Epigenetic switch drives the conversion of fibroblasts into proinvasive cancer-associated fibroblasts

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Carcinoma-associated fibroblasts (CAF) mediate the onset of a proinvasive tumour microenvironment. The proinflammatory cytokine LIF reprograms fibroblasts into a proinvasive phenotype, which promotes extracellular matrix remodelling and collective invasion of cancer cells. Here we unveil that exposure to LIF initiates an epigenetic switch leading to the constitutive activation of JAK1/STAT3 signalling, which results in sustained proinvasive activity of CAF. Mechanistically, p300-histone acetyltransferase acetylates STAT3, which, in turn, upregulates and activates the DNMT3b DNA methyltransferase. DNMT3b methylates CpG sites of the SHP-1 phosphatase promoter, which abrogates SHP-1 expression, and results in constitutive phosphorylation of JAK1. Sustained JAK1/STAT3 signalling is maintained by DNA methyltransferase DNMT1. Consistently, in human lung and head and neck carcinomas, STAT3 acetylation and phosphorylation are inversely correlated with SHP-1 expression. Combined inhibition of DNMT activities and JAK signalling, in vitro and in vivo, results in long-term reversion of CAF-associated proinvasive activity and restoration of the wild-type fibroblast phenotype.
Carcinoma-associated fibroblasts (CAF) are key components of solid tumour ecosystems, including breast, skin, head and neck, prostate and lung cancers. CAF play an active role in tumour development and their presence within the tumour stroma is associated with poor clinical outcome. Besides promoting primary tumours, CAF remodel the tumour microenvironment by generation of tensile forces within the extracellular matrix (ECM) that support favourable cues for tumour invasion. CAF-dependent excessive ECM remodelling leads to tumour fibrosis, which is associated with tumour cell metastasis and metastasis. CAF-dependent profibrotic activity is governed by constitutive activation of the Janus kinase 1 (JAK1), which leads to constitutive phosphorylation of its downstream target, the STAT3 (signal transducer and activator of transcription 3) transcription factor. We have demonstrated that the proinflammatory LIF (leukaemia inhibitory factor) cytokine initiates the sustained proinvasive stromal fibroblast phenotype observed in cancers. However, how the activated status of stromal fibroblast is sustained remains to be elucidated.

STAT3 belongs to a large family of transcription factors, and its constitutive activation has been observed in 70% of solid and haematological tumours. STAT3-dependent transcriptional activity is mediated by phosphorylation on tyrosines 705 and 727 by the JAK family kinases in response to cytokine-mediated signalling. Activated STAT3 translocates to the nucleus and binds promoter DNA sequences to regulate target gene transcription. STAT3 activity is enhanced by acetylation on lysine 685 by the histone acetyltransferases (HAT) p300/CBPs. A post-transcriptional modification that is reversed by histone deacetylases (HDACs). Acetylated STAT3 binds to the DNA methyltransferase DNMT1, which mediates targeted gene silencing by methylation of promoter DNA sequences. DNA methylation plays critical roles in control of sustained and constitutive activation of signalling pathways. Overall, both tumour and stromal cell DNA are hypomethylated; however, hypermethylation of CpG dinucleotides in gene promoters is known to silence tumour-suppressor gene expression. Among the major DNA methyltransferases (DNMTs), DNMT1 maintains pre-existing DNA methylation patterns along with DNA replication and cell division; DNMT3a and DNMT3b target unmethylated CpGs and introduce de novo methylations.

Here using combination of three-dimensional model of organotypic invasion assays of head and neck and breast tumours, and using in vitro and in vivo models of breast carcinomas, we demonstrate that an epigenetic switch initiates and maintains the proinvasive phenotype of CAF. We show that LIF induces constitutive activation of the JAK1/STAT3 signalling pathway by post-translational regulation of STAT3 acetylation by p300, in fibroblasts. Acetylated STAT3 leads to an epigenetic-dependent loss of expression of the SHP-1 tyrosine phosphatase, which is a negative regulator of the JAK/STAT pathway. Silencing of SHP-1 by promoter methylation leads to sustained constitutive phosphorylation of the JAK1 kinase and the STAT3 transcription factor that maintain the contractile and proinvasive fibroblasts abilities. Blockage of both JAK signalling and DNA methyltransferase activities both in vitro and in vivo results in long-term proinvasive phenotypic reversion of CAF. Finally, we corroborate our in vitro and in vivo findings through immunohistological analysis of STAT3 activity and SHP-1 expression in both head and neck and lung human carcinoma biopsies. Therefore, we conclude that in human carcinomas from different origins, LIF induces a sustained proinvasive activation of CAF through an epigenetic-dependent loss of SHP-1 phosphatase.

