary antibody (Dako, Ely, Cambridgeshire, UK) was diluted 1:400. pSerSTAT4 blocking peptide (Santa Cruz Biotechnology, Heidelberg, Baden-Wurttemberg, Germany) was employed at a five-fold excess to compete out the pSerSTAT4 antibody.

**Fluorescence microscopy**

Immunofluorescence and confocal microscopy were performed on cells cultured in monolayer on cover slips in 24-well plates. Cells were fixed in 2% buffered paraformaldehyde for 10 minutes at 4°C, permeabilised in ice-cold 100% methanol for 10 minutes, blocked in 1% BSA for 20 minutes at room temperature, and incubated with relevant antibody (or with isotype-matched antibody) for 1 hour at room temperature or overnight at 4°C, and subsequently with an Alexa-Fluor-546 secondary antibody for 40 minutes at 37°C. Nuclei were counterstained with TO-PRO-3 iodide. Cells were examined with the Leica TCS SP5 confocal microscope (Leica Microsystems, Milton Keynes, Buckinghamshire, UK). For quantification of STAT4 expression, Z-stacks of five sections (each 0.9 μm) from the base to the apex of cells were acquired randomly and analyzed using the SP5 software. Regions of interest (ROI) were created using the poly-line function and fluorescence intensity histograms, based on a 0–255 RGB colour code, produced for each acquisition channel. For experiments involving the pSerSTAT4 blocking peptide (Santa Cruz Biotechnology, Heidelberg, Baden-Wurttemberg, Germany), the blocking peptide was mixed with the pSerSTAT4 antibody for 30 minutes, and fixed cells were then incubated with the resulting mixture for 2 hours at room temperature.

**Immunohistochemistry of paraffin-embedded skin sections**

4μm paraffin-embedded tissue sections were dewaxed, endogenous peroxidase blocked with 0.5% H2O2 in methanol, and then washed with PBS. Nonspecific binding was blocked with avidin/biotin blocking solution (Vector Laboratories, Peterborough, Cambridgeshire, UK) followed by PBS/20% fetal bovine serum/1% bovine serum albumin. Sections were incubated for 1 hour in a humidified chamber at room temperature with relevant antibodies in PBS. After washing with PBS, sections were incubated for 30 minutes at room temperature with biotin-conjugated swine anti-rabbit antibody solution (Dako, Ely, Cambridgeshire, UK) diluted 1:400. Staining was visualised using an ABC system (Dako, Ely, Cambridgeshire, UK) and diaminobenzidine (Biogenex, San Ramon, CA, USA). Sections were counterstained with Mayer's Hematoxylin.

**Western Blotting**

Treated and untreated cells were lysed in ice-cold RIPA buffer (Cell Signaling Technology, Hitchin, Hertfordshire, UK). Lysates were then spun at 16,000×g for 30 minutes at 4°C, and the supernatant was recovered. Proteins were quantified with the Quant-iT™ protein assay kit (Molecular Probes) using a Qubit fluorometer (Invitrogen, Paisley, Renfrewshire, UK) and then stored at −80°C until use. 40 µg of protein from whole-cell lysate was added to 4x Laemmli buffer (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) and heated for 5 minutes at 95°C. Proteins were resolved by 12% SDS-PAGE, along with a molecular weight marker (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK), then transferred to nitrocellulose membranes, which were subsequently blocked with 5% milk protein before being probed for 12 hours with the specific antibody. Following incubation with horseradish peroxidase-conjugated secondary antibody, protein bands were detected using an Amersham ECL kit (GE Healthcare, Hatfield, Hertfordshire, UK), and chemiluminescent signals recorded with a ChemiDoc XRS imager (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK).

**Cell cycle synchronization**

Cells undergoing mitosis were acquired using a selective mitotic cell detachment technique as previously described 27. Cell culture synchronisation in M phase was conducted using nocodazole (Sigma-Aldrich, Gillingham, Dorset, UK), where cultured cells were incubated in medium containing 0.1 μg/ml nocodazole for 16 hours. After nocodazole incubation, cells were collected and fixed in 2% buffered paraformaldehyde for 10 minutes at 4°C.

**Flow cytometry**

Single-cell suspensions were washed in PBS, fixed in 2% paraformaldehyde for 15 minutes at room temperature, then incubated with primary antibody (or isotype-control antibody) for 1 hour at 4°C. Binding was detected by Alexa-Fluor-488 goat anti-rabbit IgG (Molecular Probes, Paisley, Renfrewshire, UK) diluted 1:200 for 40 minutes at 4°C. Fluorescence was quantified by using a FACSCalibur flow cytometer (Becton Dickinson, Cowley, Oxfordshire, UK), and data processed with the CellQuest software programme (BD Biosciences, Cowley, Oxfordshire, UK).

