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1 STAT3 potentiates RNA polymerase I-directed transcription

2 and tumor growth by activating RPA34 expression

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- 18 Short title: STAT3 & Pol I-directed transcription
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- 22

23 Abstract

24 Background: Deregulation of either RNA polymerase I (Pol I)-directed transcription

or expression of signal transducer and activator of transcription 3 (STAT3) correlates

closely with tumorigenesis. However, the connection between STAT3 and Pol I-

- 27 directed transcription hasn't been investigated.
- 28 Methods: The role of STAT3 in Pol I-directed transcription was determined using
- 29 combined techniques. The regulation of tumor cell growth mediated by STAT3 and
- 30 Pol I products was analyzed *in vitro* and *in vivo*. RNAseq, ChIP assays and rescue

assays were used to uncover the mechanism of Pol I transcription mediated bySTAT3.

Results: STAT3 expression positively correlates with Pol I product levels and cancer

- cell growth. The inhibition of STAT3 or Pol I products suppresses cell growth.
- 35 Mechanistically, STAT3 activates Pol I-directed transcription by enhancing the
- 36 recruitment of the Pol I transcription machinery to the rDNA promoter. STAT3
- directly activates Rpa34 gene transcription by binding to the RPA34 promoter, which
- 38 enhances the occupancies of the Pol II transcription machinery factors at this
- 39 promoter. Cancer patients with RPA34 high expression lead to poor survival
- 40 probability and short survival time.
- 41 **Conclusion:** STAT3 potentiates Pol I-dependent transcription and tumor cell growth

42 by activating RPA34 *in vitro* and *in vivo*.

43 Keywords: STAT3, RNA polymerase I, ribosomal rRNA expression, tumor growth,

44 RPA34

45 Background

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT 46 family that regulates numerous biological processes, including cell proliferation and 47 migration, apoptosis, angiogenesis, immunosuppression and cancer stem cell 48 maintenance [1-3]. STAT3 can be activated by several canonical signaling pathways 49 such as IL6/JAK, EGF/EGFR and ABL/SRC pathways [2, 4, 5]. After activation, 50 STAT3 is phosphorylated, dimerized and translocated to the nucleus through its 51 nuclear localization sequence, where it binds to STAT3 consensus sequences to 52 activate transcription of its target genes [2, 6, 7]. Numerous studies have shown that 53 STAT3 is also localized to mitochondria and regulates mitochondrial respiration by 54 interacting with components of the electron transport chain (ETC) [8-12]. In addition 55 to the roles in mitochondrial, another non-canonical role of STAT3 is that 56 unphosphorylated STAT3 (uSTAT3) can enter the nucleus and bind to the GAS 57 promoter sequence to modulate transcription [2, 13]. The uSTAT3 contributes to 58 cancer progression by increasing STAT3 transcription activity. Activation of Stat3 59 gene transcription by IL-6 signaling augments uSTAT3 production, which promotes 60 expression of E2f1, Met and Mras genes [15, 16]. Recently, many novel activators of 61 STAT3, including lncRNA, miRNA, circRNA and proteins, have been identified [3, 62 17-25]. Some of them have been confirmed to be promising targets for anti-cancer 63 therapy [3, 26-29]. Cai G et al reported that an inhibitor called SD-36 can act as a 64 potent and selective degrader of STAT3 to inhibit the growth of a subset of acute 65 myeloid leukemia by inducing cell cycle arrest [30]. Another inhibitor STAT3-IN-3 66 has been confirmed to repress tumor growth for breast cancer cell line 4T1 67 xenografted in mice by reducing proliferative activity [31]. It has been shown that 68 69 constitutively activated STAT3 can promote cell proliferation by increasing the expression of CyclinD1, c-Myc and Survivin [32-34]. However, how STAT3 70 activation enhances cell proliferation is not fully understood. 71 Human RNA polymerase I (Pol I) is responsible for the synthesis of 45S pre-rRNA, 72 which is instantly processed into 28S, 18S and 5.8S rRNA. Pol I products are 73 74 essential to ribosomal assembly, protein synthesis and cell growth [35, 36]. Abnormally high levels of Pol I products have been observed in a subset of cancer 75 tissues [37]. Pol I-directed transcription is tightly controlled by many factors, 76 including Pol I general transcription factors, oncogenic factors, tumor suppressors, 77 signaling pathways, chromatin modification and non-coding RNAs [38-43]. Despite 78 massive advances in the research field of Pol I-directed transcription, the regulatory 79 pathways and factors controlling this process remain to be identified. In our previous 80 work, we showed that cytoskeletal filamin A (FLNA) silencing enhanced Pol I-81 directed transcription and cell proliferation [44]. Recently, RNA-seq analysis revealed 82 that FLNA silencing reduced STAT3 mRNA expression in tumor cell lines. Whether 83 STAT3 is associated with Pol I-dependent transcription hasn't been investigated. In 84

this study, we showed that STAT3 functions as a positive factor in the regulation of
Pol I-directed transcription and tumor cell survival and growth. We investigated the
effect of an STAT3 inhibitor and Pol I-specific inhibitors on tumor cell growth *in vitro*and *in vivo* and explored the regulatory mechanisms of Pol I transcription mediated by
STAT3.

90 Materials & Methods

91 *Plasmids, cells, and reagents*

Three distinct DNA fragments encoding STAT3 shRNA molecules were 92 synthesized by Sangon Biotech (Shanghai, China) and inserted downstream of the U6 93 promoter at the pLVU6-EGFP-Puro plasmid (Inovogen, Beijing, China). STAT3 and 94 RPA34 cDNA fragments were inserted immediately downstream of the mCherry gene 95 96 at the pLVEF1a-mCherry-Puro plasmid (Inovogen, Beijing, China). The rDNA promoter along with a piece of cDNA encoding a small fragment of 45S rRNA near 97 the 5' prime was loaded into the pGL3-basic reporter vector. Cell lines, including 98 SaOS2, HeLa, 293T and HepG2, were purchased from American Type Cell Collection 99 (ATCC, USA) and cultured their corresponding medium supplied with 10% FBS 100 101 (Thermo Scientific, USA) and 1×Penicillin/ Streptomycin (GE Healthcare). After culturing 48 hours, mycoplasma contamination tests were performed and STR (short 102 tandem repeat) profiling was performed. Restriction enzymes were purchased from 103 New England Biolab (USA). Biological reagents such as transfection and Western 104 blot detection reagents were obtained from Thermo Scientific (USA). The chemicals 105 used in this study were purchased from Sigma-Aldrich (Merk). 106

107 *Transfection and cell line generation*

Three distinct double-strand siRNA fragments that interfere with STAT3 expression
were synthesized by Genewiz Co (Shuzhou, China). HeLa and HepG2 cells were
cultured for 24 hours in 12-well plates, transient transfection for cells in each well
was performed using the mixture of 2 µL Turbofect (Thermo Scientific) and 60
pmoles siRNA (20 pmoles for each siRNA). Forty-eight hours post-transfection,

113 STAT3 and ribosomal RNA expression were analyzed by Western blot and RT-qPCR,

respectively. For the generation of cell lines with STAT3 knockdown or

- overexpression, the medium containing lentiviral particles was initially prepared by
- transfecting 293T cells with 40 μ g lentiviral vectors expressing STAT3 shRNA- or

mCherry-STAT3 and packaging vectors pH1 (30 μ g) and pH2 (10 μ g). The resulting

medium was used for the transduction of HeLa, HepG2 and 293T cells. Cells were

selected with puromycin, and stable cell lines with STAT3 silencing and

120 overexpression were verified by RT-qPCR and Western blot. For the generation of the

cell lines concurrently expressing STAT3 shRNA and mCherry-RPA34, lentiviral

122 particles expressing mCherry-STAT3 were used for the transduction of the STAT3-

depleted cell lines, and the rest of the protocol followed the procedures as described

124 above.