Results
Epigenetic mechanisms sustain the proinvasive CAF phenotype. We first assessed whether, similar to CAF isolated from head and neck, lung and breast human carcinomas (HN-CAF, Lu-CAF and Br-CAF, respectively), the long-term LIF or TGFβ-activated human dermal fibroblasts (hDF-LIF or hDF-TGFβ) constitutively retain their proinvasive properties. hDF were stimulated for 7 days in culture using LIF or TGFβ in the presence or absence of a LIF blocking antibody. After 15-30- and 60-day culture in low serum concentration (Fig.1a), the hDF proinvasive ability was assessed using a three-dimensional SCC12 cell organotypic invasion assay. In these conditions LIF induced the sustained proinvasive phenotypic phenotype (Fig. 1b) and, constitutively, the JAK1/STAT3 signalling pathway (Supplementary Fig. 1a) as observed with CAF compared with primary hDF (Supplementary Fig. 1b). Interestingly, TGFβ, the major mediator in vitro and in vivo CAF activator, relied on LIF to constitutively activate the proinvasive ability of hDF (Fig. 1b) and the JAK1/STAT3 signalling pathway (Supplementary Fig. 1a). These results indicate that a 7-day LIF stimulation is sufficient to confer a long-term proinvasive CAF-like phenotype to hDF. CAF secrete procarcinogenic factors, including interleukin-6 (IL6)-family cytokines that may sustain constitutive activation of JAK1 kinase via an autocrine regulatory loop. Therefore, we investigated whether media conditioned (CM) by CAF, hDF-LIF and hDF-TGFβ can activate JAK1/STAT3 signalling in hDF. Stimulation of hDF by long-term CAF, LIF- and TGFβ-activated hDF CM failed to promote JAK1/STAT3 phosphorylation (Fig. 1c) and collagen gel contraction (Fig. 1d), while short-term TGFβ-activated CM (hDF-TGFβ6h) induced STAT3 phosphorylation and gel contraction (Fig. 1c,d). Accordingly, in hDF-LIF, abrogation of GP130 receptor or addition of a specific LIF blocking antibody failed to alter STAT3 phosphorylation, which contrasts with the abrogation of JAK1 expression (Supplementary Fig. 1c,d). Taken together, these data demonstrate that LIF confers permanent proinvasive conversion to hDF that is independent of a LIF autocrine signalling regulatory loop but dependent on JAK1 activity. We thus hypothesized that epigenetic modifications might be involved in the constitutive activation of CAF and hDF-LIF. To test this idea, HN-CAF-dependent three-dimensional matrix gel contraction assays were performed in the presence of 45 small-molecule inhibitors targeting the epigenetic and DNA-modifier cell machinery (Fig. 1e, Supplementary Fig. 1e and Supplementary Table 1). For each tested molecule, the screen was performed at optimized concentrations (Supplementary Table 1); dimethylsulphoxide was used as a control. Among the six classes of inhibitors, three HAT p300 inhibitors (Garcinol, Anacardic acid and C646) and three DNA methyltransferase inhibitors (5′-Aza-2′-deoxycytidine, Zebularine and Decitabine) blocked the CAF contractile capacity (Fig. 1e). Accordingly, the p300 activator CTPB (N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-benzamide) significantly increased CAF contractility (Fig. 1e). Inhibition of matrix remodelling by both p300 and DNMT inhibitors was further confirmed in hDF-LIF (Supplementary Fig. 1e). In light of these results, the role of p300 in CAF- and hDF-LIF-dependent SCC12 cell invasion was further investigated. Using C646, a small-molecule inhibitor, the crucial role of p300 in proinvasive activity was established (Supplementary Fig. 1fg). These results demonstrate that LIF governs the long-term activation of proinvasive fibroblasts, which also involves DNMTs and p300 activities, and further suggests that the sustained phenotypes observed in CAF rely on epigenetic modifications.
Figure 1 | Epigenetic-dependent proinvasive fibroblasts phenotype. (a) Schematic representation of experimental conditions for long-term maintenance of the proinvasive properties of LIF- and TGFβ1-activated fibroblasts. (b) Representative images of haematoxylin and eosin (H&E) coloration of paraffin-embedded sections of SCC12 in response to control hDF (Veh.) or hDF previously activated for 7 days with LIF or TGFβ1, in presence or absence of LIF blocking antibody (αLIF) after 15, 30 and 60 days of culture in 0.5% SVF media. Scale bar, 100 μm. I.I., invasion index (n=3; mean ± s.d.; ***P<0.001).

(c) Immunoblot of pJAK1, pSTAT3 and pSMAD2 in hDF cells stimulated by three CAF-CM, long-term LIF and TGFβ1-activated hDF-CM and short-term TGFβ-activated hDF-CM. Immunoblot of JAK1, STAT3, SMAD2 and tubulin as control. (d) Percentage of gel contraction by hDF stimulated by three CAF-CM, long-term LIF and TGFβ1-activated hDF-CM and short-term TGFβ-activated hDF-CM (n=3 in triplicate, mean ± s.d., ***P<0.001).

(e) Quantification of matrix remodelling by CAF in the presence of small molecules inhibitors for 6 days. (n=3 in triplicates).
P300 mediates STAT3 acetylation for fibroblast activation. Screening for small-molecule inhibitors of the epigenetic cell machinery identified p300 as a crucial regulator of proinvasive fibroblasts matrix remodelling in vitro. Indeed, in hDF, RNA interference (RNAi)-mediated silencing of p300 before LIF initiation resulted in blockage of the sustained proinvasive property displayed by hDF-LIF (Fig. 2a,b). Interestingly, silencing of p300 in HN-CAF resulted in loss of their proinvasive potential (Supplementary Fig. 2a,b), which confirmed the results obtained in the contractility screens (Fig. 1e, Supplementary Fig. 1c). Furthermore, the p300 small-molecule activator CTBP was sufficient to promote hDF-dependent matrix remodelling (Supplementary Fig. 2c) and the sustained proinvasive cell capacity (Supplementary Fig. 2d). Taken together, these data showed that p300 plays a central role in the proinvasive fibroblast activation. Besides acetylating histones, p300 also acetylates STAT3, which is crucial for STAT3 transcriptional activity.21 We then showed that in hDF, LIF induced p300 binding to activate STAT3 (Fig. 2c) and RNAi silencing of p300 or blockage of p300 activity by the C646 small-molecule inhibitor inhibited the LIF-dependent constitutive activation of JAK1 phosphorylation and both the STAT3 phosphorylation on residue tyrosine 705 and the acetylation on residue lysine K685 (Fig. 2h,d). Furthermore, activation of p300 by CTPB was sufficient to constitutively activate the JAK1/STAT3 signalling pathway in hDF cells (Fig. 2e). This observation further confirms that p300 is responsible for STAT3 acetylation on LIF-dependent proinvasive fibroblast activation and suggests that STAT3 acetylation is pivotal in this process. At this stage, the role of STAT3 acetylation in sustained fibroblast proinvasive activity was then assessed. Using organotypic invasion assays, hDF stably expressing either green fluorescent protein (GFP; empty-GFP), or the wild-type form of STAT3 (STAT3-GFP) or a non-acetylated STAT3 mutant fused to GFP (STAT3-K685R-GFP), were investigated for their LIF-dependent sustained proinvasive activity. HN-CAF overexpressing either the control or the WT-STAT3 vector were found to retain proinvasive properties, that were abrogated in cells expressing a non-acetylated form of STAT3 (Fig. 2f). Furthermore, molecular analysis of the JAK1/STAT3 signalling pathway in hDF-LIF expressing the mutant STAT3-K685R cDNA, revealed that STAT3 acetylation is compulsory for phosphorylation of the residue tyrosine 705, and that LIF-dependent JAK1/STAT3 constitutive phosphorylation requires STAT3 acetylation (Fig. 2g). HN-CAF expressing the mutant STAT3-K685R cDNA, lost proinvasive activity (Supplementary Fig. 2e) and STAT3 phosphorylation, independent of JAK1 activity (Supplementary Fig. 2f). Taken together, these data demonstrate that STAT3 acetylation on residue lysine 685 is compulsory for proinvasive fibroblast property.

