Overexpression of GSTP1 promotes colorectal cancer cell proliferation, invasion and metastasis by up-regulating STAT3

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Running title: GSTP1 promotes colorectal cancer progression by upregulating STAT3

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Abstract

Abnormal expression of glutathione S-transferase Pi 1 (GSTP1) is associated with the progression of several tumor types. However, its role and molecular mechanism in the progression of colorectal cancer (CRC) is largely unknown. In the present study, immunohistochemistry (IHC) and quantitative-reverse transcription PCR (qRT-PCR) were used to detect the expression of GSTP1 and signal transducer and activator of transcription 3 (STAT3) in CRC tissues. Western blotting was applied to detect the expression of GSTP1 and proteins of Janus kinase (JAK)-STAT3 pathway. The interaction and co-localization of GSTP1 and STAT3 were detected by co-immunoprecipitation (CO-IP) and immunofluorescence, respectively. A positive correlation was identified between the expression of GSTP1 and STAT3 in human CRC tissues. Overexpression of GSTP1 promoted the proliferation, invasion and metastasis of CRC cells by upregulating STAT3. GSTP1 and STAT3 can directly bind to and regulate each other, and can be regulated by the upstream gene which was called F-box only protein 8 (FBX8). The present study demonstrated that GSTP1 could enhance the expression of STAT3 to promote the proliferation, invasion and metastasis of CRC cells, which provides a potential therapeutic target for clinical treatment of CRC.

Keywords

GSTP1; STAT3, CRC; proliferation; invasion; metastasis

1. Background

Following the lung and breast cancer, CRC is the third most common cancer worldwide [1]. The combined application of surgery, chemotherapy and radiotherapy can control many localized tumors, however, it is limited in the restriction of the development of metastatic disease [2]. Therefore, further elucidation of the molecular mechanisms underlying the tumorigenesis and pathogenesis of CRC is urgently required for this lethal disease.

Glutathione S-transferases (GSTs) (EC 2.5.1.18) are phase II metabolic enzymes [3], which play a role in xenobiotic biotransformation [4], drug metabolism [5], protection against oxidative stress, and modulating cell proliferation and signaling pathways [6, 7]. The GST Pi 1 (GSTP1), as an isozyme of GST, is a major regulator of cell signaling in response to stress, hypoxia, growth factors and other stimuli [8]. GSTP1 is overexpressed in a variety of human cancers, including gastric cancer, pancreatic cancer and bladder cancer [9, 10]. GSTP1 is also involved in the process of proliferation and invasion in tumor cells; The overexpression of GSTP1 promotes tumor cell proliferation and inhibits apoptosis in head and neck squamous cell carcinoma (HNSCC) [11]. However, GSTP1 inhibits the proliferation of bladder cancer T24 cells and arrests these cells in the G0/G1 phase [12]. In addition, a recent study suggested that GSTP1 may be applied as an important biomarker for liquid

biopsy [13].

The signal transducer and activator of transcription (STAT) family is phosphorylated via Janus kinases (JAKs) in response to the binding of growth factors or cytokines to their corresponding receptors [14-16]. These factors are known to stimulate the activation of intracellular STAT proteins, which are phosphorylated and dimerized, and subsequently translocated to the nucleus for transactivation of a number of genes involved in numerous cellular processes [17]. Persistent activation of STAT3 has been observed in multiple human malignancies, including various stages of CRC [18-20]. Furthermore, high expression of STAT3 alters the cell cycle [21, 22] and inhibits apoptosis by upregulating anti-apoptotic signaling [23, 24] in inflammation-associated CRC and other human cancers [25]. In addition, GSTP1 negatively regulates STAT3 activation in epidermal growth factor (EGF) signaling, and is also a regulator of the cell cycle via EGF signaling in human hepatocellular carcinoma (HCC) [8]. However, the regulatory mechanisms between GSTP1 and STAT3 in the progression of CRC remain unknown.

In our previous studies, it was identified that the loss of F-box only protein 8 (FBX8) in hepatocellular carcinoma, gastric cancer and CRC was associated with poor survival of patients [26-28]. FBX8 is a metastatic suppressor downstream of miR-223 and targets mTOR degradation in CRC [27]. It was also found that FBX8 inhibits the proliferation, invasion and metastasis of CRC by promoting the degradation of GSTP1. At the same time, it was confirmed that GSTP1 can be used as an effective marker to predict the prognosis of CRC [29].

The present study revealed that overexpression of GSTP1 can promote the proliferation, invasion and metastasis of CRC cells by upregulating STAT3, and this function can be regulated by FBX8. Therefore, it may provide a potential therapeutic target for the clinical treatment of CRC.

2. Materials and methods

2.1 IHC

The sections were dewaxed and rehydrated, the endogenous peroxidase was eliminated with 3% H_2O_2. The antigen was repaired with 0.01 M, pH 6.0 sodium citrate buffer by microwave oven boiling for 5 min. After blocking with 5% goat serum at room temperature for 1 h, add anti-GSTP1(1:200) or anti-STAT3(1:100) antibody which were diluted with appropriate proportion about 50 μl and overnight at 4℃. Incubating with horseradish egg protein rabbit secondary antibody or murine secondary antibody for 90 min at room temperature. Labeling streptavidin with appropriate horseradish peroxidase, incubate for 30 min at room temperature. DAB color developer needs to be observed under the microscope.

Staining was scored in a double-blind manner by two individuals with a score of 0 (representative negative), 1 (weak), 2 (medium), and 3 (strong). Depending on the percentage of the stained area relative to the total cancerous tissue area or blood vessel, the staining range is divided into 0 points (0%), 1 point (1-25%), 2 points (26-50%), 3 points (51-75%) and 4 points (76 -100%). the sum of the dyeing strength and range was taken as the final dyeing value (0-7): (-) total score <3 points, (+) total

score 3 points, (++) total score 4 points, (+++) total score is 5 points or more, in which - or + is a low expression group, ++ and +++ are high expression groups.