125 Endogenous protein activation and repression assays mediated by CRISPR dCas9-

126 KRAB/VP48 and STAT3 inhibitor assays

127 Two DNA fragments encoding the guide RNA molecules targeting different

- positions at the *STAT3* promoter were synthesized by Sangon (Shanghai, China) and
- inserted downstream of the U6 promoter at the pLVU6-sgRNA-hUbC-dCas9-KRAB
- vector (Cat No. 71236, Addgene, USA) or the pAC2-dual-dCas9VP48-sgExpression
- vector (Cat No. 48236, Addgene, USA). The resulting vectors were transiently
- transfected into HepG2 cells; after 48 hours, cells were harvested, and STAT3 and
- ribosomal RNA were detected by Western blot and RT-qPCR, respectively. For the
- assays with a STAT3 inhibitor, two groups of HeLa or HepG2 cells were cultured for
- 135 24 hours before the STAT3 inhibitor was added into one group of cells at a final
- 136 concentration of 2 μ M, meanwhile, DMSO was added into another group of cells.
- 137After 48 hours, STAT3 expression and phosphorylation were analyzed by Western
- blot using an anti-STAT3 antibody (CST#9139, CST, USA) and an anti-p-STAT3
- antibody (CSB-PA004932LA01HU, CUSABio, China), while ribosomal RNA
 expression was detected by RT-qPCR.
- 141 *Immunofluorescence assays*

HeLa or HepG2 cells were cultured on small round coverslips (14 mm in diameter)

in the complete medium. When growing up to 60% of culturing surface, cells were
fixed for 10 min with 4% formaldehyde freshly prepared with 1×PBS solution. After

144 fixed for 10 min with 4% formaldehyde freshly prepared with 1×PBS solution. Afte 145 fixation, immunofluorescence (IF) assays were performed as described previously

- 146 [45] using the antibodies against STAT3 and RPA34 (CSB-PA006734, CUSABio,
- 147 China) and nucleolar protein markers (Fibrillarin, Ab66630, Ab4566, Abcam, UK).
- IF assays for HepG2 cell lines expressing STAT3 shRNA or control shRNA were
 performed using antibodies against RPA34 and Fibrillarin. Cell specimens were
 observed under a confocal fluorescence microscope, and images were captured with a
 60× objective lens (Olympus). The resulting images were analyzed with ImageJ
- 152 software (NIH).
- 153 *RT-qPCR and 5-ethynyl uridine assays*

HeLa and HepG2 cell lines expressing STAT3 shRNA or mCherry-STAT3 and their 154 control cell lines were cultured in 6-well plates using their corresponding culture 155 medium. At 90% confluence, cells were harvested and total RNA was extracted from 156 the cells using the RNA extraction kit (Axygen). The expression of both STAT3 and 157 ribosomal RNA genes was analyzed by RT-qPCR as described previously [44, 45]. 158 For 5-ethynyl uridine (EU) assays, HeLa or HepG2 cells were cultured and labelled 159 with EU for 2 hours; after labeling, cells were fixed with a 4 % formaldehyde solution 160 and EU-labeled cells were detected using the Cell-Light EU Apollo 555 (or 488) 161 Imaging Kit (RiboBio, Guangzhou). Cell samples were observed under a confocal 162 fluorescence microscope (Olympus, Japan), and images were captured with a 163

- 164 20×objective lens. The fluorescence intensity for nucleoli or nucleoplasm area was
- obtained with the Image J software. The relative fluorescence intensity for a nucleolus
- 166 was obtained using the following formula: (the fluorescence intensity of a nucleolus –
- the fluorescence intensity of the equal area of nucleoplasm) \times the rate of Pol I
- 168 products in total rRNA (0.983). The data from EU assays were analyzed by the
- 169 ImageJ and Graphpad Prism 8 software.

170 *Dot blotting*

171 HepG2 cell lines expressing STAT3 shRNA or meCherry-STAT3 and the

cells were harvested and nuclei were purified from the cells. Next, total RNA was 173 extracted from nuclei using an RNA extraction kit (Axygen). One microgram of total 174 RNA was loaded in individual circles on a piece of nylon membrane (5 cm \times 8 cm), 175 and the membrane was dried at 65 °C for 0.5 hour. Probes were prepared in a 40 µL 176 reaction mixture containing 10 U of Klenow enzyme; 25 pmol of biotin-labelled 177 random hexamer primers and 500 ng of template DNA amplified from the introns of 178 45S pre-rRNA. Dot blot hybridization was performed using standard procedures. 179 After hybridization, the membrane was incubated for 1 hour in a 5% skimmed milk-180 PBS solution containing 1 µL of an anti-biotin HRP-linked antibody and was washed 181 twice with 1×PBS and detected with ECL reagent. 182

corresponding control cell lines were cultured in 10 cm dishes. At 90% confluence,

Cell proliferation assays 183

172

Cell proliferation assays for HeLa and HepG2 cell lines expressing STAT3 shRNA 184 or mCherry-STAT3 were performed using different approaches, including cell 185 counting, CCK8, EdU and colony formation. Cell counting, CCK-8 and EdU assays 186 were performed as described previously [46, 47]. For colony formation, cell lines 187 expressing STAT3 shRNA or mCherry-STAT3 and their corresponding control cell 188 lines were diluted and seeded in 6-well plates. After culturing for 10 days, cell 189 colonies were fixed and then stained for 15 min with 0.02% crystal violet. After that, 190 cell samples were washed, air-dried and photographed with a camera. The number of 191 total colonies and the sizes of individual colonies were calculated and analysed 192 statistically. For the analysis of cell proliferation and colony formation under the 193 treatment with DMSO, STAT3-In-3 (500 nM), CX-5461 (50 nM), and both STAT3-194 In-3 (500 nM) and CX-5461 (50 nM), experimental procedures were the same as the 195 assays without drug treatment described above.

196

Animal models for tumor formation 197

Sixteen of five-week-old BALB/c female nude mice were obtained from the Vital 198 River Laboratory Animal Technology Co. (Beijing, China). The nude mice inhabited a 199 room under a sterile condition with controlled temperature, humidity and light. After 200 adapting for one week, mice were randomly distributed into two groups (n=8 for each 201 group). Each mouse was subcutaneously injected using 1×10^7 HepG2 cells expressing 202 STAT3 shRNA or control shRNA. After 7 days, growing tumors were measured with 203 a Vernier calliper every 3 days. Tumor volumes were calculated using the formula: V= 204

 $\frac{\pi}{5}$ × length×width². At the end of the sixth week, mice bearing a tumor were 205

euthanized under the Animal Welfare Guideline, and the tumors within the mice were 206 removed, weighed, and photographed. Tumor samples randomly picked from controls 207

- or treatments were subjected to hematoxylin and eosin (H&E) staining and 208
- immunohistochemistry analysis as described previously [48, 49]. For the tumor 209
- formation assays under the treatment with different drugs, 24 nude mice were 210
- nurtured for 1 week at a sterilized condition. After that, the mice were subcutaneously 211
- injected with 1×10^7 HepG2 cells. Five days later, mice were randomly divided into 4 212
- groups (n=6 for each group), which were injected with different drugs, including 100 213

- 214 μ L 0.9% NaCl, 100 μ L STAT3-In-3 (3 mM), 100 μ L CX-5461 (3.9 mM) and both of 215 STAT3-In-3 (100 μ L, 3 mM) and CX-5461 (100 μ L, 3.9 mM). Drug injection was
- 216 carried out every 2 days until mice were euthanized. Tumor sizes and weight were 217 analyzed as described above. Animal experiments for drug inhibitors were clearly
- analyzed as described above. Animal experiments for drug inhibitors were clearly
 labeled without blinding. Mouse model experiments were approved by the Animal
- and Medical Ethics Committee in the School of Life Science and Health at Wuhan
- 220 University of Science and Technology. All animal experiments were conducted
- according to the Animal Welfare Guidelines (China).
- 222 Messenger RNA-seq analysis

HepG2 cell lines expressing STAT3 shRNA or control shRNA were cultured in 223 10-cm dishes in triplicates. At 85% confluence, cells were harvested and total RNA 224 was extracted with a Qiagen RNeasy kit and sent to Frasergen Gene Information Co. 225 (Wuhan, China) for mRNA-seq analysis. RNA libraries were constructed and loaded 226 on a Novaseq 6000 instrument according to the manufacturer's instructions 227 (Illumina, San Diego, USA). DNA sequencing was performed using a 2×150bp 228 paired-end (PE) configuration and sequence data were obtained by the HiSeq 229 Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina). The raw data 230 231 containing adapter, PCR primers and other fragments less than 20 bases were trimmed with Trimmomatic (v0.30) so that high-quality clean data were achieved. 232 The clean data were aligned to the human reference genome (Hg38) using software 233 Hisat2 (v2.0.1). Differential expression analysis was performed using the DESeq 234 Bioconductor package. GO-TermFinder was used to identify Gene Ontology (GO) 235