DNMTs govern LIF-dependent fibroblasts activation. Screening using the small-molecule inhibitor library also identified hypomethylating agents (DNA methylation inhibitors) as the most potent molecules blocking HN-CAF and hDF-LIF matrix remodelling (Fig. 1e, Supplementary Fig. 1c). We disclosed that 5'-azacytidine (5'-Aza), decitabine and zebularine, pyrimidine nucleoside analogues of cytidine incorporated into DNA, where it reversibly inhibits DNMT, interfered with CAF-dependent SCC12 cell collective invasion (Fig. 3a). Therefore, we next assessed the role of the DNMT protein family in LIF-dependent constitutive fibroblast activation in three-dimensional in vitro organotypic invasion assays. Specific RNAi-dependent silencing of DNMT1 expression in fibroblasts (Supplementary Fig. 3a) demonstrated that DNMT1 expression is required for CAF-dependent matrix remodelling (Fig. 3b) and invasion of SCC12 cells (Fig. 3c). DNMT1 is primarily implicated in the maintenance of CpG methylation throughout development and cell divisions, while DNMT3 role consists in de novo CpG methylation.31 Therefore, to assess a possible involvement of DNMT3 in initiation of the contractile and proinvasive process of the LIF-dependent fibroblast activation, we investigated the messenger RNA (mRNA) steady-state level of the DNMT family after short-term LIF stimulation of hDF. We first found that mRNA steady-state level of DNMT3b, but not that of DNMT1 or 3a, was significantly induced after short-term LIF stimulation of hDF (Fig. 3d). We second observed that DNMT3b binds to STAT3 active form upon LIF stimulation (Fig. 3e); therefore, the potential role for DNMT3b in induction of the constitutive proinvasive activation by LIF in hDF was investigated. Specific deletion of DNMT3b expression before LIF stimulation resulted in blockage of LIF-dependent activation of a proinvasive phenotype in hDF (Fig. 3f,g); whereas loss of DNMT3b expression in CAF and in long-term activated hDF-LIF had no effect on gel contraction (Supplementary Fig. 3b,c). Taken together, these results demonstrate that DNMT3b governs the LIF-dependent proinvasive fibroblast activation, which in HN-CAF is sustained by DNMT1. This explains why in HN-CAF, which have undergone the activation step, mediated by a LIF/DNMT3b signalling, targeting DNMT3b is ineffective, while targeting DNMT1 blocks the proinvasive phenotype.

JAK1/STAT3 sustained activity is mediated by loss of SHP-1. Having shown that restriction of the DNMTs catalytic activities inhibits both the establishment and the maintenance of the proinvasive phenotype of carcinoma- and LIF-activated fibroblast, we first investigated the molecular mechanisms underlying the DNMT-dependent proinvasive fibroblast activation. The effect of 5'-Aza on the JAK1/STAT3 constitutive signalling pathway activity was thus assessed after treatment of HN-CAF and LIF-activated fibroblast. Inhibition of both STAT3 phosphorylation and acetylation was observed (Fig. 4a) potentially consequent to inhibition of JAK1 phosphorylation on residue tyrosine 1022 (Fig. 4a, Supplementary Fig. 4a), which reflects its kinase activity.32 JAK1 tyrosine 1022 phosphorylation is controlled by a balance between JAK kinase transactivation and dephosphorylation.33 To address the functional role of 5'-Aza in the JAK1/STAT3 signalling activity and by consequence in HN-CAF proinvasive ability, we hypothesized that a DNMT-dependent epigenetic silencing regulates JAK1 kinase expression. Thus HN-CAF were exposed to 5'-Aza and then to orthovanadate, a broad phosphatase inhibitor. Under such experimental conditions JAK1 phosphorylation and both STAT3 phosphorylation and acetylation were rescued (Supplementary Fig. 4b), which validated the working hypothesis. Since tyrosine-protein phosphatase non-receptor type 6 (PTPN6), also known as SHP-1 (Src homology region 2 domain-containing phosphatase-1), is known to dephosphorylate a number of tyrosine kinases including JAK1 (ref. 34), the possibility that SHP-1 could regulate the proinvasive activity of HN-CAF in our system was assessed. Indeed, specific SHP-1 inhibition by PTP inhibitor 1 (PTP inh 1) restored the proinvasive property blocked by the 5'-Aza treatment (Fig. 4b). Subsequent analyses at the molecular level demonstrated that SHP-1 re-expression induced by 5'-Aza treatment correlated with decreased JAK1/STAT3 activity in HN-CAF, and that inhibition of SHP-1 phosphatase activity restored a constitutive JAK1/STAT3 signalling activity (Fig. 4c). Furthermore, in both HN-CAF and hDF-LIF, inhibition of both the proinvasive capacity (Supplementary Fig. 4c) and JAK1/STAT3 signalling (Supplementary Fig. 4d,e) induced by
loss of DNMT1 expression consequent to RNAi treatment was rescued by specific RNAi targeting of SHP-1 expression. Taken together, these data demonstrate that long-term activation of proinvasive fibroblast activity is sustained by constitutive activation of the JAK1/STAT3 signalling pathway through a DNMT1-dependent downregulation of the SHP-1 phosphatase expression. At the initiation phase, LIF induces an enhanced expression of DNMT3b and the loss of SHP-1 expression, which
results in constitutive JAK1/STAT3 signalling pathway activity. Accordingly blockage of DNMT3b expression, prior to LIF stimulation, leads to inhibition of JAK1/STAT3 phosphorylation and rescue of SHP-1 protein expression (Fig. 4d). Consistently with our previous data, inhibition of p300 catalytic activity or loss of p300 expression consequent to RNAi treatment before LIF...
Figure 4 | Epigenetic-dependent sustained JAK1/STAT3 signalling in CAF. (a) Immunoblot of pJAK1, pSTAT3 and AcSTAT3 in CAF after 7-days treatment in the presence or absence of 5′-Aza inhibitor at 10 and 3 μM final concentration. Immunoblot of JAK1, STAT3 and tubulin as control. (b) Representative images of H&E coloration of paraffin-embedded sections of SCC12 in response to CAF following 5′-Aza inhibitor alone or in combination with PTP inhibitor 1 (n = 3, mean ± s.d., ***P < 0.001). (c) Immunoblot of DNMT1, pSHP-1, PTP1, pJAK1, pSTAT3 and AcSTAT3 in CAF following 5′-Aza inhibitor alone or in combination with PTP inhibitor 1. Immunoblot of JAK1, STAT3 and tubulin as control. (d) Immunoblot of DNMT3b, pSHP-1, pJAK1, pSTAT3 and AcSTAT3 in hDF activated or not by LIF transfected with siRNA control (siLuc) or siRNA targeting DNMT3b (siDNMT3b#1, #2). Immunoblot of JAK1, STAT3 and tubulin as control. (e) Schematic illustration of the PTPN6 gene structure and the regions analysed by bisulfite sequencing. The PTPN6 gene has two alternative promoters. We restricted our analysis to Promoter 1 since Promoter 2 is mostly active in the haematopoietic lineage. Vertical lines in Region 1 and Region 2 indicate the position of the CpG dinucleotides. (f) Bisulfite-sequencing results in two regions of the PTPN6 promoter (R1 and R2) in hDF transfected with control RNAi (siLuc) and RNAi targeting DNMT3b (siDNMT3b#1 and #2). Immunoblot of JAK1, STAT3 and tubulin as control. Donut charts summarize the global proportion of methylated CpG with the percentage in the center (n = 2, P < 0.0001, two-tailed Fisher’s exact test). (g) Representative images of H&E coloration of paraffin-embedded sections of SCC12 in response to CAF overexpressing control vector (GFP) or SHP-1 gene (SHP-1_GFP) (n = 3, mean ± s.d., ***P < 0.001). (h) Immunoblot of SHP-1, pJAK1, pSTAT3 and AcSTAT3 in CAF overexpressing control vector (GFP) or SHP-1 gene (SHP-1_GFP). Immunoblot of JAK1, STAT3 and tubulin as control.
stimulation inhibits the loss of SHP-1 expression in hDF (Supplementary Fig. 4f,g). A possible explanation is that LIF, through a DNMT-dependent pathway, regulates the methylation status of the SHP-1 promoter. To address this possibility, we analysed by bisulphite sequencing the two regions R1 and R2 of the PTPN6 promoter 1 (ref. 35) rich in CpG dinucleotides (depicted in Fig. 4e). Strikingly, long-term LIF-treated hDF compared with untreated controls, presented hypermethylation of the PTPN6 promoter, a DNMT3b– (Fig. 4f, Supplementary Fig. 4h) dependent modification that was hampered by siRNA-mediated knockdown of DNMTs during LIF stimulation. Accordingly, transgenic expression of SHP-1 in HN-CAF was sufficient to block the proinvasive capacity of these cells (Fig. 4g) and to inhibit their JAK1/STAT3 signalling pathway activity (Fig. 4h). Accordingly, SHP-1 was undetectable in CAF from multiple carcinoma origins compared with three primary hDF (Supplementary Fig. 4i) and regions 1 and 2 of PTPN6 promoter 1 of human CAF were hypermethylated compared with the hDF PTPN6 promoter (Supplementary Fig. 4j). These results establish that a LIF/p300/DNMT signalling governs the initial step of JAK1/STAT3 constitutive activation through SHP-1 silencing. Once this epigenetic repression is established, targeting DNMT3b has no effect, while targeting DNMT1 inhibits the proinvasive phenotype of CAF.