2.2 Immunofluorescence

For immunofluorescence of cells seeded at a density of 0.5×10^4 cells on Confocal NEST dish glass bottom Petri dishes. After 24 h, cells were fixed in 4% paraformaldehyde, permeabilized with 0.02%Triton-X/1 × PBS, and blocked in 1 × PBS + 10% fetal bovine serum and 1% BSA. Primary GSTP1 (1:200) and STAT3 (1:100) antibodies were incubated overnight at 4℃ at the dilutions listed below in 1× PBS. Secondary antibodies coupled to Alexa Fluor 488 or 594 (Invitrogen) was incubated 2 h at room temperature. Nuclear DNA was stained with 4′, 6-diamidino-2-phenylindole (DAPI). Confocal images were taken by Olympus inverted fluorescence microscope and were outputted by PV10-ASW 1.7 viewer software.

2.3 Co-IP

In brief, the extracts of SW620 cells were blocked with IgG or protein A/G-agarose 2 h at 4°C to get rid of unspecific protein binding and then they were incubated with anti-FBX8 or anti-GSTP1 antibody overnight at 4°C. The protein A/G-agarose was separated out by centrifugation at 4℃, 2500 rpm. PVDF membranes were blocked with 5% skim milk 1 h at room temperature and incubated with GSTP1 (1:200) and STAT3 (1:100) antibodies overnight at 4℃ at the dilutions listed below in 5% skim milk. Protein bands were visualized using enhanced chemiluminescence kit HRP (FD bio-femto ECL Kit).

2.4 Glutathione S-transferase (GST) pull-down assay

The interaction of truncated GSTP1 with STAT3 was examined in HCT116 and SW620 cells by GST-mediated pull-down assays (Thermo Scientific, Rockford, IL). Recombinant GST-STAT3-CCD (218-400), GST-STAT3-DBD (401-564), GST-STAT3-Linker (565-663) and GST-STAT3-SH2 (664-768) proteins were expressed and purified. Purified GST-STAT3-CCD (218-400), GST-STAT3-DBD (401-564), GST-STAT3-Linker (565-663) and GST-STAT3-SH2 (664-768) fragments were bound to glutathione resin as a GST-fusion protein and incubated with GSTP1 at 4°C for 2 h. After extensive washing with assay buffer, the complex was eluted with 5 mM reduced glutathione and the bound protein complexes were disrupted. Then, the proteins were separated on SDS-PAGE and Western blotting.

2.5 Statistical analysis

All statistical analyses were performed by SPSS version 22.0 (IBM, USA). The results were presented as mean ± SD. Pearson correlation analysis was applied to analyze the correlation between GSTP1 and STAT3. For experiments among/between sample groups or three comparisons were analyzed by one-way ANOVA or independent samples T-test. Before the analysis of variance, Levene test was used for variance. A two-tailed $P < 0.05$ was considered as statistically significant in all tests.

Detailed methods about Plasmids and siRNA transfection, Cell proliferation assay(CCK8), Cell invasion assays in vitro, Western blotting, qRT-PCR analysis were described in Appendix A−Supplementary data.

3. Results

3.1 The expression levels of GSTP1 and STAT3 are positively correlated in human CRC tissues

STAT3, widely recognized as a cancer gene, is typically associated with poor prognosis of various human malignancies and promotes cancer progression or metastasis [30-33]. The direct interaction between GSTP1 and STAT3 can promote HCC progression [8], and our previous study found that GSTP1 can be ubiquitinated by FBX8, thus inhibiting its function in promoting CRC proliferation, invasion and metastasis [29]. Therefore, IHC was used to detect the expression of GSTP1 and STAT3 in 20 human CRC tissues. The results demonstrated that the expression levels of GSTP1 and STAT3 in human CRC tissues were positively correlated (Figure 1A). Western blotting and qRT-PCR were used to detect the expression levels of GSTP1 and STAT3 in 8 paired fresh CRC tissues (Figure 1B and 1C). As shown in (Figure 1D), the expression of GSTP1 was also positively correlated with STAT3 in paired fresh CRC tissues (Pearson's r =-0.8781, P = 0.0006).

3.2 Overexpression of GSTP1 promotes the proliferation, invasion and metastasis of CRC cells dependent on STAT3

The expression of GSTP1 is positively correlated with STAT3. Combined with previous studies, we predicted GSTP1 may play a role in the progression of CRC by regulating STAT3. CRC cell lines with stable knockdown of GSTP1 were used in the previous study [29] (SW620/shGSTP1 and HCT116/shGSTP1 cell lines), and STAT3

was overexpressed in these cells to perform relevant recovery experiments. As shown in (Figure 2A and 2B), overexpression of STAT3 could significantly promote the invasion and proliferation of cells in the GSTP1-knockdown group in vitro. In addition, the expression levels of STAT3 and GSTP1 were detected in subcutaneous tumors, in situ implants and liver metastases of CRC in nude mice, which were obtained from a previous study. The results demonstrated that in these three tumor tissues, the expression of STAT3 was significantly upregulated in the GSTP1 overexpressed group (Figure 3A and 3B), which indicated that the expression of GSTP1 and STAT3 in mice tissue samples are consistent.

3.3 GSTP1 and STAT3 can directly bind and regulate each other

It was further hypothesized that GSTP1 could interact with STAT3 in CRC cells. As expected, Co-IP analyses and immunofluorescence identified the interaction between the two proteins. The existence of GSTP1 was detected in the immunoprecipitates obtained with an antibody against STAT3 (Figure 4A). Immunofluorescence demonstrated that GSTP1 and STAT3 exhibited co-localization in the cytoplasm of SW620 cells (Figure 4B). The present study cloned four truncated constructs of STAT3: CCD (218-400), DBD (401-564), Linker (565-663) and SH2 (664-768) (Figure 4C), and then identified an interaction between the CCD domain of STAT3 and GSTP1 by GST pull-down (Figure 4C). It was identified that the CCD domain of STAT3 was essential for the interaction with GSTP1.