- terms that annotate a list of enriched genes where their P-values were less than 0.05.
- 237 Computer codes for the volcano plotting of DEGs and dot plot for pathway
- enrichment analysis were stored in laboratory computer and are available on request.
- The upstream analysis for the RNA-seq data was performed by Frasergen GeneInformation Co.
- 241 Western blot
- HeLa and HepG2 cells including control or treatment cells (knockdown and
 overexpression) were cultured in 6-well plates. At 90% confluence, cells were
- harvested and lysed with 200 μ L of 1xSDS loading buffer (50 mM Tris-HCl, 2% SDS,
- 245 0.1% Bromophenol blue, 10% Glycerol, 100mM DTT). After boiling for 10 min at
- 100° C within a heat block, $10 \,\mu$ L samples were used for Western blot analysis using
- the antibodies against STAT3 (CST#9139, CST, USA), UBF (ab244287, Abcam, UK),
- TBP (SC-421, Santa Cruz Biotech, USA), TAF1A (SC-393600, Santa Cruz Biotech,
- USA), RPA34 (CSB-PA004932LA01HU, CUSABio, China) and RPA49 (CSB-
- 250 PA050039, CUSABio, China).
- 251 Reporter assays and chromatin immunoprecipitation assays
- Reporter assays were performed as described previously [45] using HeLa or HepG2
- cell lines expressing STAT3 shRNA and their control cell lines, where the reporter
- vectors driven by the RPA34 promoter and the β -galactase-expressing vectors were
- co-transfected into these cell lines. For ChIP assays, HepG2 cells or HepG2 cell lines
- stably expressing STAT3 shRNA or control shRNA were cultured in 10-cm dishes,
- fixed with 10 mL 1% formaldehyde-containing PBS solution and harvested for

chromatin immunoprecipitation (ChIP) analysis. ChIP assays were performed using 258 the protocol described previously [44] except that antibodies for ChIP assays were 259 replaced. The DNA from each ChIP assay was eluted with 40 µL ddH₂O after 260 chromatin de-crosslinking and DNA purification, and 1 µL of ChIP DNA sample was 261 used for a qPCR reaction, where 0.5 ng genomic DNA (0.02% input) acted as a 262 positive control in the assay. Relative enrichment was obtained by calculating the 263 percentage for the relative quantity of promoter DNA from 1/40 ChIP DNA samples 264 in that from 0.02% input. 265 Pearson' correlation, Kaplan Meier Plotting and Statistical analysis 266

Pearson correlation analysis between STAT3 and RPA34 expression in normal 267 tissues or clinical cancer samples based on the dataset deposited at The Cancer 268 Genome Atlas (TCGA) was performed using the GEPIA online tool 269 270 (http://gepia2.cancer-pku.cn/#index). Kaplan-Meier Plotting showing the relationship between RPA34 expression levels and survival probability or survival time was 271 performed using the Kaplan-Meier Plotter online tools (www.kmplot.com) and the 272 RNA-seq data of liver hepatocellular carcinomas (LIHC) and kidney renal carcinoma 273 (KIRC) deposited at the TCGA. Violin plots were obtained by Graphpad Prism 8 274

based on the expression data of cancer samples deposited at the TCGA.

The experiments in this study, including RT-qPCR, proliferation assays, ChIP 276 assays and reporter assays, were carried out with the samples of three biological 277 replicates or three independent experiments at least. All data generated in the 278 experiments were used for statistical analysis without exclusion. The means, standard 279 deviations (SD) and histograms for the data of cell proliferation, tumor growth, RT-280 qPCR, luciferase assays, and ChIP assays were calculated with the GraphPad Prism 281 8.0 software. P values were obtained by student's t test or two-way ANOVA wherever 282 it is appropriate. 283

284

285 **Results**

286 STAT3 acts as a positive factor to regulate Pol I-directed transcription

287 It has been shown that cytoskeletal FLNA silencing can stimulate Pol I-directed

transcription [44]. Recently, we performed RNA-seq analysis using FLNA-depleted
cell line (SRA accession number: SRP318361,

290 <u>https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA726417</u>) and found that

FLNA silencing reduced the expression of both STAT3 mRNA and protein (Fig. S1).

In addition, transcription factor STAT3 has been shown to regulate cell proliferation,

which is associated with Pol I product levels. Based on this information, we

hypothesized that STAT3 maybe is required for the regulation of Pol I-directed

transcription. To support this hypothesis, we determined the effect of STAT3

expression change on the synthesis of Pol I products in SaOS2 cells. Unexpectedly,

297 STAT3 siRNA transfection reduced Pol I product expression rather than stimulating

this process in SaOS2 cells (Fig. S2A and B). Consistent results were obtained when

similar assays were performed using HeLa and HepG2 cells (Fig. S2C-F). These

results suggest that STAT3 is required for normal transcription directed by Pol I and 300 possibly plays a positive role in this process. This result is opposite to that observed in 301 Fig. S1, where FLNA knockdown reduced the expression of STAT3 (Fig. S1) and 302 stimulated the synthesis of Pol I products (44). To clarify the role of STAT3 in Pol I-303 dependent transcription, we generated several cell lines (HepG2, HeLa, 293T) stably 304 expressing STAT3 shRNA or control shRNA (Fig. 1A, Fig. S2G and I). However, we 305 failed to get the SaOS2 cell line stably expressing STAT3 shRNA. The reason for this 306 outcome is because SaOS2 cells grew extremely slow and many cells died after 307 STAT3 silencing. Analysis of rRNA expression by RT-qPCR showed that STAT3 308 shRNA stable expression significantly reduced the synthesis of Pol I products (Fig. 309 1B, Fig. S2H and J), indicating that STAT3 expression positively correlates with Pol 310 I-directed transcription. 311 To validate the positive role of STAT3 in Pol I-directed transcription, we prepared 312 several cell lines (HepG2, HeLa, 293T) stably expressing mCherry-STAT3 and 313 analyzed the effect of STAT3 overexpression on rRNA synthesis. Evidently, STAT3 314 overexpression enhanced the synthesis of Pol I products in these cell lines (Fig. 1C 315

and D, Fig. S3A-D). Since 5-ethynyl uridine (EU) can be incorporated into the RNA

- newly synthesized, we next examined the effect of STAT3 expression alteration on
 rRNA synthesis by performing EU assays using HeLa and HepG2 cell lines
- established above. Noticeably, STAT3 silencing reduced nucleolar fluorescence
- intensity (Fig. 1 E and F, Fig. S2K and L). In contrast, STAT3 overexpression
- augmented nucleolar fluorescence intensity when compared to control cell lines (Fig.
 1G and H, Fig. S3E and F). Next, we verified these results using a more direct method
- 323 (Dot blot). The results from Dot blot assays showed that STAT3 silencing reduced the 324 synthesis of pre-rRNA (Fig. 1I and J). Conversely, STAT3 overexpression enhanced
- this process (Fig. 1K and L). Collectively, these results indicate that STAT3 plays a
- positive role in the regulation of Pol I-directed transcription in tumor cells.

327 Both CRISPR dCas9 activation or repression systems and a STAT3 inhibitor

328 *confirmed the positive role of STAT3 in Pol-I directed transcription*

In order to gain further evidence to support that STAT3 functions as a positive 329 factor in Pol I-mediated transcription, we utilized the CRISPR dCas-9 systems to 330 activate or inhibit endogenous STAT3 expression and observed the effect of STAT3 331 activation or inhibition on rRNA synthesis. We show that endogenous STAT3 332 333 inhibition dampened the synthesis of Pol I products (Fig. 2A-C), while endogenous STAT3 activation enhanced the expression of Pol I products (Fig. 2D-F). Previous 334 studies showed that STAT3 has to be phosphorylated before entering a nucleus [2]; 335 STAT3-IN-3 can impede the phosphorylation of STAT3 at its Tyr⁷⁰⁵ and Ser⁷²⁷ sites 336 [31], which is required for the entry of STAT3 into nuclei. Thus, we determined the 337 effect of STAT3-IN-3 on Pol I-dependent transcription in HeLa or HepG2 cells 338 cultured in the medium containing 5 µmol/L of STAT3-IN-3. Interestingly, the 339 presence of STAT3-IN-3 did not affect STAT3 expression but down-regulated STAT3 340 phosphorylation levels and the synthesis of Pol I products in both HeLa and HepG2 341 cells (Fig. 2G-J), suggesting that STAT3 phosphorylation is required for Pol I-directed 342

transcription. STAT3 and Pol I products have an abnormally high expression in a

subset of cancer types [1, 2, 37]. Thus, we next determined whether the expression of
STAT3 and Pol I products in HeLa and HepG2 cells is higher than that in their
corresponding normal cell lines using Western blot and RT-qPCR techniques. As

- expected, HeLa and HepG2 cells showed higher levels of STAT3 and Pol I products
- than their normal cell lines, including HUCEC and HL-7702 cells (Fig 2K and L, Fig.
- 349 S4). These results further confirmed that STAT3 functions as a positive regulator in
- 350 Pol I-dependent transcription in human cancer cells.
- 351 *STAT3 may regulate tumor cell growth in vitro and in vivo by affecting Pol I-directed* 352 *transcription*