Phenotypic and molecular constitutive reversion of CAF. Ruxolitinib, an FDA approved JAK1/2 pharmacological inhibitor, targets proinvasive tumour microenvironment modifications by transiently inhibiting human CAF contractility. Having demonstrated here that DNMT1 supports constitutive JAK1/STAT3 activation and proinvasive activity of HN-CAF, we hypothesized that treatment of activated fibroblasts with 5′-Aza could permanently revert the activated phenotype. To test such an idea, activated fibroblasts were cultured for 7 days in the presence of either Ruxolitinib or 5′-Aza. After removal of the inhibitors, the molecular activity of the JAK1/STAT3 signalling pathway was measured for 5 days, and the contractile activity of the treated cells was monitored for additional 45 days in collagen gel (Fig. 5a). HN-CAF treated with Ruxolitinib restored STAT3 phosphorylation 2 days after drug removal, while SHP-1 expression was not affected (Supplementary Fig. 5a). 5′-Aza treatment resulted in a transient 3-day decrease of STAT3 phosphorylation, SHP-1 expression was first induced on DNMT1 inhibition, but restoration of the STAT3 activity correlated with the loss of SHP-1 protein (Supplementary Fig. 5b). Taken together, these data indicate that in HN-CAF the effects of both Ruxolitinib and 5′-Aza are transient. Because both drugs act at different signalling levels to regulate the JAK1/STAT3 signalling pathway, possible synergistic activity of the two compounds was explored. Combined 7-day Ruxolitinib and 5′-Aza treatment of activated fibroblasts resulted in long-term abrogation of the JAK1/STAT3 phosphorylation and rescued SHP-1 protein expression (Supplementary Fig. 5c). Further, such long-term abrogation of the JAK1/STAT3 signalling pathway activity was confirmed using hDF_LIF (Supplementary Fig. 5d) by dual inhibitor treatment. We next assessed whether the combined treatment could revert activated fibroblasts into a permanent low-contractile and non-proinvasive hDF phenotype. While HN-CAF and hDF_LIF treated with one inhibitor initially showed significant reduction of contractile capacity, no significant difference was observed at day 15 compared with the untreated counterparts (Fig. 5b, Supplementary Fig. 5e). On the contrary, on combined treatment, the activated fibroblasts completely and permanently lost all capacity to contract the collagen lattices (50-day assays) that became indistinguishable from the hDF counterparts (Fig. 5b, Supplementary Fig. 5e). Similar abrogative effects were observed after a 21-day treatment (Supplementary Fig. 5f,g). Interestingly, abrogation of the proinvasive phenotype was observed with carcinoma- LIF- and TGFβ-activated fibroblasts even after removal of the two inhibitors. In cells exposed to only one of the two inhibitors, the abrogative effect was reversed 5 days after the drug removal (Fig. 5c). Also, in an organotypic model of breast carcinoma, Br-CAF promoted collective invasion capacity in the MDA-MB-468 carcinoma cell line that was abrogated by the combined 5′-Aza and Ruxolitinib treatment of the Br-CAF (Fig. 5d). Taken together, these data demonstrate that in hDF, LIF induces an epigenetic-dependent constitutive activation of the JAK1/STAT3 signalling pathway that governs the proinvasive CAF cell ability. Such epigenetic activation is abrogated on the long term by treatments combining JAK1 and DNMT inhibitors. The potential therapeutic effect of targeting the DNMT and JAK pathway during breast carcinogenesis was evaluated in vivo. Thus, mouse dermal fibroblasts (mDF), isolated from back skin of Balb/C mice, were activated in vitro by LIF, then submitted to treatment with either one of each inhibitors or a combination of both. Treated mDF were co-implemented in the forth mammary fat pad of BALB/c female mice together with the 67NR mouse mammary carcinoma cell line, that neither invades from the primary tumour, nor activates fibroblasts in vitro and in vivo (Fig. 6a). Thirty days after co-implantation, the mice were killed, and the 67NR cell-invasive capacity and tumour ECM remodelling were analysed by immunohistchemistry. 67NR cells, from the primary tumour bulk (T) invaded the adjacent tissue exclusively when co-implanted with LIF-activated mDF, which contrasted with the behaviour of 67NR injected either alone or co-implanted with control mDF (mDF_Veh.). Treatment of mDF_LIF with one inhibitor reduced the onset of a proinvasive microenvironment, while treatment combining both inhibitors restored the non-invasive 67NR cell phenotype (Fig. 6b,c, left panels). The invasive behaviour of 67NR cells correlated with an excessive tumour ECM remodelling disclosed by Picrosirius Red staining and orthogonal light visualization (Fig. 6c, middle and right panels, and Fig. 6d). Taken together, these data indicate that LIF-activated fibroblasts induce onset of a proinvasive tumour microenvironment in vivo, in a syngenic and orthotopic mice model of breast cancer.