**Pharmacological inhibition**

Cultured HeLa and HaCaT cells were treated separately with a variety of pharmacological inhibitors; these included 100 μM lisofylline (Enzo Life Sciences, Exeter, Devon, UK) and 20ng/ml rapamycin, 1μM alsterpaullone, 20mM SP600125, 10mM U0126, 20 mM SB203580 (each from Calbiochem, Beeston, Nottinghamshire, UK). Cells were then washed, collected by trypsinisation and fixed in 70% ice cold ethanol for 10 min before pelleting for FACS analysis. For confocal microscopic analysis, cells grown on coverslips were treated directly with lisofylline and fixed in 2% paraformaldehyde after washing.

**SiRNA Transfection**

For silencing of STAT4, Silencer Select Predesigned STAT4-specific siRNA sequences (Ambion, Paisley, Renfrewshire, UK) were used at 5nM concentration for 24, 48 and 72 hours; these included s13533 sense CAACGAUUCUUCUUCAAAAtt, s13533 antisense UUUUGAAGAAGAAUCGUUGcc, s13531 sense CAGAGGCCGUUGGUACUUAtt, s13531 antisense UAAGUACCAACGGCCUCUGag and siRNA Negative Control #1 siRNA. SiRNA transfectioninto HeLa cells was carried out using Lipofectamine 2000(Invitrogen, Paisley, Renfrewshire, UK) according to the manufacturer's instructions.

**Time lapse microscopy**

Cell cultures were examined on an Olympus CK2 inverted microscope (Olympus Optical, Hounslow, Middlesex, UK), and images were recorded at 5 minute intervals for 3 hours using an Olympus 3040Z digital camera. In each of three separate experiments, three fields per well were imaged and analysed from three treated and three untreated wells.

**Statistical analysis**

Data are expressed as mean ± s.e.m. of at least three independent experiments. Data were analyzed using a non-parametric *t* test (Mann-Whitney) in which *p* < 0.05 was considered statistically significant. Calculations were performed using Prism 5.0 GraphPad software (San Diego, CA, USA).

**Results**

**Mitotic keratinocytes contain abundant cytoplasmic pSerSTAT4**

IL-12 is well recognised as an immunomodulatory cytokine for peripheral blood mononuclear cells, inducing T cell proliferation and IFN-gamma production and increasing NK cell cytotoxicity 28. In addition, IL-12 has been reported to possess other non-immunomodulatory activities, such as its ability to suppress ultraviolet-radiation induced apoptosis of keratinocytes 29. During investigations in our laboratory, in which IL-12 was added to HaCaT keratinocytes, we made an unexpected observation that a number of HaCaT keratinocytes in the cell cultures were brightly positive for pSerSTAT4 (Fig. 1a), with equal numbers of pSerSTAT4-bright cells noted in the presence or absence of IL-12. The pattern of pSerSTAT4 expression in these pSerSTAT4-bright cells, coupled with linear areas of less intense staining within the cell, led us to hypothesise that these cells were in mitosis and that pSerSTAT4 might play a role in normal cell division. To ensure that the anti-pSerSTAT4 antibody recognised pSerSTAT4 alone, and was not binding to another unrelated protein in the HaCaT keratinocytes, immunoblotting was performed. As expected, Western blotting demonstrated the presence of pSerSTAT4 in peripheral blood mononuclear cells which had been stimulated with IL-12 and in Epstein-Barr Virus (EBV)-transformed B lymphocytes (Fig. 1b). A similar band was observed in HaCaT keratinocytes, with no evidence of binding by the anti-pSerSTAT4 antibody to other proteins in these cells (Fig. 1b). To confirm that the pSerSTAT4-bright cells were undergoing mitosis, HaCaTs were immunostained for pSerSTAT4, alpha-tubulin and chromosomal DNA (TO-PRO-3) and viewed by confocal immunofluorescence microscopy. The pattern of immunofluorescence by the alpha-tubulin and TO-PRO-3 indicated that the pSerSTAT4-bright cells were in various stages of mitosis and that pSerSTAT4 was present throughout the cytoplasm of these cells during this phase of the cell cycle (Fig. 1c). By contrast, in non-mitotic cells, pSerSTAT4 was confined to the nucleus (Fig. 1c). No pSerSTAT4 signal was identified in cells stained with isotype control antibody (data not shown). Tyrosine phosphorylation precedes serine phosphorylation during STAT4 intracellular signaling from IL-12 5, therefore HaCaTs were triple stained for phosphotyrosine STAT4 (pTyrSTAT4), alpha-tubulin and chromosomal DNA. Surprisingly, pTyrSTAT4 co-localised with the DNA compartment in dividing cells (Fig. 1d), with no obvious pTyrSTAT4 staining in the cytoplasm of mitotic cells, indicating that the serine phosphorylated form of STAT4 throughout the cytoplasm during mitosis is not concomitantly tyrosine phosphorylated. To obtain further supporting evidence for the presence of increased pSerSTAT4 in the cytoplasm of mitotic HaCaTs, these cells were also triple stained with an antibody which recognises total STAT4 (including unphosphorylated, tyrosine-phosphorylated and serine-phosphorylated forms of STAT4), anti-alpha-tubulin and TO-PRO-3. Consistent with the observation of more abundant pSerSTAT4 in cells undergoing mitosis, staining for total STAT4 showed an increase in STAT4 throughout the cytoplasm in mitotic cells (Fig. 1e). Furthermore, adsorption of the anti-pSerSTAT4 antibody with a specific pSerSTAT4 blocking peptide, prior to immunofluorescence staining, abolished the pSerSTAT4 signal seen in mitotic cells (Fig. 1f).