Because STAT3 expression change affected Pol I product synthesis, and Pol I 353 product levels correlate closely with cell growth [35, 36]; it is necessary to determine 354 the effect of STAT3 upregulation or downregulation on cell proliferation. To this end, 355 the proliferative activity of several cell lines, including HeLa, HepG2 and 293T cell 356 lines with STAT3 depletion or overexpression, was initially analyzed by cell counting 357 and CCK-8 methods. Apparently, STAT3 silencing reduced cell proliferative activity 358 for these cell lines (Fig. 3A and B, Fig. S5A-D). In contrast, STAT3 overexpression 359 enhanced cell proliferative activity (Fig. 3C and D, Fig S6 A-D). The incorporation of 360 5-ethynyl-2'-deoxyuridine (EdU) into genomic DNA is widely utilized to assess the 361 activity of cell proliferation. EdU assays showed that STAT3 downregulation reduced 362 the rate of EdU positive cells, while STAT3 overexpression augmented the rate of 363 EdU-labelled cells (Fig. 3E-H, Fig. S5E and F and Fig. S6E and F). Consistent results 364 were obtained using HepG2 cells with endogenous STAT3 inhibition or activation by 365 a dCas-9 system (Fig. S5G and H, Fig. S6 G and H). To further understand how 366 STAT3 expression alteration affects cancer cell growth, we performed colony 367 formation assays using HepG2 cell lines with STAT3 depletion or overexpression. 368 Analysis of the colony number and size revealed that STAT3 downregulation reduced 369 the number of total colonies and the sizes of individual colonies (Fig. S7A-C), while 370 STAT3 overexpression enhanced them (Fig. S7D-F). These data suggest that STAT3 371 promotes cell growth by reducing cell death and increasing proliferative activity. We 372 showed that STAT3 can concurrently promote cell proliferation and activate Pol I 373 product synthesis. Therefore, we determined whether the increase of Pol I products 374 induced by STAT3 overexpression contributes to the promotion of cell proliferation. 375 Cell proliferation assays were performed in the presence and absence of CX-5461 (a 376 Pol I transcription inhibitor) using HeLa and HepG2 cell lines stably expressing 377 mCherry-STAT3. Strikingly, the presence of CX-5461 inhibited the enhancement of 378 cell proliferation and the activation of Pol I-directed transcription induced by STAT3 379 overexpression (Fig. 3I and J, Fig. S8). These data indicate that the increase of Pol I 380 products contributes to the promotion of cell proliferation induced by STAT3 381 overexpression although the contribution of other pathways cannot be excluded. 382 To understand if alteration of Pol I products by STAT3 silencing affects cell growth 383

in vivo, we performed tumor formation assays using nude mice (n=8 for each group) subcutaneously injected with 1×10^7 HepG2 cells stably expressing STAT3 shRNA or control shRNA. Analysis of tumor sizes and weights showed that the tumors with STAT3 silencing showed the reduction in sizes and weights compared to those without STAT3 silencing (Fig. 4A-C). Further assays revealed that tumor tissues
formed in nude mice possessed the morphology of liver cancer tissues (Fig. 4D) and
retained the original features of HepG2 cells before injection (Fig. 4E-G). These data
indicate that STAT3 silencing can inhibit tumour growth *in vivo*, which is associated
with the reduction of Pol I products.

393 The presence of both STAT3-IN-3 and CX-5461 shows additive effect on the

inhibition of tumour cell growth in vitro and in vivo

STAT3-IN-3 has been reported to suppress breast cancer cell growth [31]. Thus, 395 we next evaluated the effect of STAT3-IN-3 on the proliferative activity of HeLa and 396 HepG2 cells. Notably, the presence of STAT3-IN-3 repressed the proliferative activity 397 of these two cell types (Fig. 5A and B, Fig. S9). Since the presence of CX-5461 (a Pol 398 I-specific inhibitor) suppresses the proliferation activity of HeLa and HepG2 cells 399 400 (Fig. 3I and J, Fig. S8), we next investigated whether the combination of STAT3-IN-3 and CX-5461 can cause greater inhibition to cell proliferation than the application of a 401 single drug. Interestingly, the treatments with both CX-5461 and STAT3-IN-3 showed 402 greater inhibition to HepG2 cell proliferation than the treatments with CX-5461 or 403 STAT3-IN-3 (Fig. 5C and D). Whether the combination of STAT3-IN-3 and other Pol 404 405 I inhibitors such as actinomycin D and BMH-21 can cause the same effect as observed above is unclear. Thus, HepG2 cells were treated with STAT3-IN-3 and 406 actinomycin D (or BMH-21); and the results confirmed that the treatments with two 407 drugs still showed additive effect on cell growth compared to the treatments with one 408 drug (Fig. S10A-D). Next, we determined how these drugs inhibit cell growth by 409 initially analyzing expression of a cell proliferation marker (CDKN1B) and apoptosis 410 related factors (Caspas-3 and cleaved Caspase-3) in HepG2 cells by Western blot. The 411 treatments with both STAT3-IN-3 and CX-5461 increased expression of CDKN1B 412 and cleaved caspase-3 and reduced expression of Caspase-3; however, the treatments 413 with a single drug had little effect on expression of these proteins (Fig. S10E and F), 414 suggesting that these two drugs may inhibit cell growth by affecting cell proliferation 415 and apoptosis. To verify this result, we performed colony formation assays; and the 416 results showed that all treatments with drugs reduced the number of total colonies and 417 the sizes of individual colonies compared to the DMSO treatment, indicating that both 418 inhibitors can induce cell death and inhibit cell proliferation. Furthermore, the 419 treatments with both CX-5461 and STAT3-IN-3 showed greater inhibition to the 420 421 number and sizes of colonies than the treatments with a single drug (Fig. 5E-G), indicating that the application of two drugs has additive effect on the inhibition of 422 colony formation. 423

To determine whether these results can be reproduced in vivo, we injected HepG2 424 cells into nude mice (n=6 for each group) to allow them form tumors for 5 days. The 425 mice bearing a tumor were treated with different combinations of drugs. Analysis of 426 tumor sizes revealed that the average size of the tumors from the mice treated with 427 drugs was significantly smaller than that from the mice treated with 0.9% NaCl. 428 Furthermore, drug treatments did not affect the weights of mice significantly (Fig. 5H 429 and I). Strikingly, the treatments with both of CX-5461 and STAT3-IN-3 exhibited 430 greater inhibition to tumor volumes and weights compared to the treatments with CX-431

5461 or STAT3-IN-3 only (Fig. 5H, J and K). Collectively, these data indicate that the
application of both CX-5461 and STAT3-IN-3 has additive effect on the suppression
of HepG2 cell growth *in vitro* and *in vivo* compared to the application of CX-5461 or
STAT3-IN-3.

Messenger RNA-seq revealed the regulation of RPA34 expression by STAT3 436 To understand how STAT3 regulates Pol I-directed transcription, we first 437 determined whether STAT3 can be localized to the nucleoli of human cells. 438 Immunofluorescence (IF) assays were performed using HeLa and HepG2 cells and 439 the antibodies against STAT3 or Fibrillarin (a nucleolar protein marker). 440 Unexpectedly, STAT3 couldn't be observed in the nucleoli of these cells (Fig. S11), 441 suggesting that STAT3 indirectly regulates Pol I-mediated transcription. To gain a clue 442 about how STAT3 modulates Pol I-directed transcription, we performed RNA-seq 443 444 analysis using the total RNA extracted from HepG2 cell lines stably expressing STAT3 shRNA or control shRNA. RNA-seq analysis showed that STAT3 silencing 445 caused expression downregulation of 1223 genes and expression upregulation of 931 446 genes (Fig. 6A). Analysis of gene ontology (GO) and pathways revealed that 447 significant differential expression genes (DEGs) induced by STAT3 silencing in 448 449 HepG2 cells contain ribosome-related GO terms or pathways (Fig. S12A and B), indicating that STAT3 expression is associated with ribosome pathway. Indeed, Pol I 450 product alteration has been shown to affect ribosome biogenesis [35, 36]. 451 Unexpectedly, among significant DEGs (log₂ fold change>0.6), the genes encoding 452 any of the Pol I transcription machinery factors couldn't be found. Next, we examined 453 all expression dataset by removing the threshold of significant difference. 454 Consequently, the expression of three genes encoding Pol I machinery factors such as 455 RPA12, RPA34 and TAF1C showed reasonable reduction after STAT3 silencing (Fig. 456 6B). RT-qPCR confirmed that RPA34 mRNA expression was affected by both STAT3 457 silencing and overexpression in both HeLa and HepG2 cells, whereas alteration of 458 RPA12 and TAF1C expression showed inconsistency between HeLa and HepG2 cell 459 lines or between STAT3 depletion and overexpression (Fig. 6C-F). Western blotting 460 confirmed that STAT3 silencing reduced RPA34 protein expression in both HepG2 461 and HeLa cells, whereas TAF1C expression was not affected by STAT3 knockdown in 462 both cell types. Unexpectedly, RPA12 expression was affected by STAT3 silencing in 463 HepG2 cells but not in HeLa cells (Fig. 6G and H). Since RPA34 is usually located in 464 the nucleoli of human cells, we next examined whether alteration of STAT3 465 expression affects RPA34 levels in the nucleoli by performing immunofluorescence 466 (IF) staining. IF data showed that STAT3 silencing reduced the RPA34 levels in the 467 nucleoli of HepG2 cells compared to the control cell line (Fig. S13). Taken together, 468 these results indicate that STAT3 can positively regulate RPA34 expression at both 469 RNA and protein levels in HepG2 and HeLa cells. 470 Cancer patients with RPA34 abnormal high expression lead to low survival 471 probability 472 473 The results obtained above (Fig. 6) suggest a positive regulatory relationship