STAT3 and SHP-1 inverse expression in human carcinomas. To confirm the in vitro findings with ex vivo observations, an immunohistological analyses of STAT3 phosphorylation and acetylation together with SHP-1 expression were performed in 100 human carcinoma samples from head and neck (n = 50) and lung (n = 50) cancers (Fig. 7a, Supplementary Fig. 6a). The levels of AcSTAT3 detected in lung (Fig. 7b) and head and neck (Fig. 7c) was consistently opposite to SHP-1 expression, which establishes a strong inverse correlation between STAT3-K685 acetylation and SHP-1 expression (r² = −0.87 for head and neck; r² = −0.55 for lung carcinomas; Fig. 7d). Thus the ex vivo data are consistent with the in vitro evidence revealing that STAT3 acetylation leads to SHP-1 downregulation in stromal fibroblasts. No significant difference between phosphorylation and acetylation of STAT3 in the tumour stroma of both head and neck and lung tumours biopsies was observed, which corroborates the in vitro evidence that STAT3 acetylation is compulsory for phosphorylation of the STAT3 tyrosine 705 residue (Supplementary Fig. 6a–c). Next, the quick scores obtained from each variable were considered for the design of a predictive model. A principal component analysis was done to highlight dispersed values and build a clean set of measurements avoiding
Figure 5 | Phenotypic and molecular reversion of CAF. (a) Schematic representation of the experimental conditions for long-term phenotypic and molecular reversion of the proinvasive property of activated fibroblasts. After inhibitors treatment for 7 or 21 days, cells are embedded in matrix for a 50-day gel contraction assay, assayed every day for a period of 5 days for molecular analysis, and assayed at day 5 after inhibitors removal for proinvasive ability. (See also Supplementary Fig. 5). (b) Percentage of gel contraction of hDF and CAF for a period of 50 days following a 7-day treatment of either Ruxolitinib or 5'-Aza or both inhibitors together. (c) Representative images of H&E coloration of paraffin-embedded sections of SCC12 in response to CAF, hDF_LIF and hDF_TGFβ1, 5 days after a period of 7-day treatment of Ruxolitinib or 5'-Aza or both inhibitors together (n = 3, mean ± s.d., ***P < 0.001). Scale bar 100 μm. (d) Representative images of H&E coloration of paraffin-embedded sections of MDA-MB-468 human breast cancer cell line in response to breast CAF 5 days after a period of 7-day treatment of Ruxolitinib or 5'-Aza or both inhibitors together (n = 3, mean ± s.d., ***P < 0.001).
Figure 6 | Stromal DNMT and JAK favour tumour invasion in vivo. (a) Schematic representation of the experimental conditions used for in vivo cell co-implantation into the mammary fat pad of 8-week-old BALB/c female mice. 67NR mouse breast carcinoma cells were co-injected together with LIF preactivated mDF and treated with inhibitors. (b) Graphic representation of tumour formation induced by 67NR cells alone or in the presence of LIF-preactivated mDF. Total numbers of mice bearing tumours after single or combined inhibitor treatment are shown. (c) H&E coloration of paraffin-embedded sections of primary tumours isolated from mice (left panels) showing 67NR cells invading from the primary tumour (T) into the adjacent tissue (aT) (black arrow). Picrosirius Red staining visualized by both parallel (middle panels) and orthogonal (right panels) light showing tumour ECM remodelling at the areas invaded by the tumoral 67NR cells. Scale bars, 200 and 100 μm for ×20 and ×40X magnifications, respectively. (d) Quantification of Picrosirius Red staining using ImageJ software. Percentage of threshold area is shown (mean ± s.d.; *P<0.05).
these values. Twenty six values were eliminated from the set. Scatter-plots were made to calculate the correlation matrix for each couple of variables for both the complete and the cleaned set of measurements (Supplementary Table 2A). Correlation coefficients are widely acceptable especially for the cleaned set, suggesting the possibility to define a reliable predictive model. Using linear multivariate logistic regressions three predictive models were thus obtained (Supplementary Table 2B).

In silico values generated from these models are highly correlated with the ex vivo values. Furthermore, the ‘SHP-1 ~ ac-STAT + p-STAT’ model resulted to be relevant and able to predict the SHP-1 level by scoring STAT3 phosphorylation and acetylation only with high level of confidence (Supplementary Fig. 6d).