**pSerSTAT4 is increased in mitotic keratinocytes**

To determine whether the diffuse cytoplasmic staining of pSerSTAT4 in mitotic keratinocytes was due to an increase in pSerSTAT4 rather than simply due to a redistribution of pSerSTAT4 from the nuclear compartment, mitotic HaCaT cells were collected by mitotic detachment, in which gentle shaking of cell cultures causes release of cells undergoing mitosis into the supernatant 27. Because of limitations in obtaining large quantities of mitotic cells at a single time point with this method, HaCaTs and HeLas were treated in separate experiments for 16 hours with 0.1 µg/ml nocodazole, which disassembles microtubules and synchronises cells in mitosis 30. The resulting cells from both procedures underwent triple staining for pSerSTAT4, alpha-tubulin and chromosomal DNA, and this confirmed the mitotic nature of the cells obtained by both techniques (Fig. 2a,b). Flow cytometry was conducted as per Uzel et al.31 to assess the relative amounts of pSerSTAT4 in the unsynchronised and mitotic cell populations, and demonstrated a rise in pSerSTAT4 in the cells captured by mitotic detachment (mean fluorescence intensity (MFI) 337.76 in one of three representative experiments) in comparison with the cells growing routinely in normal cell culture (MFI 24.40) (Fig. 2c). pSerSTAT4 was similarly much higher in mitotic cells synchronised with nocodazole (MFI 227.96) than in unsynchronised cell cultures (MFI 35.06) (Fig. 2d). An increase in pTyrSTAT4 was also detected in nocodazole-synchronised cells (increase in MFI from 56.04 in unsynchronised cells to 94.70 in nocodazole-treated cells) (Fig. 2e), but this relative increase was lower than that seen with pSerSTAT4.

**pSerSTAT1, pTyrSTAT1, pSerSTAT3 and pTyrSTAT3 levels in mitotic cells**

In addition to the ability of STAT4 to form homodimers 32, STAT4 can form heterodimers with STAT119 and separately with STAT3 8,33. Therefore, we examined whether mitotic cells contained alterations in the distribution and/or amount of pSerSTAT1, pTyrSTAT1, pSerSTAT3 and pTyrSTAT3 similar to those seen in pSerSTAT4. A small amount of positive immunofluorescence signal was detected for pSerSTAT1, pTyrSTAT1, pSerSTAT3 and pTyrSTAT3 in the cytoplasm of mitotic cells on confocal microscopy (Fig. 3a,c,e,g). However, by comparing the nuclear signal in non-mitotic cells to the cytoplasmic signal in mitotic cells on confocal microscopy with that seen with pSerSTAT4, it was noted that the relative cytoplasmic to nuclear signal was not as dramatically increased in the case of pSerSTAT1, pTyrSTAT1, pSerSTAT3 and pTyrSTAT3 as that in pSerSTAT4 (compare red signals in Fig. 3a,c,e,g with Fig. 1c). Although some elevation was noted with flow cytometry in the levels of pSerSTAT1, pTyrSTAT1, pSerSTAT3 and pTyrSTAT3 following synchronization with nocodazole (MFIs 7.14 and 9.13, 5.01 and 12.06, 51.90 and 69.25, 54.05 and 100.32 for pSerSTAT1, pTyrSTAT1, pSerSTAT3 and pTyrSTAT3 in non-synchronised and nocodazole-synchronised cells respectively), none of these increased to the same extent as that seen with pSerSTAT4 (compare Fig. 3b,d,f,h with Fig. 2d). These results suggest that the elevation of pSerSTAT4 throughout mitosis is not accompanied by a similar rise in phosphorylated forms of STAT1 or STAT3, and suggest that pSerSTAT4 does not dimerise with either of these STATs during cell division.