between STAT3 and RPA34. To further confirm this observation, we performed the analysis of Pearson correlation between STAT3 and RPA34 based on the RNA-seq

data of cancer samples deposited at The Cancer Genome Atlas (TCGA). Interestingly, 476 positive correlation between STAT3 and RPA34 expression was observed in several 477 cancer types, including liver hepatocellular carcinoma (LIHC, R=0.3), kidney renal 478 clear cell carcinoma (KIRC, R=0.5), kidney renal papillary cell carcinoma (KIRP, 479 R=0.65), thymoma (THYM, R=0.77), diffuse large B-cell lymphoma (DLBC, 480 R=0.69) and thyroid carcinoma (THCA, R=0.63) (Fig. 7A-C, Fig. S14). Further, 481 strong positive correlation (R=0.79) between STAT3 and RPA34 expression was also 482 observed in normal tissues when Pearson correlation analysis was performed using 483 the RNA-seq data of liver, cervix and kidney tissues deposited at the TCGA (Fig. 7D). 484 Next, we analyzed the expression difference of RPA34 between cancer cells and 485 normal cells by Western blot. Clearly, both HeLa and HepG2 cells have higher RPA34 486 expression than their normal cell lines, HUCEC and HL-7702, respectively (Fig. 487 S15A and B). Interestingly, the presence of STAT3-IN-3 dampened RPA34 expression 488 in both HeLa and HepG2 cells (Fig.S15C and D). We next determined whether the 489 expression difference of RPA34 between cancer and normal tissues is similar to that 490 between tumor and normal cell lines. Thus, RPA34 expression was analyzed based on 491 the RNA-seq data in the TCGA database, and the results were presented in Fig 7E and 492 493 F. Apparently, both liver hepatocellular carcinomas (LIHC) and kidney renal carcinomas (KIRC) showed higher RPA34 expression than their normal tissues. We 494 then addressed whether high levels of RPA34 expression can affect cancer patient 495 survival rate. To this end, we performed Kaplan-Meier plotting using the RNA-seq 496 dataset of liver hepatocellular carcinomas (LIHC) and kidney renal carcinoma (KIRC) 497 obtained from the TCGA database. We showed that the patients with RPA34 high 498 expression levels in liver hepatocellular carcinomas (LIHC) or kidney renal 499 carcinomas (KIRC) exhibited lower survival probability and shorter survival time 500 when compared to the patients with low RPA34 expression levels. Taken together, 501 cancer patients with high levels of RPA34 expression may lead to low survival rate, 502 suggesting that RPA34 may act as a biomarker of poor prognosis in a subset of 503 504 cancers.

505 *STAT3 modulates the recruitment of the Pol I transcription machinery components to* 506 *the rDNA promoter by controlling RPA34 expression*

507 Apart from RPA34, whether alteration of STAT3 expression affects the expression 508 of other factors related to Pol I transcription at the protein level is unclear. Thus, we 509 analyzed the expression of a few factors related to Pol I transcription apparatus by 510 Western blot using cell lines with STAT3 silencing or overexpression.

511 Immunoblotting results showed that both STAT3 upregulation and downregulation

- affected RPA34 expression in HeLa and HepG2 cells. However, the expression of
- 513 UBF, TAF1A, and RPA49 was variable between two cell types or between STAT3 514 knockdown and overexpression samples (Fig. 8A-D, Fig. S16). Since STAT3
- 515 positively regulates the synthesis of Pol I products, we determined whether STAT3
- 516 binds to the rDNA promoter by performing ChIP assays. ChIP qPCR data showed
- 517 that STAT3 does not bind to the rDNA promoter (Fig. 8E). This result is consistent
- 518 with that obtained in IF assays (Fig. S11). We next investigated whether alteration of
- 519 STAT3 expression affects the assembly of the Pol I transcription machinery factors

at the rDNA promoter by performing ChIP assays using HepG2 cells. We showed 520 that STAT3 silencing reduced the occupancies of the Pol I transcription machinery 521 factors at the rDNA promoter, while STAT3 overexpression enhanced the 522 occupancies of these factors at the promoter (Fig. 8F and G), suggesting that STAT3 523 can modulate the recruitment of the Pol I transcription machinery factors to the 524 rDNA promoter by affecting RPA34 expression. Next, we addressed whether 525 alteration of STAT3 expression can affect the rDNA promoter (rDNAP) activity. To 526 achieve this goal, we amplified the rDNA promoter along with the DNA fragment 527 encoding about 300 nt 45S pre-rRNA immediately downstream of the promoter, the 528 resulting DNA was inserted into the pGL3-basic. The promoter-driven reporter 529 vectors were transfected into HeLa and HepG2 cell lines. RT-qPCR was used to 530 detect the expression of a 'reporter' gene using the primers as indicated in Fig. 8H. 531 The results showed that STAT3 knockdown inhibited the rDNAP activity; whereas 532 STAT3 overexpression activated the rDNAP activity in both of cell types (Fig. 8I-L). 533 Collectively, these data indicate that STAT3 can modulate the recruitment of 534 components of the Pol I transcription machinery to the rDNA promoter by 535 controlling RPA34 expression, which consequently affects the transcription activity 536 of the rDNA promoter. 537

538 STAT3 regulates Rpa34 gene transcription by binding to the Rpa34 promoter
539 To determine whether RPA34 is required for the regulation of Pol I transcription

- mediated by STAT3, we performed rescue experiments by expressing mCherry-540 RPA34 in HepG2 and HeLa cell lines with STAT3 depletion. The results from the 541 rescue experiments showed that mCherry-RPA34 expression reversed the inhibition of 542 Pol I-directed transcription induced by the STAT3 silencing (Fig. 9A and B, Fig. S17 543 A and B) and alleviate the repression of HepG2 cell growth caused by STAT3 544 silencing (Fig. 9C and D, Fig. S17 C and D), indicating that RPA34 participates in the 545 regulation of Pol I-directed transcription mediated by STAT3. We then determined 546 whether RPA34 expression alteration affects the synthesis of Pol I products by in 547 HepG2 and HeLa cells using a lentiviral expression system. We showed that RPA34 548 silencing reduced the synthesis of Pol I products (Fig. 9E and F, Fig. S17 E and F). In 549 contrast, RPA34 overexpression increased Pol I product expression (Fig. 9G and H, 550 Fig. S17 G and H), indicating RPA34 positively regulates the synthesis of Pol I 551 products. To understand how STAT3 regulates RPA34 expression, we searched for the 552 STAT3-binding motif in the Rpa34 gene promoter. Surprisingly, the Rpa34 promoter 553 contains two putative STAT3 consensus sequences upstream of the transcription start 554 site (Fig. 9I). ChIP assays confirmed that STAT3 can bind to the *Rpa34* promoter (Fig. 555 9J). Next, the *Rpa34* promoter was inserted into the pGL3-basic reporter vector and 556 the Rpa34 promoter activity was examined by performing luciferase assays. We 557 showed that STAT3 silencing reduced the *Rpa34* promoter activity, while STAT3 558 overexpression enhanced its activity (Fig. 9K and L, Fig. S17I and J). Mutations of 559 STAT3 binding sites blunted the activity of the Rpa34 promoter (Fig. 9M and N), 560 indicating that STAT3 controls *Rpa34* gene expression at the transcription step. To 561 understand how STAT3 regulates Rpa34 gene transcription, we performed ChIP 562
- assays using HepG2 cell lines expressing STAT3 shRNA or control shRNA. ChIP-

qPCR showed that STAT3 silencing inhibited the assembly of the RNA polymerase II
transcription machinery factors at the *Rpa34* promoter (Fig. 9O). These data suggest
that STAT3 regulates *Rpa34* gene transcription by affecting the recruitment of Pol II
transcription machinery factors to the *Rpa34* promoter.