**Discussion**

CAF are responsible for the onset of the proinvasive tumour microenvironment, which favours carcinoma cell dissemination from the primary tumour. Interestingly, the proinvasive properties of CAF remain activated even after their isolation from tumours and propagation in vitro. Here, we show that such permanently activated proinvasive status of CAF, isolated from head and neck, lung and breast carcinomas, results from the LIF-mediated persistent activation of the JAK1/STAT3 signalling pathway, which regulates acto-myosin contractility and confers on them the proinvasive capacity of digging tracks within the ECM that are subsequently used by the tumour cells to collectively invade. Constitutive activation of the JAK1/STAT3 signalling pathway is a process frequently observed in tumour cells. It involves either genetic mutations activating the GP130-IL6ST receptor or the JAK2 kinase or secretion of growth factors and cytokines mediating an autocrine activation of the signalling pathway. While occurrence of oncogenic mutations in CAF is still matter of debate, in our case, any possible involvement of secreted factors from both CAF and LIF-activated fibroblasts can be excluded. Activated fibroblasts isolated form tumours or fibrotic tissues present a globally hypomethylated DNA profile that strikingly contrasts with a hypermethylated pattern of the promoters of tumour-suppressor genes such as Thy1, tglbr2 or PTEN. Moreover, epigenetic modifications are considered a crucial step in the constitutive fibroblast activation in a mouse model of kidney.

![Diagram](https://example.com/diagram.png)

**Figure 7 | Inverse correlation of AcSTAT3 and SHP-1 expression.** (a) AcSTAT3, pSTAT3 and SHP-1 immunohistological staining in human head and neck (n = 50) and lung (n = 50) carcinomas. Scale bar, 100 μm. (b) Quantification of mean Quick Score (QS) for SHP-1, both in the stroma and the tumour bulk, in lung carcinomas decorated with high- and low-acetylated STAT3 shown in a. (c) Quantification of mean Quick Score (QS) for pSTAT3, in the bulk of the tumour, in lung carcinomas decorated with high and low acetylated STAT3 shown in a. (d) Quantification of mean QS for SHP-1, both in the stroma and the tumour bulk, in head and neck carcinomas decorated with high- and low-acetylated STAT3 tumours shown in a. (e) Quantification of mean Quick Score (QS) for pSTAT3, in the tumour bulk, in head and neck carcinomas decorated with high- and low-acetylated STAT3 shown in a. (f) Plot of mean QS form AcSTAT3 (x axis) and SHP-1 (y axis) showing a negative correlation between Acetylated STAT3 and SHP-1 detection in lung (blue) and head and neck (red) carcinomas.
fibrosis. Consistent with such observations, we have identified the DNMT family epigenetic modifiers as regulators of the proinvasive CAF activity that acts on the JAK1 and STAT3 constitutive activation. In this context, we provide evidence that LIF induces an initiation phase that requires DNMT3b transcriptional expression and its association with AcSCAT3 to promote methylation of the PTPN6 tyrosine phosphatase promoter. Subsequently, the DNMT1-dependent replication methylation of the PTPN6 promoter maintains silent the SHP-1 expression, which causes constitutive tyrosine phosphorylation of JAK1 resulting in fibroblast activation. Epigenetic regulation of the JAK1/STAT3 pathway has been suggested in tumour cells. However, we demonstrate for the first time that in cancer epigenetic downregulation of SHP-1 sustains the proinvasive activity of the stromal fibroblasts. Interestingly, this observation is corroborated by the strong inverse correlation existing between detection of acetylated STAT3 and expression of SHP-1 observed in human tissues obtained from head and neck and lung tumour specimens. In light of our results, we propose that PTPN6, a tumour suppressor gene in leukaemia and lymphoma, acts as a fibrotic suppressor gene in CAF from head and neck, lung and breast cancers.

We further disclose a crucial role for p300 HAT in proinvasive HN-CAF phenotype (Fig. 2). Indeed, the transcriptional co-activator p300 has been implicated in diverse biological functions, including tissue fibrosis, and its catalytic acetyltransferase activity is enhanced in fibroblasts in response to TGFβ (ref. 44) and in tumour cells by the LIF or IL6 cytokines. In our system, specific inhibition of p300 abolishes the LIF- and carcinoma-associated proinvasive fibroblast activity, suggesting that, likewise in several human diseases, in stromal fibroblasts p300 and its homologue CBP possess distinct functions. Experimental evidence indicates a clear role for both p300 and CBP in tumorigenesis as tumour-suppressor genes in haematological tumours and through regulation of tumour suppressing signalling pathways, such as TGFβ and p53, in cancer cells. Our results disclose a procarcinogenic function for p300. Indeed, p300 activation by inflammatory cytokines leads to permanent JAK1/STAT3 signalling activation through the DNMT3b-dependent SHP-1 tyrosine phosphatase silencing and proinvasive stromal fibroblast initiation.

DNA epigenetic modification is a reversible process. Reversion can be induced by treatments with the 5′-Azacytidine that degrades DNMT. However, in patients with myelodysplastic syndromes, re-expression of tumour-suppressor genes consequent to treatment with 5′-Azacytidine or decitabine is transient and concomitant with reversion of the methylation profiles. In our system, a 7-day treatment on both HN- and Br-CAF or hDF, LIF combining both 5′-Azac and the JAK1/2 inhibitor Ruxolitinib reverts the proinvasive properties of activated fibroblasts on the long term. Association of both compounds is mandatory for such a long-term effect, because treatments using each single-chemical compounds only transiently inhibit the CAFT-dependent matrix remodelling, SCC12 cells invasion and SHP-1 protein expression. It is conceivable that the passive DNA demethylation induced by 5′-azacytidine is not sufficient to permanently revert the CAF phenotype, whereas active genomic demethylation, through replacement of modified cytosine in the absence of cell division may explain such a prolonged effect of the combined inhibitor treatment. Indeed, BMP7-induced inhibition of kidney fibrosis is mediated by an active genomic Tet3-dependent Rasall promoter demethylation. Hesson et al. suggested that nucleosome reassembly is a crucial step that dictates the transient efficacy of DNMT inhibitors. We therefore hypothesize that inhibition of DNMT activity, together with JAK inhibition may lead to histone modification, such as acetylation and methylation, resulting in the inhibition of nucleosome reassembly within the SHP-1 promoter.