Thus, it was examined whether GSTP1 could activate the STAT3 signaling pathway

199 in CRC cells, and it was identified that exogenous expression of GSTP1 further
200 increased the protein expression of phosphorylated (p)-STAT3, STAT3 and the
201 downstream STAT3 targets cyclin D1 and CDC25A in SW480 cells (Figure 4D).
202 However, there was no appreciable effect on the upstream components of the STAT3
203 signaling pathway, such as JAK2 and p-JAK2 (Figure 4D). By contrast, depletion of
204 GSTP1 in SW620 cells decreased the levels of p-STAT3, STAT3, cyclin D1, and
205 CDC25A (Figure 4D). Notably, this regulation was not one-way; it was identified that
206 ectopic expression of STAT3 could also upregulate the protein level of GSTP1 and
207 induce higher levels of the downstream STAT3 targets cyclin D1 and CDC25A. At the
208 same time, silencing STAT3 could decrease the expression levels of GSTP1, cyclin
209 D1 and CDC25A, but not the expression of p-JAK2 (Figure 4E). In addition, when
210 AG490 (100 μM) was used to block the JAK2-STAT3 pathway, a significant decrease
211 was observed in the expression of JAK2, p-JAK2，STAT3，p-STAT3 and GSTP1 24 h
212 after treating LoVo cells (Figure 4F).
213

214 ## 3.4 The interaction between GSTP1 and STAT3 is regulated by FBX8

215 As a downstream target of FBX8, GSTP1 can interact with STAT3, therefore, we
216 predicted that FBX8 could regulate the interaction between GSTP1 and STAT3. Co-IP
217 assays demonstrated that the existence of GSTP1, in FBX8-expressing SW620 and
218 SW480/FBX8 cells, was detected to a higher extent in the immunoprecipitates
219 obtained with an antibody against STAT3 compared with the control cells (Figure 5A
220 and 5B). However, the presence of GSTP1 demonstrated the opposite results; it was

detected to a lesser extent in the immunoprecipitates obtained with an antibody against STAT3 (Figure 5A and 5B). These results indicated that FBX8 was a suppressive factor for the combination of GSTP1 and STAT3.

4. Discussion

Previously, we identified GSTP1 as the downstream target of FBX8 by Co-IP and mass spectrometry analyses, and confirmed that GSTP1 can promote the proliferation, invasion and metastasis of CRC [29]. In addition, it was identified that GSTP1 could regulate STAT3 to affect the development of HCC [8]. Therefore, we hypothesized that GSTP1 may be involved in the progression of CRC by regulating STAT3.

The present study detected GSTP1 and STAT3 in human colorectal tissues and found that GSTP1 expression was positively correlated with STAT3 expression. This result revealed that GSTP1 may be able to regulate the expression of STAT3 to play a role in the progression of CRC. Recent evidence suggests GSTP1 is involved in tumor cell proliferation and invasion; overexpression of GSTP1 increased cell proliferation in HNSCC [11]. In comparison, GSTP1 arrests bladder cancer T24 cells in the G0/G1 phase and upregulates p21 expression [12]. The present study investigated the effect of GSTP1 on the proliferation and invasion of CRC cells in vitro by recovery experiments. The results demonstrated that overexpression of STAT3 could significantly promote the proliferation and invasion of CRC cells after GSTP1 downregulation. This indicated that GSTP1 promoted the proliferation and invasion of CRC cells depending on STAT3. In addition, the immunohistochemical results of

subcutaneous tumors, *in situ* implanted tumors and liver metastases of CRC in mice also confirmed the aforementioned conclusion.

Mechanistically, the present studies served as a proof-of-concept that GSTP1 and STAT3 can form a complex, and that upregulation of GSTP1 led to activation of the STAT3 pathway (Figure 6). Meanwhile, STAT3 can positively regulate the protein expression of GSTP1. STAT3 is phosphorylated via JAK, then dimerized and subsequently translocated to the nucleus for transactivation of a number of genes involved in numerous cellular processes [14-17]. In addition, the overexpression of STAT3 can affect the cell cycle [21, 22] or inhibit apoptosis by enhancing anti-apoptotic signaling [23, 24] in CRC. Therefore, identifying the association between GSTP1 and the STAT3 pathway is an important way to illustrate the molecular mechanisms of GSTP1 in CRC. The present results confirmed the interaction of GSTP1 and STAT3, and demonstrated that GSTP1 positively regulated STAT3 signaling, resulting in the alteration of p-STAT3 and STAT3, as well as the targeted genes such as cyclin D1 and CDC25A. STAT3 siRNA significantly abolished the increase of STAT3, cyclin D1 and CDC25A, and decreased the protein expression of GSTP1, but there was no change in p-JAK2. Exogenous STAT3 exhibited the adverse results. In addition, western blotting revealed a concentration-dependent decrease in the level of JAK2, p-JAK2，STAT3，p-STAT3 and GSTP1 after 24 h of treating LoVo cells with the specific inhibitor (AG490) of JAK2. These results demonstrated GSTP1 interacted with STAT3 without involvement of JAK2. Combined with previous research that FBX8 can degrade the expression of GSTP1

[29], we speculated that FBX8 could affect the association of GSTP1 and STAT3. Subsequent experiments confirmed that FBX8 was a restraining factor for the combination of GSTP1 and STAT3.