Based on the data obtained in this study, we proposed a model by which STAT3 568 regulates Pol I-directed transcription. Specifically, after phosphorylation, STAT3 569 enters nuclei and directly binds to the Rpa34 promoter to modulate Rpa34 gene 570 transcription. After translation in cytoplasm, RPA34 enters the nucleoli of human cells 571 and binds to the rDNA promoter along with other factors of the Pol I transcription 572 machinery. Consequently, STAT3 modulates Pol I-directed transcription by 573 controlling RPA34 expression and the assembly of the Pol I transcription machinery 574 575 at the rDNA promoter (Fig. 10).

576 **Discussion**

Previous studies showed that STAT3 can be activated by canonical signaling 577 pathways. Upon activation, STAT3 is phosphorylated and forms a homodimer to 578 enter the nucleus, where phosphorylated STAT3 regulates the transcription of target 579 genes directed by RNA polymerase II [1, 2]. In this study, however, we found that 580 STAT3 can positively regulate 45S ribosomal RNA expression. Thus, we identified a 581 novel role of STAT3 in transcriptional regulation in this work. This finding seems 582 contradiction with the initial observation in FLNA-depleted SaOS2 cells, where 583 FLNA silencing reduced STAT3 expression (Fig. S1) but increased expression of Pol 584 I products [44]. This discrepancy may be because thousands of differential 585 expression genes were downregulation and upregulation in FLNA-depleted SaOS2 586 cells [50], and STAT3 might not play a key role in this situation; instead, FLNA acts 587 as a key regulator in Pol I-directed transcription and regulates it by a sequestration 588 mode [44]. In recent years, many novel factors, including non-coding RNA and 589 proteins, have been shown to regulate cancer development by affecting STAT3 590 signaling [3, 25, 51, 52]. Thus, the function of STAT3 identified in this study extends 591 the understanding of the regulatory mechanism of gene transcription and cancer 592 development mediated by STAT3. We showed that STAT3 can activate RPA34 593 expression but not expression of Pol I general transcription factors (Fig. 6, Fig. 8), 594 and STAT3 enhances the recruitment of the Pol I transcription machinery to the 595 rDNA promoter by increasing RPA34 expression (Fig. 8). Furthermore, STAT3 596 activates Rpa34 gene transcription by binding to the Rpa34 promoter, and RPA34 597 silencing affected the synthesis of Pol I products [Fig. 9], indicating that STAT3 598 regulates Pol I-dependent transcription by controlling RPA34 expression, This result 599 is distinct from the previous findings in which the oncogenic factor MYC regulates 600 Pol I-dependent transcription by interacting with the ribosomal DNA promoter rather 601 than Pol I subunit [39, 53]. This study provides a novel mechanism by which the 602 oncogenic factor STAT3 modulate Pol I-dependent transcription 603 STAT3 has become an appealing target for anti-cancer therapy due to its activating 604 role in cancer development for a subset of cancers [1, 3]. In this work, we found that 605

506 STAT3 has higher expression in HeLa and HepG2 cells than it does in normal cells,

and STAT3 promotes proliferation activity for these cell types. Additionally, abnormal 607 high expression of its downstream factor RPA34 in a subset of cancers was observed, 608 and cancer patients with high expression of RPA34 have lower survival rate and 609 shorter survival time compared those with low expression of RPA34 (Fig 7). These 610 data suggest that STAT3 may modulate cancer development by influencing the 611 expression of its downstream factor RPA34, and RPA34 can act as a biomarker of 612 poor prognosis in subset of cancer types. Intriguingly, the presence of STAT3-IN-3 613 can severely inhibit cell proliferation and induce cell death (Figs. 2, 3 and 5; Figs. S4-614 8, Fig. S10). Additionally, the Pol I-specific inhibitor CX5461 represses proliferation 615 activity for these cell types by inhibiting the increase of Pol I products induced by 616 STAT3 overexpression (Fig. 3I and J). These results suggest that tumor cell growth 617 can be concurrently inhibited by STAT3-IN-3 and CX-5461. Indeed, the tumor cells 618 619 treated with both STAT3-IN-3 and CX-5461 (or BMH-21/Actinomycin D) led to additive effect on cancer cell deaths or cell growth suppression in vitro and in vivo 620 when compared to the cells were treated with either of the inhibitors (Fig. 5, Fig. 621 S10A-D). Currently, multiple drugs are often used for anti-cancer research as well as 622 cancer therapy in the clinic [54, 55]. Thus, the result of inhibitor assays has profound 623 medical significance because STAT3-IN-3 and Pol I transcription inhibitors would act 624 as combined drugs in cancer therapy in the future. 625

626 **Conclusions**

In this study, we identified a positive role of STAT3 in Pol I-directed transcription 627 in human tumor cells. STAT3 positively regulates cancer cell survival and growth in 628 vitro and in vivo. The presence of both of STAT3 and Pol I transcription inhibitors has 629 a greater inhibitory effect on tumor cell growth than the application of either 630 inhibitors. STAT3 activates RPA34 transcription by binding to the Rpa34 promoter, 631 which consequently controls Pol I-directed transcription by affecting the Pol I 632 transcription machinery assembly at the rDNA promoter. RPA34 has s abnormal high 633 expression in subset of cancer types, and cancer patients with RPA34 high expression 634 exhibits poor prognosis. Our findings provide a novel insight into Pol I-directed 635 transcription and a promising prospect that STAT3 and Pol I-specific inhibitors may 636 act as combined drugs in cancer therapy. 637

638 Abbreviations

639 STAT3, signal transducer and activator of transcription 3; Pol I, RNA Polymerase I;

Pol II, RNA polymerase II; RPA34, DNA-directed RNA Polymerase I subunit RPA34;

EU, 5-ethynyl uridine; EdU, 5-ethynyl-2'-deoxyuridine, p-STAT3, phosphorylated

642 STAT3; mCherry-STAT3, mCherry-tagged STAT3 fusion protein; GAPDH,

643 glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA box-binding protein;

644 RPA40, DNA-directed RNA polymerase I subunit RPA40; UBF, upstream binding

645 factor; TAF1A, TBP-associated factor 1A; RPA49, DNA-directed RNA polymerase I

subunit RPA49; FLNA, Filamin A; STAT3-IN-3, STAT3 inhibitor 3; CX-5461, 2-(4-

647 methyl-1,4-diazepan-1-yl)-N-[(5-methylpyrazin-2-yl)methyl]-5-oxo-

648 [1,3]benzothiazolo[3,2-a][1,8]naphthyridine-6-carboxamide (Pol I-mediated rRNA

- 649 synthesis inhibitor), BMH-21 (Pol I elongation inhibitor), N-[2-
- 650 (dimethylamino)ethyl]-12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-
- 651 carboxamide.
- 652 Additional Information
- 653 Acknowledgements
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- 655 **Authors' contributions**:
- 656 CZ performed most work in Figures 1-8 and in the supplementary file; JW validated
- data and mentored researchers; YS and DY performed cell culture and cell line
- screening; YP and BG performed gene cloning; HD prepared CRPSR dCas9
- expression system, designed experiments and performed a part of supervision work;
- 660 XY performed RPA34 shRNA cloning; S Zhang and S Zhao performed most of the
- supervision work, processed data, and edited the manuscript; WD acquired the fund of
- this work, designed experiments, processed data, and wrote the manuscript.