In our conclusion, we establish that acquisition and long-term maintenance of head and neck, lung or breast CAF proinvasive ability relies on a two-step mechanism. In hDF, LIF initiates this process through STAT3 acetylation by p300 and DNMT3b-dependent methylation of the PTPN6 promoter, which is followed by a long-term maintenance phase mediated by a DNMT1-dependent loss of SHP-1 expression and JAK1 kinase constitutive activity. In light of our results obtained by combining the use of 5′-Azac and Ruxolitinib, both drugs approved by the Food and Drug administration, we demonstrate the potential of molecular reversion of activated fibroblasts into normal fibroblast-like cells and we propose that such treatment might represent an attractive approach to inhibit the onset of the proinvasive tumour microenvironment in cancer.

**Methods**

**Cell culture.** Human primary dermal fibroblasts (hDF), MDA-MB-468 and human HEK293 Phoenix cells were maintained in DMEM supplemented with 10% FCS (fetal calf serum). Human CAF isolated from patients with head and neck, lung and breast cancers were cultured in DMEM supplemented with 10% FCS and insulin-transferrin-selenium (#41400-045; Invitrogen, Carlsbad, CA). SCC12 cells were cultured in FAD media, as described in Gaggioli et al.

Long-term LIF and TGFβ-activated fibroblasts (hDF, LIF and hDF_TGFβ1) have been performed in DMEM supplemented with 0.5% FCS containing 2 ng ml−1 final concentration of human recombinant proteins for 7 days. Next, activated-hDF was cultured for 15 days (otherwise stated) in 0.5% FCS media before experiments (as described in Supplementary Fig. 1a).

**Cytokines and neutralizing antibodies and inhibitors.** TGFβ1 was purchased from PeproTech (Rocky Hill, NJ) and was used at 2 ng ml−1; recombinant human LIF was purchased from Millipore (Woburn, MA) and was used at a concentration of 2 ng ml−1. LIF neutralizing antibody (AB-250-NA, R&D, Minneapolis, MN) was used at 10 μg ml−1. Neutralizing antibodies were incubated for 1 h with CM before experiments. The inhibitors/antibodies used in these inhibitors were used in the following study: Pyrroden (420909, Calbiochem, Los Angeles, CA) was used at 5 μM, Ruxolitinib (#1398, Axon medchem, Groningen, The Netherlands) at 10 μM, 5′-Azac (#A2385, Sigma, Saint Louis, USA) was used daily at 2.5 μM, TGFβ1 (#540200, Millipore, Billerica, MA) at 10 μM, Dexamethasone (D5754, Millipore, Billerica, MA) at 1 mM, CM (SM002, Sigma, Saint Louis, USA) and CTPB (5-μ-202538, Santa Cruz, Biotechnology, Santa Cruz, CA) at 25 μM. A list of the concentrations used for the inhibitors/activators in Fig. 1d is provided in Supplementary Table 1.

**RNAi transfections.** Cells were plated at 60% confluence and subjected to transfection the following day using Dranarmfect 3 (#F-1002-02; Dramacon, Inc., Lafayette, CO) at 100 nM final concentration of RNAi. For long-term activation studies, fibroblasts were transfected once at day 0, stimulated with recombinant cytokines from day 1 to day 7, retransfected at day 8, and maintained in 0.5% FCS until day 15 for extracellular matrix remodelling, organotypic invasion assays and protein extraction. RNAi sequences are listed below:

- p300 #1−5′-GGACUAACCCCUAAAGAUAUGA-3′
- p300 #2−5′-GCACGGGAAUAAUGCGA-3′
- HDAC3 #3−5′-ACCAAGAUAACUGAUAAGU-3′
- HDAC3 #2−5′-GGAUGUUGGUCUAAAGUC-3′
- HDAC3 #3−5′-GACCCGACGACGAAUACUA-3′
- HDAC3 #4−5′-AAGCCGUGUGGAAGUUAA-3′
- DNMT1 #1−5′-GCACCCUAUUGCCGAAUA-3′
- DNMT1 #2−5′-AUAUAUGAUGGUGAUAUG-3′
- DNMT1 #3−5′-CCUGAGCCUUGACCGAUAUG-3′
- DNMT1 #4−5′-CCGAGCCGUCGACGAUCAA-3′
- DNMT3b #1−5′-ACGCACAGCGUAGCAGCU-3′
- DNMT3b #2−5′-UUUACCCCUAGCUGAAUA-3′
- DNMT3b #3−5′-GGAUAACACACACGUC-3′
- DNMT3b #4−5′-GCGCUUACCCUACACAGCU-3′

**Organotypic invasion assays and matrix remodelling assays.** For Organotypic invasion assays, 5 × 104 fibroblasts were embedded in 1 ml matrix gel. After 1 h at 37°C, matrix gel were overlaid with 5 × 104 SCC12 cells and lifted at the cell-air interface 24 h later for 5 days, the cultures were then fixed, embedded in paraffin blocks, sectioned and stained for invasion index quantification using ImageJ. For gel contraction assay, 25 × 105 fibroblasts were embedded in 100 μl of matrix gel and seeded in triplicate into 96-well plates. After 1 h at 37°C, matrix gels were overlaid with 100 μl of 0.5% FCS medium (with indicated cytokines or inhibitors) and
changed every 2 days. At day 6, the relative diameter of the well and the gel were measured using ImageJ. The percentage of gel contraction was calculated using the formula 100 × (well diameter – gel diameter)/well diameter.

**Antibodies.** Antibodies against STAT3 (α139; 1/150, pY705-STAT3 (α145; 1/1,000), ac-K685 STAT3 (α252; 1/1,000), SMAD2 (α312; 1/1,000), pS465/467-SMAD2 (α3108; 1/1,000), pY1022/1023-JAK1 (α333; 1/200), JAK1 (α3332; 1/500), ML2C (α3672; 1/500) and pThr18/19-MLC2 (α3674; 1/500) were purchased from Cell Signalling (Cell Signaling Technology, Beverly MA), α-tubulin fromSigma (T4026, Sigma, Saint Louis, MO; 1/5,000); SHP-1 (#sc-7289, 1/500 for western blot analysis), DNMT1 (#sc-20701; 1/1,000), DNMT3b (#sc-130740; 1/1,000) from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA) and SHP-1 (#ab2020; 1/200 for immunohistochemical staining) from Abcam (#ab2020, Abcam, Cambridge, UK) and performed on a Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA) and performed on a Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA).