5. Conclusions

In summary, GSTP1, as the downstream effector of FBX8, was identified as an important promoter and a useful prognostic marker for CRC. GSTP1 could interact with STAT3 and upregulate the expression of STAT3, as well as its related downstream molecules [34], to promote the proliferation, invasion and metastasis of CRC. Therefore, the present study provided a potential new molecular target for the treatment of CRC metastasis.

List of abbreviations

GSTP1	glutathione S-transferase Pi 1
CRC	colorectal cancer
STAT3	signal transducer and activator of transcription 3
FBX8	F-box only protein 8
IHC	immunohistochemistry
qRT-PCR	reverse transcription-quantitative PCR
CO-IP	co-immunoprecipitation

Declaration of ethics approval and consent to participate

Fresh CRC tissues were obtained from the department of pathology in nanfang hospital, and prior approval was obtained from the Southern Medical University Institutional Board (Guangzhou, China). Informed consent was obtained from each patient. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Southern Medical University.

Declaration of availability of data and materials

All data and models generated or used during the study appear in the submitted article.

Declaration of competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Feifei Wang, Ceng Zhang, Xiaohui Zhu and Dan Zhang: Experiments. Shunjie Ni, Zhizhi Wang and Professor Yanqing Ding: Statistical analysis. Zhaowen Zhang, Shuyi Xu and Xiaoliang Lan: Collecting tissue samples. Professor Li Liang: Conceived experiments. Analyzed data. Feifei Wang, Xiaohui Zhu and Li Liang: Writing the paper. All authors read and approved the final manuscript.

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438

439 **Figure 1. GSTP1 expression is positively correlated with the expression of STAT3**
440 **in human colorectal cancer tissues**

441 **(A)** The expression levels of GSTP1 and STAT3 in human CRC tissues were detected
442 by IHC. Scale bars represent 50 μm (left) and 20 μm (right). (**B)** Western blot analysis
443 was performed to detect the expression levels of GSTP1 and STAT3 in 8 paired fresh
444 tissue samples of human CRC. (**C)** The relative mRNA expression levels of GSTP1
445 and STAT3 in 8 paired fresh tissue samples of human CRC were detected by
446 qRT-PCR. All samples were tested in triplicate. Bars represent the mean ± SD. (**D)**
447 The correlation analysis between GSTP1 and STAT3 was performed by Pearson's
448 correlation analysis (Pearson's r =-0.8781, P = 0.0006).

449 **Figure 2. Overexpression of STAT3 can reverse the inhibition of invasion and**
450 **proliferation induced by downregulating GSTP1**

(A) Effects of overexpression of STAT3 on SW620/shGSTP1 and HCT116/shGSTP1 cell invasion were detected by invasion assay. Scale bars represent 50 μm. All samples were analyzed in triplicate. Bars represent the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$. (**B)** Effects of overexpression of STAT3 on SW620/shGSTP1 and HCT116/shGSTP1 cell proliferation were detected by CCK8 assay. All samples were tested in triplicate. Bars represent the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$.

Figure 3. Overexpression of GSTP1 can upregulate the expression of STAT3 in vivo

(A) The expressions of GSTP1 and STAT3 in subcutaneous tumors of nude mice were detected by immunohistochemistry. Scale bars represent 50 μm (left) and 20 μm (right). (**B)** The expression of GSTP1 and STAT3 in orthotopic implantation of colorectal tumors and liver metastases in nude mice were detected by immunohistochemistry. Scale bars represent 50 μm (left) and 20 μm (right).

Figure 4. GSTP1 and STAT3 can interact with each other

(A) The interaction between GSTP1 and STAT3 was detected by a co-immunoprecipitation assay with the SW620 cell line. (**B)** The co-location between GSTP1 and STAT3 was detected by an immunofluorescence assay with the SW620 cell line. Scale bars represent 10 μm. (**C)** The direct interaction site between GSTP1 and STAT3 was detected by a GST pull-down assay. **(D)** Western blot analysis was performed to detect the expression of JAK/STAT3 signaling pathway-related proteins in SW620/siGSTP1 and SW480/GSTP1 cell lines. (**E)** Western blot analysis was performed to detect the expression of JAK/STAT3 signaling pathway-related proteins

in SW620/siSTAT3 and LoVo/STAT3 cell lines. **(F)** Western blot analysis was performed to detect the expression of GSTP1 and JAK/STAT3 signaling pathway-related proteins following AG490 treatment of LoVo cells.

Figure 5. The interaction between GSTP1 and STAT3 is regulated by FBX8

(A) The interaction between GSTP1 and STAT3 was detected by a CO-IP assay after AG490 treatment of SW620 cells. (**B)** The interaction between GSTP1 and STAT3 was detected by a CO-IP assay with SW480/FBX8 cells.