663 Ethics approval and consent to participate

- Animal experiments were approved by the Animal and Medical Ethics Committee
- of School of Life Science and Health at Wuhan University of Science and
- 666 Technology. The animal protocols abided by the Animal Welfare Guidelines (China).
- 667 **Consent for publication**
- 668 Not applicable.
- 669 Data availability
- The RNA-seq data about SaOS2 cell FLNA silencing were deposited in the NCBI
- 671 repository (SRA: SRP318361,
- 672 <u>https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA726417</u>). The RNA-seq data
- about HepG2 cell STAT3 silencing were deposited in the NCBI Gene ExpressionOmnibus (GSE201548,
- 675 <u>https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201548</u>). The RNA-seq
- data used for Pearson correlation analysis, Kaplan Meier plotting and Violin plotting
- 677 were obtained from the TCGA database (<u>www.tcgaportal.org</u>).
- 678 **Competing interests**
- The authors declare no potential conflicts of interest.
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853 Figure legends



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855 Figure 1. Alteration of STAT3 expression affected Pol I-directed transcription. A

and **B**) STAT3 shRNA stable expression reduced Pol I-directed transcription in

HepG2 cell lines. STAT3 expression was detected by Western blot (A), while Pol I 857 products were detected by RT-qPCR (B). C and D) mCherry-STAT3 stable expression 858 enhanced Pol I-directed transcription in HepG2 cells. mCherry-STAT3 (C) and Pol I 859 products (D) were analyzed by Western blot and RT-qPCR, respectively. E and F) EU 860 assay results for HepG2 cells with STAT3 silencing. EU assays were performed using 861 the cell lines as indicated, and images were captured under a confocal fluorescence 862 microscope (E). The scale bars in the images represent 5 µm. Relative fluorescence 863 intensity for nucleoli in the images was calculated using ImageJ software (F). G and 864 H) EU assay results for HepG2 cells with STAT3 overexpression. EU assays were 865 performed using the cell lines as indicated. Images (G) and relative fluorescence 866 intensity for nucleoli (H) were obtained as described in E and F. I and J) Dot blot 867 results for the expression of pre-RNA in HepG2 cell lines expressing STAT3 shRNA 868 or control shRNA. J represents the quantified result for the dot blots obtained in I. K 869 and L) Dot blot results for the expression of pre-RNA in a HepG2 cell line expressing 870 mCherry-STAT3 (mCH-STAT3) or its control cell line. K represents the quantified 871 result for the dot blots obtained in L. Each column in histograms represents the 872 mean±SD of three independent experiments (n=3). *, P<0.05; **, P<0.01. P values 873

874 were obtained by Student's *t* test, performed with control and treatment groups.

Fig.2



875

Figure 2. The positive role of STAT3 in Pol I-directed transcription was

confirmed by a dCas9 activation and repression system as well as a STAT3 877 inhibitor. A) A scheme showing the guide RNA (gRNA) and dCas9-KRAB that 878 target the STAT3 promoter region. B) STAT3 immunoblotting analysis in HepG2 cells 879 transfected with the vectors expressing both STAT3 gRNAs and dCas9-KRAB or 880 dCas9-KRAB only. C) Analysis of Pol I products by RT-qPCR using the cells 881 obtained in B. D) A scheme showing guide RNA (gRNA) and dCas9-VP48 that target 882 the STAT3 promoter region. E) STAT3 expression analysis in HepG2 cells transfected 883 with the vectors expressing both STAT3 gRNAs and dCas9-VP48 or dCas9-VP48 884

- only by Western blot. F) Detection of Pol I products by RT-qPCR using the cells
- obtained in E. G) Analysis of STAT3 expression and phosphorylation by Western blot
- using HepG2 cells in the presence or absence of STAT3-IN-3 (2 μ M). H) The
- presence of STAT3 inhibitor reduced Pol I product expression in HepG2 cells. I)
- 889 Analysis of STAT3 expression and phosphorylation by Western blot using HeLa cells
- cultured in the medium with or without STAT3-IN-3 (2 μ M). J) The presence of
- 891 STAT3 inhibitor inhibited Pol I product expression in HeLa cells. **K**) Analysis of
- 892 STAT3 expression and phosphorylation by Western blot using HepG2 cells and its
- primary (normal) cells (HL-7702). L) Comparison of Pol I product levels between
- HL-7702 and HepG2 cells. Pol I products in H, J and L were detected by RT-qPCR.
- Each column in histograms represents the mean±SD of three independent experiments
- 896 (n=3). *, P < 0.05; **, P < 0.01. P values were obtained by Student's t test,
- 897 performed with control and treatment groups.

Fig.3



Figure 3. STAT3 promotes cancer cell proliferation. A and B) STAT3 knockdown
reduced HepG2 cell proliferative activity. HepG2 cell lines expressing STAT3 shRNA
or control shRNA were used to analyze proliferative activity by cell counting (A) and
CCK-8 (B) methods. C and D) STAT3 overexpression enhanced HepG2 cell

- 903 proliferative activity. Proliferation assays were performed by cell counting (C) and
- 904 CCK-8 (D) methods using a HepG2 cell line stably expressing mCherry-STAT3 and

its control cell line. E) Representative images for EdU assays using HepG2 cell lines 905 stably expressing STAT3 shRNA or control shRNA. EdU specimens were observed 906 and imaged under a fluorescence microscope, the scale bars represent 50 µm. F) 907 Statistical analysis of the EdU-labeled cells based on the EdU assays described in (E). 908 The rate of EdU positive cells represents the number of EdU-labeled cells in the 909 number of total cells counted in the images. G) Representative images for EdU assays 910 using a HepG2 cell line expressing mCherry-STAT3 and its control cell line. Scale 911 bars in all images represents 50 µ m. H) Statistical analysis of the EdU-labeled cells 912 based on the EdU assays described in G. The rate of the EdU positive cells was 913 obtained as for F. I) CX-5461 inhibited the enhancement of HepG2 cell proliferation 914 caused by STAT3 overexpression. Cell proliferation assays were performed using a 915 HepG2 cell line stably expressing mCherry-STAT3 and its control cell line, which 916 were cultured with or without CX-5461 (5 μ M). J) CX-5461 inhibited the activation 917 of Pol I-directed transcription caused by STAT3 overexpression. HepG2 cell lines 918 treated with an inhibitor for 2 days were harvested for the analysis of Pol I products. 919 Each point/column in graphs represents the mean±SD of three independent 920 experiments (n=3). *, P<0.05; **, P<0.01. P values in A-D and I were obtained by 921 two-way ANOVA, P values in F, H and J were obtained by Student's t test, performed 922

923 with control and treatment groups.

Fig.4



Figure 4. STAT3 downregulation inhibited tumor cell growth in vivo. A) STAT3 925 knockdown reduced the sizes of tumors formed in nude mice. HepG2 cell lines 926 expressing STAT3 shRNA or control RNA were subcutaneously injected into the back 927 of nude mice (n=8). One week post-injection, tumors formed in mice were measured 928 every 3 days until the mice were euthanized; the resulting data were subjected to 929 statistical analysis. B) A image showing the effect of STAT3 downregulation on the 930 tumor sizes formed in nude mice. C) STAT3 downregulation significantly reduced 931 tumor weight. The tumors obtained in B were weighed and subjected to statistical 932 analysis. D) Comparison of hematoxylin and eosin staining between tumor tissue and 933 the normal tissues as indicated. The tissues from the tumor, liver or skin were fixed, 934 sectioned and used for Hematoxylin and eosin staining. The scale bars in images 935 represent 100 µm. E) Immunohistochemistry images showing the difference of 936

- 937 STAT3 expression between the tissues expressing STAT3 shRNA and control shRNA.
- 938 F) Immunoblotting analysis of STAT3 expression in the tissues expressing STAT3
- 939 shRNA or control shRNA. G) Analysis of Pol I products by RT-qPCR in the tissues
- 940 expressing STAT3 shRNA or control shRNA. Each point/column in histograms
- represents the mean \pm SD of 8 biological replicates (n=8). *, P<0.05; **, P<0.01. P
- values obtained by two-way ANOVA (A) or Student's *t* test (C and G).

Figure 5. STAT3-IN-3 and CX-5461 showed an additive effect on the inhibition of
tumor cell growth *in vitro* and *in vivo*. A and B) The presence of STAT3-IN-3

- 945 tumor cell growth *in vitro* and *in vivo*. A and B) The presence of STAT3-IN-3
 946 decreased HepG2 cell proliferation *in vitro*. Cell proliferation assays were performed
- 947 using cell counting (A) and CCK-8 (B) methods. C and D) Effect of STAT3-IN-3 and

CX-5461 on tumor cell growth in vitro. HepG2 cell proliferation was measured with 948 cell counting (C) and CCK-8 (D) methods. E-G) Effect of STAT3-IN-3 and CX-5461 949 on the colony formation of HepG2 cells. Colony formation assays were performed 950 using HepG2 cells treated with drugs as indicated. After 10 days, cells were subjected 951 to fixation, staining and imaging (E); the number (F) and sizes (G) of colonies in the 952 images were analysed statistically. H) A plot showing the volumes of tumors 953 measured during tumor formation in the mice treated with different drugs. I) A graph 954 showing the weights of the mice treated with different drugs after tumors were 955 removed. J) An image showing the tumors obtained from the mice treated with 956 different drugs. K) Statistical analysis of the tumors obtained from the mice treated 957 with different drugs. Each point/column in histograms represents the mean±SD of 958 three independent experiments (A-D) or 6 biological replicates (H, I and K). *, 959 P<0.05; **, P<0.01. P values were obtained by two-way ANOVA (A-D and H) or 960 Student's *t* test (F, G and K). 961