**Cytoplasmic pSerSTAT4 is seen in multiple cell types undergoing mitosis in vitro**

To establish whether the cytoplasmic distribution of pSerSTAT4 is a generalised feature of mitosis in multiple skin cell types, confocal microscopy was conducted with triple immunofluorescence for pSerSTAT4, alpha-tubulin and chromosomal DNA on a variety of cell types, including primary human keratinocytes, primary human dermal fibroblasts, RPMI-7951 melanoma cells and HeLa cells. Intense diffuse cytoplasmic pSerSTAT4 staining was observed in the keratinocytes (Fig. 4a), fibroblasts (Fig. 4b) and RPMI-7951 melanoma cells (Fig. 4c). A similar pattern of pSerSTAT4 staining was detected in HeLa cells were the pattern of pSerSTAT4 staining was then examined in more detail in each of the stages of mitosis, and as exemplified in Fig. 4d, cytoplasmic pSerSTAT4 was noted during prometaphase, metaphase, anaphase and telophase with the intensity of immunofluorescence highest in metaphase.

**pSerSTAT4 is distributed throughout the cytoplasm during mitosis in vivo**

The biological relevance of the increased cytoplasmic pSerSTAT4 during mitosis was investigated by detailing the pattern of murine pSerSTAT4 and human pSerSTAT4 localisation during cell division in vivo. Herrada and Wolgemuth 10 have previously reported that murine total STAT4 is expressed in haploid male germ cells, and we detected nuclear pSerSTAT4 in spermatids within the seminiferous tubules of mouse testis. However, immunostaining of testis also demonstrated strong cytoplasmic pSerSTAT4 in spermatogonia which were clearly undergoing cell division (Fig. 5a). The existence of total STAT4 in human skin, which was more strongly detected in the granular layer of the epidermis, has been described by Nishio et al14 and we also noted the presence of cytoplasmic pSerSTAT4 in suprabasal keratinocytes in normal human skin, which was most confluent in the upper part of the epidermis (Fig. 5b). Cytoplasmic pSerSTAT4 was absent in basal keratinocytes, however, cytoplasmic pSerSTAT4 was seen in cells undergoing mitosis in this layer (Fig. 5c). In some normal skin samples, mitotic cells undergoing cytokinesis were noted, and as had been observed in our cell culture experiments, the levels of cytoplasmic pSerSTAT4 were reduced in these cells. To examine hyperproliferative states in vivo, psoriasis and cutaneous squamous cell carcinoma (SCC) were investigated. Interestingly, in psoriasis the majority of nuclei exhibited strong pSerSTAT4 staining throughout the entire epidermis (Fig.5e), suggesting that activated nuclear STAT4 within keratinocytes may play a role in the pathogenesis of this condition. In addition, multiple cells in mitosis were noted in the basal and suprabasal layers in lesional psoriasis as has been previously reported 34, and the majority of these dividing cells contained abundant cytoplasmic pSerSTAT4 (Fig.5e). In cutaneous SCCs, strong nuclear pSerSTAT4 was seen in poorly differentiated SCCs whereas this feature was not observed in well differentiated tumours (Fig. 5f). Mitotic cells containing pSerSTAT4 throughout the cytoplasm were also frequently detected in all SCCs irrespective of their differentiation status. Thus, the in vivo data suggests that pSerSTAT4 plays a role in cell division in normal and hyperproliferative skin, including in mitotic cells within inflammatory and neoplastic conditions.