Figure 6. Schematic diagram of the role between GSTP1 and STAT3 in CRC

Figures

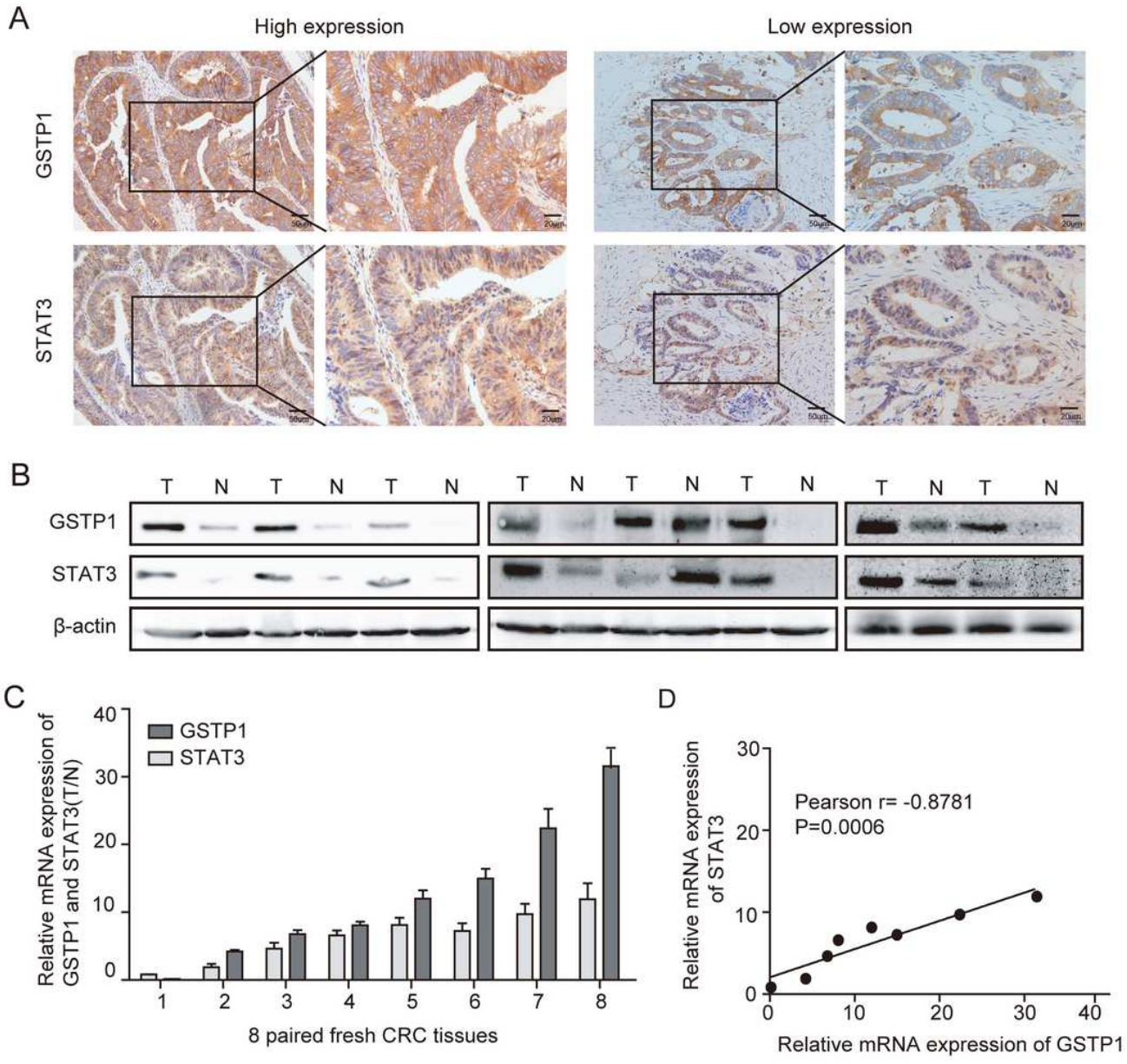


Figure 1

GSTP1 expression is positively correlated with the expression of STAT3 in human colorectal cancer tissues (A) The expression levels of GSTP1 and STAT3 in human CRC tissues were detected by IHC. Scale bars represent 50 μm (left) and 20 μm (right). (B) Western blot analysis was performed to detect the expression levels of GSTP1 and STAT3 in 8 paired fresh tissue samples of human CRC. (C) The relative mRNA expression levels of GSTP1 and STAT3 in 8 paired fresh tissue samples of human CRC were detected by qRT-PCR. All samples were tested in triplicate. Bars represent the mean ± SD. (D) The correlation analysis between GSTP1 and STAT3 was performed by Pearson's correlation analysis (Pearson's r =-0.8781, P = 0.0006).