Figure 6. STAT3 expression positively correlates with RPA34 expression at both 963 RNA and protein levels. A) A volcano plot showing the number of upregulated and 964 downregulated differential expression genes (DEGs) based on the mRNA-seq data of 965 HepG2 cell lines expressing STAT3 shRNA or control shRNA. The significant DEGs 966 were defined by the differential expression that is over 1.5-fold between the reads of 967 STAT3 shRNA and control shRNA. B) Analysis of mRNA expression (FPKM) for the 968 genes encoding Pol I transcription machinery factors. The expression of genes 969 presented in the graph was analysed from the mRNA dataset detected by mRNA-seq, 970 where differential expression genes were underscored by red lines. C and D) RT-971 qPCR was used to verify the effect of STAT3 silencing on mRNA expression of 972 RPA12, RPA34 and TAF1C in HepG2 (C) and HeLa (D) cells. E and F) RT-qPCR 973 was used to analyze the effect of STAT3 overexpression on the expression of RPA12, 974 RPA34 and TAF1C in HepG2 (E) and HeLa (F) cells. G and H) Western blot results 975 showing the effect of STAT3 knockdown on the expression of RPA12, RPA34 and 976 TAF1C in HepG2 (G) and HeLa (H) cells. Each point/column in digital graphs 977 represents the mean±SD of three biological replicates (A, B) or three independent 978 experiments (C-F). *, P<0.05; **, P<0.01. P values were obtained by Student's t test, 979 performed with control and treatment groups. 980

Fig. 7

981

982 Figure 7. The relationship between RPA34 expression levels and survival

probability and survival time in cancers. A-C) Pearson correlation analysis based 983 on the TCGA dataset of clinical cancer samples, including liver hepatocellular 984 carcinoma (LIHC), kidney renal clear cell carcinoma (KIRC) and kidney renal 985 papillary cell carcinoma (KIRP), using the GEPIA online tool (http://gepia2.cancer-986 pku.cn/#index). D) Pearson correlation analysis based on the TCGA dataset of normal 987 tissues, including liver, cervix and kidney tissues using the GEPIA online tool 988 (http://gepia2.cancer-pku.cn/#index). E and F) Violin plots showing RPA34 989 expression differentiation between normal tissues and liver hepatocellular carcinomas 990 (LIHC) or Kidney renal carcinomas (KIRC). G and H) Kaplan-Meier Plots showing 991 the relationship between RPA34 expression levels and survival probability and 992

993 survival time based on the TCGA dataset of liver hepatocellular carcinomas (LIHC) 994 and Kidney renal carcinoma (KIRC). Low: RPA34 low expression, High: RPA34 high 995 expression. High and Low expression levels were determined by the expression 996 median of cancer samples. ***, P < 0.001, P values were obtained by Student's *t* test 997 performed by normal and cancer samples (E, F).

Figure 8. STAT3 regulates the assembly of components of the Pol I transcription

machinery at the rDNA promoter by affecting RPA34 expression. A and B) Effect 1000 of STAT3 knockdown on the expression of the Pol I-related factors was analyzed by 1001 Western blot using HepG2 cells expressing STAT3 shRNA or control shRNA and 1002 antibodies as indicated (A). The quantified result of Western blots (n=3) is shown in 1003 B. C and D) Effect of STAT3 overexpression on the expression of Pol I-related factors 1004 1005 by Western blot using HepG2 cells with STAT3 overexpression. D represents the quantified result of Western blots in C (n=3). E) STAT3 does not bind to the rDNA 1006 promoter. HepG2 cells were used for ChIP assays using an anti-STAT3 antibody, 1007 where the DNA recovered from the chromatin immunoprecipitation was analyzed by 1008 qPCR. Relative enrichment was obtained by comparing the relative quantity of target 1009 DNA in 1 µL of ChIP samples to that from 0.025% input. F) STAT3 downregulation 1010 reduced the occupancies of the Pol I transcription machinery factors at the rDNA 1011 promoter. ChIP assays were performed using HepG2 cell lines expressing STAT3 1012 shRNA or control shRNA and antibodies against the factors as indicated. G) STAT3 1013 upregulation increased the occupancies of the Pol I transcription machinery factors at 1014 the rDNA promoter. ChIP assays were performed using a HepG2 cell line expressing 1015 mCherry-STAT3 and its control cell line in which antibodies against factors used for 1016 the assays were as indicated. H) A scheme showing the cloning of the rDNA promoter 1017 (rDNAP) with the reporter vector pGL3-basic. 45SF: 45S DNA fragment; Luc: 1018 1019 luciferase. I and J) STAT3 knockdown inhibited the rDNA promoter activity. The 'Reporter" gene expression was detected by RT-qPCR using the primers as indicated 1020 in H after transfection of the rDNA promoter (rDNAP)-driving reporter vectors into 1021 HeLa (I) or HepG2 (J) cell lines. Rel exp: relative expression. K and L) STAT3 1022 overexpression inhibited the rDNA promoter activity. The "Reporter" gene expression 1023 was monitored by RT-qPCR after transfecting the rDNA promoter-driving reporter 1024 vectors into HeLa (K) and HepG2 (L) cell lines. Each column in histograms 1025 1026 represents the mean \pm SD of three independent experiments. *, P<0.05; **, P<0.01. P values were obtained by Student's t test. 1027

1029 Figure 9. STAT3 modulates Pol I-directed transcription by controlling RPA34

transcription. A) Generation of HepG2 cell lines stably expressing both STAT3 1030 1031 shRNA and mCherry-RPA34. Western blot was used to verify HepG2 cell lines expressing STAT3 shRNA only or both STAT3 and mCherry-STAT3 and the control 1032 cell line using antibodies as indicated. **B**) Analysis of Pol I products by RT-qPCR 1033 using the cell lines established in A. C and D) Cell proliferation assays for the cell 1034 lines established in (A). Cell proliferation assays were performed using cell counting 1035 (C) and CCK-8 (D) methods. E and F) Effect of RPA34 silencing on Pol I-directed 1036 transcription in HepG2 cells. RPA34 and Pol I products were analyzed by Western 1037 blot (E) and RT-qPCR (F), respectively. G and H) Effect of RPA34 overexpression on 1038 Pol I-directed transcription in HepG2 cells. RPA34 and Pol I products were analyzed 1039

1040	by Western blot (G) and RT-qPCR (H), respectively. I) A cartoon showing putative
1041	STAT3 binding elements in the <i>Rpa34</i> promoter. Red letters represent STAT3
1042	consensus bases (WT), while blue letters represent the mutations of STAT3 consensus
1043	bases. J) A ChIP result showing the STAT3 occupancy at the <i>Rpa34</i> promoter in
1044	HepG2 cells. K and L) STAT3 inhibited the <i>Rpa34</i> promoter activity in HeLa and
1045	HepG2 cells. Luciferase assays were performed by transfecting the RPA34P-driving
1046	reporter vectors into HeLa and HepG2 cell lines expressing STAT3 shRNA or control
1047	shRNA. Relative luciferase activity (RLA) was obtained by comparing the luciferase
1048	activity of treatment samples to that of control samples, where the activity of control
1049	samples was arbitrarily set as 1. M and N) Mutations of the STAT3 consensus bases
1050	suppressed the <i>Rpa34</i> promoter activity. The reporter vectors driven by the wild type
1051	RPA34P or by its mutant containing STAT3-binding site mutations were transfected
1052	into HeLa and HepG2 cells. RLA, relative luciferase activity. O) STAT3
1053	downregulation inhibited the occupancies of the Pol II transcription machinery factors
1054	at the Rpa34 promoter. ChIP assays were performed using HepG2 cell lines
1055	expressing STAT3 shRNA or control shRNA and the antibodies against the factors as
1056	indicated. The relative occupancy was obtained as described in Fig. 6A. Each column
1057	in the histograms represents the mean \pm SD of three independent experiments. *,
1058	P<0.05; **, P<0.01. P values were obtained by two-way ANOVA (B-D) or Student's t
1059	test (F,H,J-O).

Fig.10

1060

1061 Figure 10. A proposed model by which STAT3 modulates Pol I-directed

1062 transcription. After phosphorylation, p-STAT3 enters nuclei and binds to the *Rpa34*

1063 promoter to transcribe RPA34 mRNA. After translation, RPA34 protein enters

nucleoli and binds to the rDNA promoter to initiate Pol I-directed transcription.