**Pharmacological inhibition with lisofylline**

Two complementary approaches (pharmacological inhibition and small interference RNA (siRNA)), were used to investigate the role of STAT4 in cell division. Previous reports have shown that rapamycin can suppress IL-12 signaling through Jak2/Stat4 in murine dendritic cells 35, and that lisofylline can inhibit IL-12 induced phosphorylation of Stat4 in splenocytes from nonobese diabetic mice 36, therefore these agents were used in the pharmacological inhibition experiments. In addition, alsterpaullone has been documented to inhibit serine phosphorylation of STAT3 37, therefore we wondered whether it might similarly prevent serine phosphorylation of STAT4 in dividing cells. As documented by confocal microscopy and flow cytometry, rapamycin (up to 20ng/ml) and alsterpaullone (up to 1µM) failed to prevent serine phosphorylation of STAT4 in mitotic cells at 30 minutes, 24, 48 and 72 hours of culture with these agents (supplemental Fig. 1a,b) and were not employed in further experiments. Conversely, culture with 100µM lisofylline for 30 minutes suppressed STAT4 serine phosphorylation in cells undergoing mitosis (Fig. 6a,b). Viewing the cells under confocal immunofluorescence suggested that a higher number of cells were in telophase in the lisofylline treated group, and quantification of cell numbers indicated that there were more cells in this phase of mitosis in the lisofylline-treated cells than in the control cells, although this difference did not reach statistical significance (p=0.0765; Fig. 6c,e). Live cell imaging was then conducted to look at the numbers of cells completing mitosis in the lisofylline-treated and untreated groups within a 3 hour time period, and showed that the number of cells completing cell division was reduced in the lisofylline-treated cultures as compared with untreated cells (Fig. 6d,f). Certain members of the mitogen-activated family of protein kinases (MAPKs) may mediate IL-12-induced serine phosphorylation 38 therefore MAPK inhibitors, including SP600125 (JNK inhibitor II), UO126 (MEK1 inhibitor) and SB203580 (p38 kinase inhibitor), were employed to determine whether any of these signaling pathways were involved in the serine phosphorylation of STAT4 during mitosis. No effect on pSerSTAT4 expression during mitosis was detected upon culture with any of these agents (supplemental Fig. 2a,b,c).

**SiRNA inhibition of STAT4**

STAT4 inhibition was also performed using a siRNA approach which targeted STAT4. Confocal microscopy demonstrated some variability in the reduction in pSerSTAT4 in the nuclei of non-dividing cells, but cytoplasmic pSerSTAT4 was visibly absent in dividing cells using this approach (Fig. 7a,b,c). Again, a greater quantity of cells seemed to be in telophase in the cell cultures where pSerSTAT4 was absent, and quantification showed a significantly higher number of cells in telophase in the STAT4-siRNA treated group than in the untreated cultures (p=0.0003; Fig. 7c,e). Live cell imaging over a 3 hour period demonstrated that significantly greater numbers of cells failed to complete mitosis during the observation period in the STAT4-siRNA treated group than in the untreated cultures (p<0.0001;Fig. 7d,f). Consistent with this, a significantly lower number of cells were noted to complete mitosis by separating into two daughter cells during this period (p=0.0158). Similar results were seen with two separate STAT4 siRNAs, indicating that these effects on mitosis were likely to be mediated by STAT4 inhibition.

**Discussion**

The role of STAT4 in a variety of immune processes, including dendritic cell activation and differentiation of Th1 and Th17 cells is well recognised 15. Although inhibition of STAT4 has been reported to affect cell growth of immune cells following stimulation with cytokines such as IL-1239, our observation of altered pSerSTAT4 in cells undergoing cell division has led to the identification of a novel role for STAT4 in facilitating timely completion of mitosis. STAT4 has previously been shown to function in mediating proliferative and anti-proliferative signals, for example STAT4 with STAT1 mediates IL-35 induced suppression of conventional T cells 19, and STAT4 suppresses the proliferation of connective tissue-type mast cells 40. Furthermore, STAT4 mediates IL-12-induced vascular smooth muscle cell proliferation in a pigeon model of atherosclerosis 41. However, our data suggests that the role of STAT4 is greater than one which simply mediates signals from extracellular stimuli to promote initiation of cell division. Although Stat4 deficient mice are grossly indistinguishable from wild-type mice, suggesting that Stat4 fulfils a redundant function in mice 42,43 , our data suggests that STAT4 plays a role in helping cells to complete mitosis and that inhibition of STAT4 may delay this process.