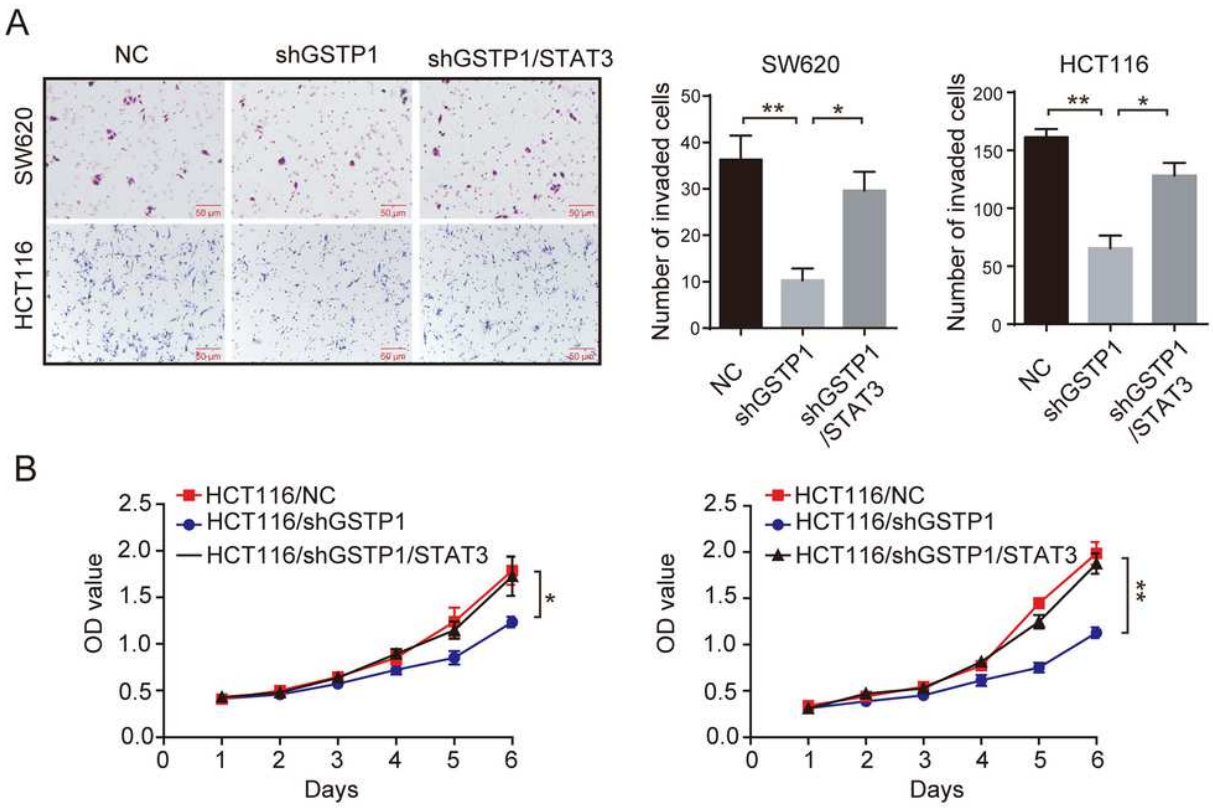
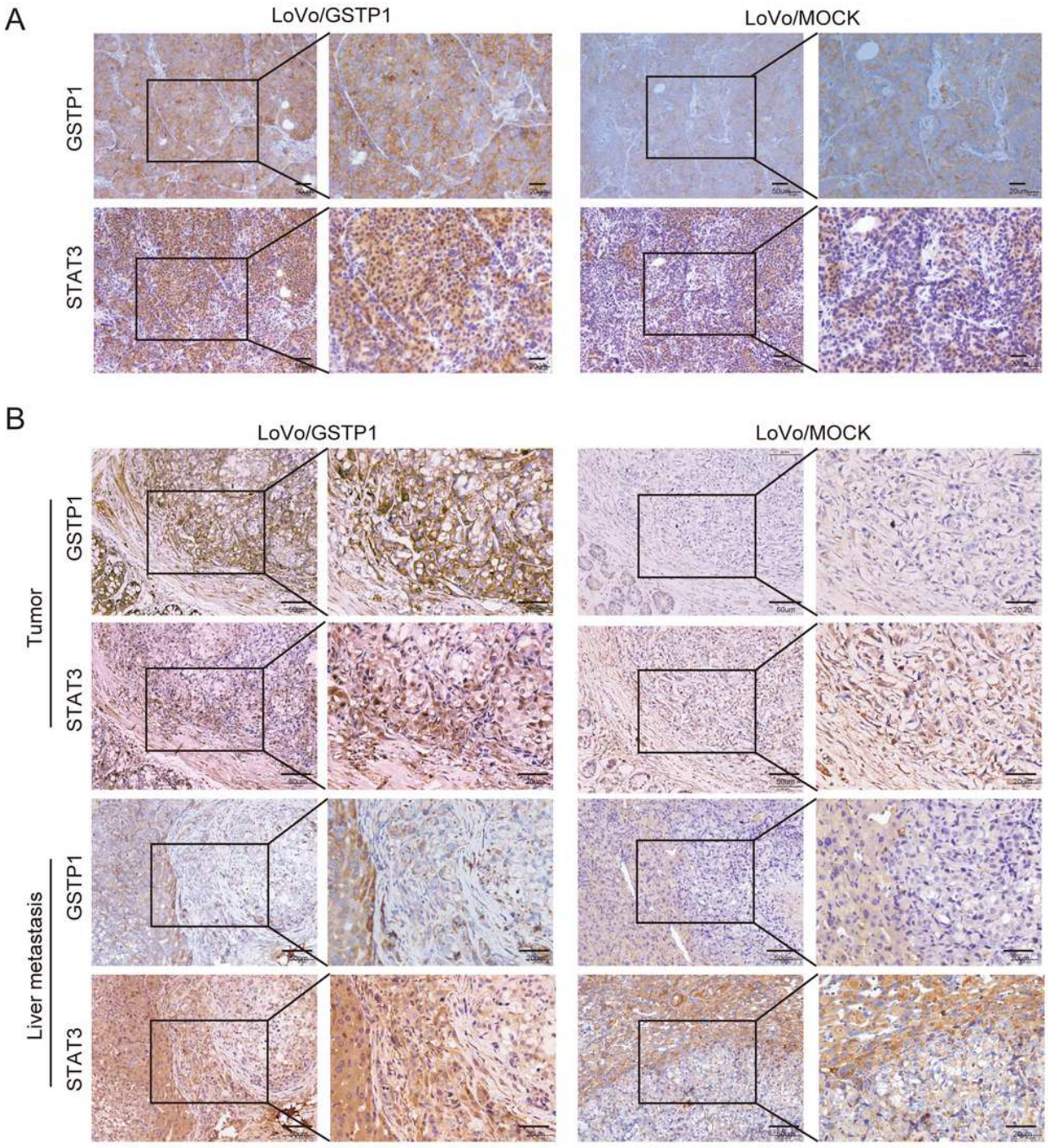


Figure 2

Overexpression of STAT3 can reverse the inhibition of invasion and proliferation induced by downregulating GSTP1 (A) Effects of overexpression of STAT3 on SW620/shGSTP1 and HCT116/shGSTP1 cell invasion were detected by invasion assay. Scale bars represent 50 μm. All samples were analyzed in triplicate. Bars represent the mean ± SD. *P<0.05, **P<0.01. (B) Effects of overexpression of STAT3 on SW620/shGSTP1 and HCT116/shGSTP1 cell proliferation were detected by CCK8 assay. All samples were tested in triplicate. Bars represent the mean ± SD. *P<0.05, **P<0.01.