**Western blots and Co-immunoprecipitation analysis.** For immunoblotting analysis, the whole protein lysates were loaded on SDS-PAGE, separated by SDS–PAGE, and transferred to a PVDF membrane. The membrane was blocked in 5% nonfat dry milk in TBST (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The membranes were incubated with primary antibodies diluted in 5% milk–TBST overnight at 4°C. After three washes in TBST, the membranes were incubated with biotinylated secondary antibody (1:1,000 Protein A, Vector, Burlingame, CA) for 1 h at room temperature. The membranes were washed with TBST and complete media was added. The day after, media was replaced with a heat-inactivated serum medium and cells were moved to 32°C for 24 h. Forty-eight hours post transfection clonal supernatants were collected and used to infect either HDF or CAF. Retrovirus infection was performed in the presence of 5 μg/ml polybrene. Stably transduced cells were selected with 5 μg/ml puromycin.

**In silico linear multivariate logistic regression.** Quick score experimental values were used for the in silico linear multivariate logistic regression achieving to determine the model. Statistical analysis was performed using the Quick Change II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The sequence of the oligonucleotide primers were used: STAT3-K685R-Forward: 5′-GAGAGGATCTTCAATGATTAGCC CAGG-3′; and STAT3 K685R-Reverse: 5′-CTCGGGCCTACAGTACCTTCCAAAT GCCTCCTC-3′ and constructs were then coupled to GFP. Following constructs verification by nucleotide sequencing, the STAT3 (wild-type and K685R) fragments were excised from pcDNA5 by Pme1 and BamH1 digestion and directionally cloned into pBabe retroviral expression vectors (containing the puromycin resistance gene as a selectable marker) digested with NaeI and BamH1. Cloned DNA was directionally cloned into the FRT expression vector (which was a gift from the laboratory of Dr. Müller-Newen using the Quick Change II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The sequence of the oligonucleotide primers were used: STAT3-K685R-Forward: 5′-GAGAGGATCTTCAATGATTAGCC CAGG-3′; and STAT3 K685R-Reverse: 5′-CTCGGGCCTACAGTACCTTCCAAAT GCCTCCTC-3′ and constructs were then coupled to GFP. Following constructs verification by nucleotide sequencing, the STAT3 (wild-type and K685R) fragments were excised from pcDNA5 by Pme1 and BamH1 digestion and directionally cloned into pBabe retrovirus expression vectors (containing the puromycin resistance gene as a selectable marker) digested with Nael and BamH1. SHP-1 cDNA was isolated from Kp by RT–PCR and subcloned into a pBabe retrovirus expression vector.

**Statistical analysis.** Student’s t-test was performed for statistical analysis of invasion assay, gel contraction assay, and quantitative PCR (qPCR) results. ***P < 0.001, **P < 0.01 and *P < 0.05. Error bars are ± s.d. Pearson’s correlation coefficient was used to assess the relationship between pSTAT3, aC3STAT3 and SHP-1 quick score within human samples.

**DNMT activity assay.** Episecker DNMT activity quantification kit (pab#131437, Abcam, Cambridge, UK) was used to assess DNMT activity on 10 μg of nuclear proteins (nuclear extracts were prepared using the Episecker Nuclear Extraction Kit (pab#131474, Abcam, Cambridge, UK) following manufacturer’s specifications.

**Plasmids constructs.** pcDNAs-mSTAT3-K685R (in which K685 is mutated from AAG (K) to AGG (R)) was derived from the wild-type pcDNAs-mSTAT3-GFP-FRT expression vector (which was a gift from the laboratory of Dr. Müller-Newen using the Quick Change II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The sequence of the oligonucleotide primers were used: STAT3-K685R-Forward: 5′-GAGAGGATCTTCAATGATTAGCC CAGG-3′; and STAT3 K685R-Reverse: 5′-CTCGGGCCTACAGTACCTTCCAAAT GCCTCCTC-3′ and constructs were then coupled to GFP. Following constructs verification by nucleotide sequencing, the STAT3 (wild-type and K685R) fragments were excised from pcDNA5 by Pme1 and BamH1 digestion and directionally cloned into pBabe retrovirus expression vectors (containing the puromycin resistance gene as a selectable marker) digested with Nael and BamH1. SHP-1 cDNA was isolated from Kp by RT–PCR and subcloned into a pBabe retrovirus expression vector.

**Production of recombinant retroviruses.** Phoenix cells were transiently transfected with 1 μg of the previously described vectors using calcium phosphate mediated transfection using classical procedures. Six hours after transfection, cells were washed with PBS and complete media was added. The day after, media was replaced by a heat-inactivated serum medium and cells were moved to 32°C for 24 h. Forty-eight hours post transfection clonal supernatants (retroviral particles) were collected and used to infect either HDF or CAF. Retrovirus infection was performed in the presence of 5 μg/ml polybrene. Stably transduced cells were selected with 5 μg/ml puromycin.

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Bisulfite sequencing. Genomic DNA was extracted with the QIAamp DNA Blood Mini Kit (Qiagen). Bisulfite sequencing was conducted with the EZ DNA Methylation kit (Zymo Research). Briefly, 200 ng of genomic DNA in CT Conversion Reagent buffer was denatured for 8 min at 98 °C and incubated for 3.5 h at 64 °C. After on-column desalination, DNA was eluted in 10 μl elution buffer, and subjected to 40 cycles of PCR amplification (95 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min) following an initial denaturation step at 95 °C for 10 min. Primers for the SHP-1 (PTPN6) promoter (LOU1398, LOU1399, LOU1400 and LOU1401) were designed using the MethPrimer online software (www.uoregon.org/methprimer/index1.html). Their specificity for bisulfite converted DNA was verified by PCR amplification of bisulfite-treated and -untreated genomic DNA. PCR products were gel-purified and cloned into pGEM-T (pGEM-T Easy Vector System, Promega). Transformants containing recombinant plasmids were selected by blue/white colony screening and randomly picked up for Sanger sequencing.

Analysis was performed using the QUMA online software26 with the default conditions (exclusion of sequences with a bisulfite conversion rate below 95% or with an identity to the reference sequence lower than 90%). Statistical analysis was performed using the GraphPad Prism 6 software.

Oligos:
Region 1, fwd: LOU1398
5′-GTTTTGATAGGGGTATTTTTT-3′
Region 1, rev: LOU1399
5′-CACCATAAATCCACCTAAACCTCA-3′
Region 2, fwd: LOU1400
5′-AGTTTACTGGGAGTTTTGTGT-3′
Region 2, rev: LOU1401
5′-GAAAAACCCCTAATATATTCCT-3′.

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