The different intracellular localisation of pSerSTAT4 to that of pTyrSTAT4 in the current study signifies that one group of STAT4 molecules are serine phosphorylated whereas another group are tyrosine phosphorylated during mitosis, and it seems likely that phosphorylation of these separate STAT4 molecules would occur in a coordinated manner to ensure that adequate amounts of pSerSTAT4 and pTyrSTAT4 are present during mitosis in these separate intracellular compartments. The existence of pSerSTAT4 without concomitant tyrosine phosphorylation is perhaps not surprising because serine phosphorylation of STAT3, STAT5A and STAT5B in the absence of tyrosine phosphorylation has been reported in response to certain growth factors such as insulin and prolactin 44,45. In addition, Shi et al. 37 in 2006 have shown that serine phosphorylation of STAT3 by cyclin-dependent kinase 1 is critical for nocodazole induced mitotic arrest. Our observations of pSerSTAT4 elevation in cells collected by mitotic arrest, as well as those halted in mitosis by nocodazole, and the presence of cytoplasmic pSerSTAT4 in the various cell types in vitro and in vivo, indicates that pSerSTAT4’s role in mitosis is not simply related to nocodazole/pharmacologically-induced mitotic arrest. Furthermore, the lack of an obvious effect on pSerSTAT4 by alsterpaullone, a cyclin-dependent kinase 1 inhibitor, signifies that the role of STAT4 in mitosis is quite different to that of STAT3 permitting mitotic arrest after nocodazole treatment. In fact, our overall data demonstrates that STAT4 promotes progression through mitosis rather than inducing arrest during this phase of the cell cycle.

In the time lapse experiments, a high proportion of mitotic cells underwent complete division into two daughter cells despite an almost total lack of pSerSTAT4 during lisofylline exposure, and separately in the absence of STAT4 following treatment with siRNA. It is possible that a minimal amount of pSerSTAT4 was present in the lisofylline-treated cells and that this amount was sufficient for progression of mitosis in some of the cells, but the viability of STAT4 null mice 46,47 suggests that there exists an amount of redundancy in this system, and that cells can complete mitosis in the absence of STAT4. The latter may be either because the absence of STAT4 simply leads to a delay in the completion of mitosis or that other cellular factors can substitute for STAT4 and undertake this role when necessary.

It can be seen from the in vivo data that diffuse cytoplasmic pSerSTAT4 is expressed during cell division in a variety of different cell types. The fact that cytoplasmic pSerSTAT4 was detected in suprabasal keratinocytes in normal skin raises the question of whether pSerSTAT4 is also involved in keratinocyte differentiation. Cytoplasmic pSerSTAT4 was not seen in “non-dividing” keratinocytes during cell culture, but this may be due to the fact that keratinocytes in monolayer are not differentiated to the same extent as those in the upper layers of the epidermis. Conversely, nuclear pSerSTAT4 was observed in the majority of keratinocytes throughout lesional psoriatic skin, which suggests that increased STAT4 transcriptional activation may play a role in this disease. The STAT4 rs7574865 single nucleotide polymorphism T allele has been reported to be associated with psoriasis 48, and T cells isolated from psoriatic skin exhibit increased sensitivity to interferon-alpha which activates STAT4 49. It has also previously been shown that IL-23 is expressed in psoriatic skin 50, and as IL-23 activates STAT4, it is possible that the nuclear pSerSTAT4 in keratinocytes noted in our study is a result of the higher IL-23 and/or interferon-alpha signaling in this condition. Similar to that seen in the vast majority of mitotic cells, cytoplasmic pSerSTAT4 was present in mitotic keratinocytes in psoriasis, which (as confirmed in Fig. 5c) is known to contain many mitotic cells within the suprabasal as well as basal layers of the epidermis. While it is acknowledged that targeting STAT4 might have effects on normal cell division, antiproliferative agents such as methotrexate are currently used as treatment for psoriasis, and it is possible that novel therapeutic agents which inhibit pSerSTAT4 might be beneficial in psoriasis because they would not only target the higher cell proliferation but also the increased nuclear pSerSTAT4 throughout the epidermis.

Not surprisingly, cytoplasmic pSerSTAT4 was also observed in mitotic cells within SCCs. It is widely recognised that higher proliferation within neoplastic tumours confers a poorer prognosis in terms of recurrence and/or metastases 51,52. However, we also noted a greater amount of nuclear pSerSTAT4 in less differentiated SCCs, which are known to have a worse prognosis 53. Although further work is required to determine whether this nuclear pSerSTAT4 is due to increased STAT4 signaling in the less differentiated tumours and whether this nuclear pSerSTAT4 plays a role in, or is simply a consequence of, the tumourigenesis process, our observations raise the question of whether STAT4 might similarly constitute a useful therapeutic target in this neoplasm, particularly in more proliferative and less differentiated cases.