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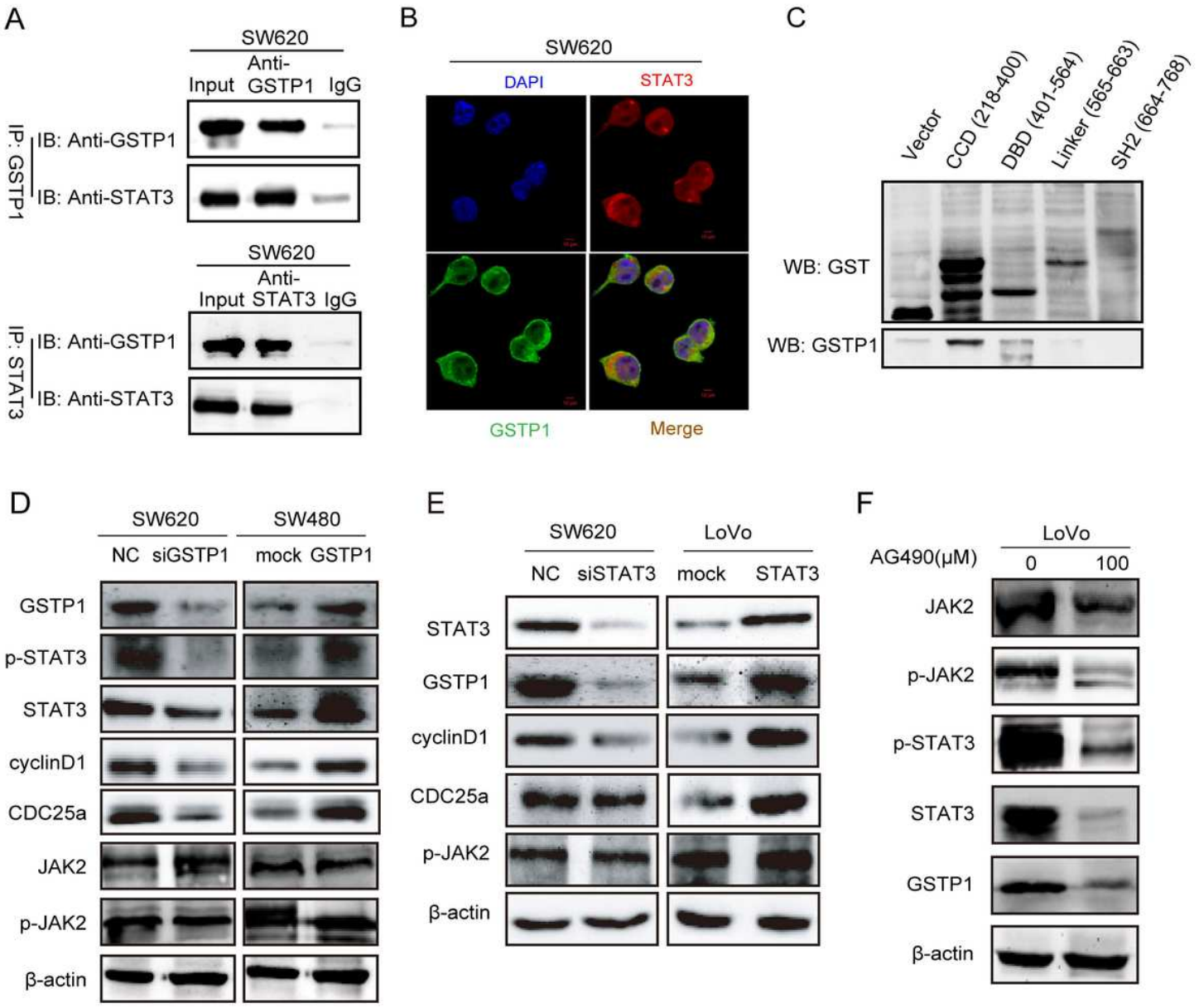

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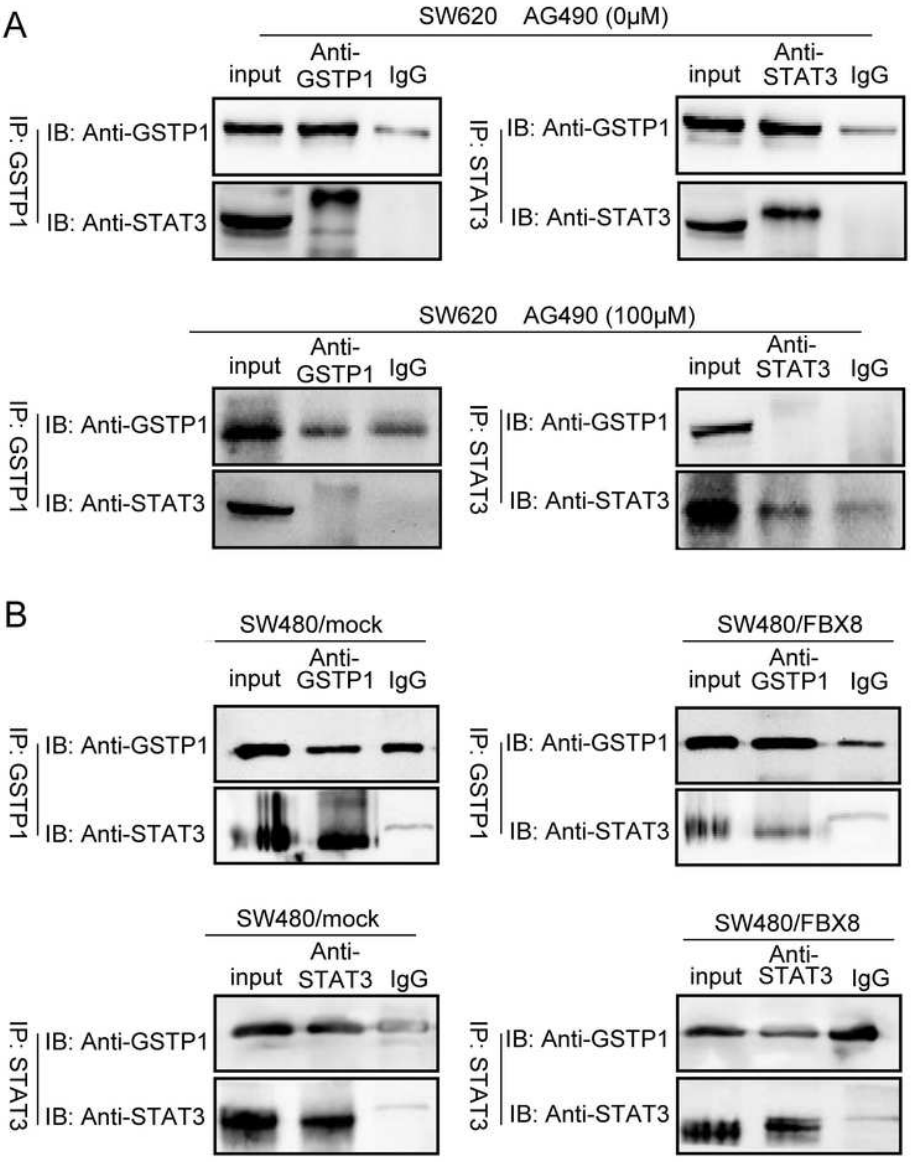