In summary, our results show that pSerSTAT4 is increased in mitotic cells in vitro and in vivo, and that STAT4 plays a role in assisting progression through telophase, such that mitosis and separation into two daughter cells is completed in a timely manner. We would hypothesize, as clinical implications of our results, that therapeutic agents which inhibit pSerSTAT4 may be beneficial in diseases of the skin (and possibly of other organs) which have high levels of proliferation, for example in skin cancer and in psoriasis..

**Acknowledgements**

We are grateful to Anton Page and Dave Johnston for assistance with confocal microscopy. CF was supported by a travelling fellowship from the University of Cagliari. CL and SA were supported by the National Institute for Health Research (NIHR) as NIHR Academic Clinical Fellows.

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**Figure Legends**

**Fig. 1. Cytoplasmic pSerSTAT4 is present in cells undergoing mitosis.** (a) pSerSTAT4 (green) was determined by immunofluorescence microscopy on subconfluent monolayer cultures of HaCaT keratinocytes and shows presence of pSerSTAT4 bright cells (arrows). (b) Western Blot with same anti-pSerSTAT4 antibody as used in A demonstrates single pSerSTAT4 protein band at 89 kDa in IL-12 stimulated PBMC, EBV immortalized B-cells and unstimulated HaCaTs. (c-f) Confocal microscopy of HaCaTs shows (c) pSerSTAT4 (red) in the cytoplasm of cells in mitosis, whereas (d) pTyrSTAT4 (red) is present at the site of the chromosomes. (e) Total STAT4 is increased throughout the cytoplasm in mitotic cells. Nuclei/chromosomes (blue), alpha tubulin (green). (f) No pSerSTAT4 cytoplasmic staining is detected in mitotic cells when anti-pSerSTAT4 is pre-absorbed with blocking peptide. Nuclei/chromosomes (blue), alpha tubulin (green). Scale bar: 22 μm.

**Fig. 2. Enrichment of mitotic cells shows that pSerSTAT4 is increased in the majority of the mitotic cells.** Confocal microscopy of HaCaTs in mitosis (a) obtained by mitotic detachment and (b) synchronised by nocodazole, and simultaneously stained for pSerSTAT4 (red), alpha tubulin (green), nuclei/chromosomes (blue). Photographs are representative of 3 separate experiments and demonstrate high levels of pSerSTAT4 throughout the cytoplasm in the vast majority of cells. Scale bar: 22 μm. Flow cytometric analysis of HaCaT cells synchronised by (c) mitotic detachment and (d) nocodazole confirm that pSerSTAT4 levels are raised in mitotic cells (red line) in comparison with normal asynchronous cell culture (black line). (e) pTyrSTAT4 is also elevated in mitotic cells (red line) but not to the same extent as pSerSTAT4. Graphs in (c-e) are each representative of 3 separate experiments.

**Fig. 3. Confocal microscopy and flow cytometry of phosphorylated forms of STAT1 and STAT3 show mild elevation during mitosis.**  Confocal microscopy (left hand column) and FACS analysis (right hand column) of pSerSTAT1 (a,b), pTyrSTAT1 (c,d), pSerSTAT3 (e,f) and pTyrSTAT3 (g,h) in HaCaT cells show minimal/mild rise in pSerSTAT1, pTyrSTAT1, pSerSTAT3 and pTyrSTAT3 during mitosis. For confocal microscopy, nuclear/chromosomal compartment (blue), alpha tubulin (green) and (a) pSer-STAT1 (red), (c) pTyr-STAT1 (red), (e) pSerSTAT3 (red), (g) pTyrSTAT3 (red). Scale bar: 22 μm. For FACS analysis, cells synchronised in mitosis (red line), normal asynchronous cell culture (black line). Each photograph and graph is representative of 3 separate experiments. Note that the levels of pSerSTAT4, as depicted in figure 2, are higher than the levels of pSerSTAT1, pTyrSTAT1, pSerSTAT3 and pTyrSTAT3 during mitosis.

**Fig. 4. Cytoplasmic pSerSTAT4 is increased during mitosis in a variety of different cell types in vitro.** Subconfluent monolayer cultures of (a) primary human keratinocytes, (b) primary human fibroblasts, (c) RPMI-7951 melanoma cells, show high levels of cytoplasmic pSerSTAT4 during mitosis. Scale bar: 22 μm. (d) Alteration in pSerSTAT4 during the course of mitosis in HeLa cells. Confocal images on cells immunostained for simultaneous detection of pSerSTAT4 (red), alpha tubulin (green) and DNA/chromosomes (blue). Each photograph is representative of 3 separate experiments. Scale bar: 10 μm.