Figure 5

The interaction between GSTP1 and STAT3 is regulated by FBX8 (A) The interaction between GSTP1 and STAT3 was detected by a CO-IP assay after AG490 treatment of SW620 cells. (B) The interaction between GSTP1 and STAT3 was detected by a CO-IP assay with SW480/FBX8 cells.

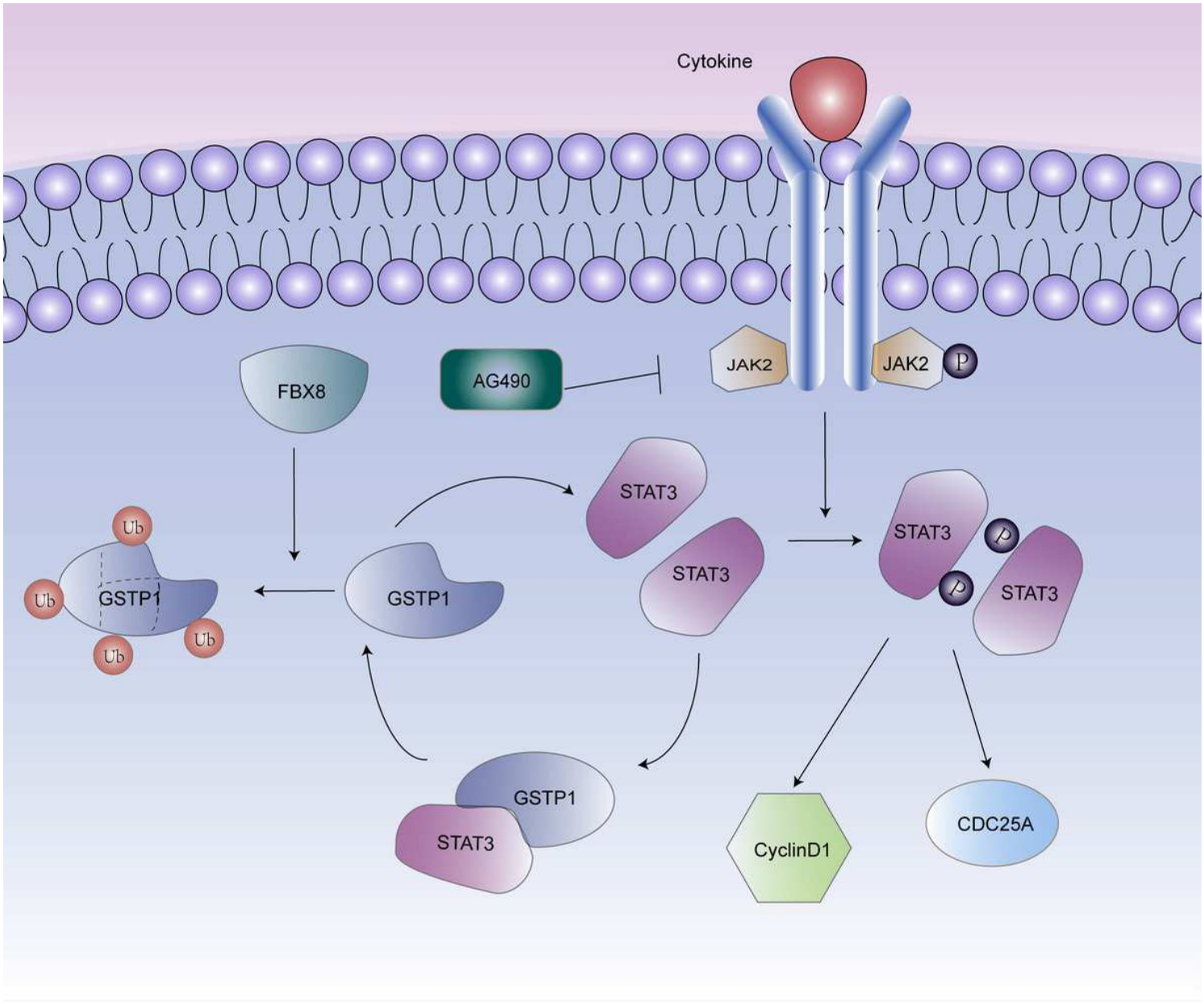

Figure 6

Schematic diagram of the role between GSTP1 and STAT3 in CRC

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Attachment1.pdf