**Fig. 5. Cytoplasmic pSerSTAT4 is elevated during cell division in vivo.** Detection of pSerSTAT4 by immunoperoxidase staining (brown) of mouse and human tissues. Although nuclear pSerStat4 is present in spermatids (black arrow in (a)), strong cytoplasmic pSerSTAT4 (white arrows) is seen in mitotic cells in (a) testis (n=3), (b) normal skin (n=10), (c) psoriatic skin (n=6) and (d) squamous cell carcinoma (n=10). In (d), pSerSTAT4 is no longer elevated in a dividing cell which is in the final stage of mitosis (black arrow). Mouse (a), human (b-d). Scale bar: 60 μm.

**Fig. 6. Reduced expression of pSer-STAT4 following treatment with lisofylline reduces the telophase to cytokinesis transition.** Addition of lysofylline to (a) HaCaT cells decreases the levels of intracellular pSerSTAT4 in comparison with (b) untreated HaCaTs. Graphs in A and B show mean fluorescence intensity (quantitative fluorescence microscopy) of pSerSTAT4 (red) and DNA (blue) along region of interest (ROI) in mitotic cells from corresponding confocal microscopy images (representative of 3 separate experiments). (c) Examples of cell in telophase in lysofylline-treated HaCaTs (left) and cell undergoing cytokinesis in untreated HaCaTs (right) and (d) quantification of cells in telophase following treatment with lisofylline in comparison with untreated cells showing more lysofylline-treated cells in telophase; data in (d) displays mean (and s.d.) of two individual experiments with 100 mitosis counted in each experiment. (e) Representative examples of live-cell time-lapse microscopy of HaCaTs treated with lisofylline or left treated demonstrate delayed cytokinesis in the lisofylline treated cells. (f) Quantification of the proportion of cells which have completed cell division and the proportion which have failed to complete mitosis in lisofylline-treated and untreated cells; data represents mean (and s.e.m.) of three separate experiments. Confocal images in (a), (b) and (c) show pSerSTAT4 (red), alpha tubulin (green) and DNA/chromosomes (blue). Scale bar 22 μm.

**Fig. 7. Inhibition of pSerSTAT4 expression by STAT4-specific siRNA is associated with a reduction in cells completing the telophase to cytokinesis transition.** 48 hours after transfection with STAT4-specific siRNA, HeLa cells have decreased levels of pSerSTAT4 (b) in comparison with HeLa cells transfected with scrambled siRNA (a); mean fluorescence intensity of pSerSTAT4 (red) and DNA (blue) from region of interest (ROI) in cells undergoing mitosis in confocal microscopy images are illustrated in graphs in a and b (representative of 3 separate experiments). (c) More cells were observed in telophase in STAT4 siRNA-treated HeLa cells (right) than in the scrambled siRNA-treated cells (left). (d) Quantification of cells in telophase after STAT4-siRNA demonstrates higher numbers of cells in telophase compared with those treated with the scrambled siRNA; mean (and s.e.m.) of three separate experiments, 100 mitosis counted in each experiment. (e) Representative images of live-cell time-lapse microscopy of HeLa cells exhibit delayed cytokinesis after STAT4-siRNA. (f) Quantification of the proportion of cells which have failed to complete and cells which have completed cell division; data represents mean (and s.e.m.) of three separate experiments. Confocal images of cells in (a), (b) and (c) show pSerSTAT4 (red), alpha tubulin (green) and DNA/chromosomes (blue). Scale bar 22 μm.

**Supplementary Fig. 1. Rapamycin and alsterpaullone do not alter the presence or distribution of cytoplasmic pSerSTAT4 during mitosis.** Confocal images of HaCaT keratinocytes immunostained for pSerSTAT4 (red), alpha tubulin (green) and DNA/chromosomes (blue) in (A) untreated, (B) rapamycin-treated, and (C) alsterpaullone-treated cells. No alteration is seen in pSerSTAT4 following culture with rapamycin and alsterpaullone. Each photograph is representative of 3 separate experiments. Scale bar: 40 μm.

**Supplementary Fig. 2. MAPK inhibitors do not prevent phosphorylation of STAT4 during mitosis.** Addition of SP600125 (JNK inhibitor II), UO126 (MEK1 inhibitor) and SB203580 (p38 kinase inhibitor) to HaCaT cells does not block phosphorylation of STAT4 during cell division. Confocal images show pSerSTAT4 (red), alpha tubulin (green) and DNA/chromosomes (blue); representative examples from 3 separate experiments. Scale bar 40 μm.