**Supplementary Data**

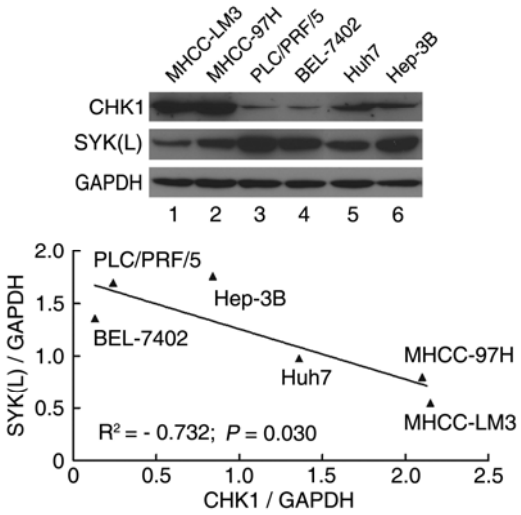
# CHK1 Targets Spleen Kinase (L) for Proteolysis in Human Hepatocellular Carcinoma

Jian Hong[1,2,6], Kaishun Hu[1,3,6], Yunfei Yuan[1,2*], Yi Sang[1,3], Qiangui Bu[4] , Guihua Chen[5], Longjun Yang[1,2], Binkui Li[1,2] , Pinzhu Huang[1,2], Dongtai Chen[1], Yi Liang[1,3], Ruhua Zhang[1,3], Jingxuan Pan[4] , Yi-Xin Zeng[1,3], and Tiebang Kang[1,3*]

Running title: Regulation of SYK(L) by CHK1

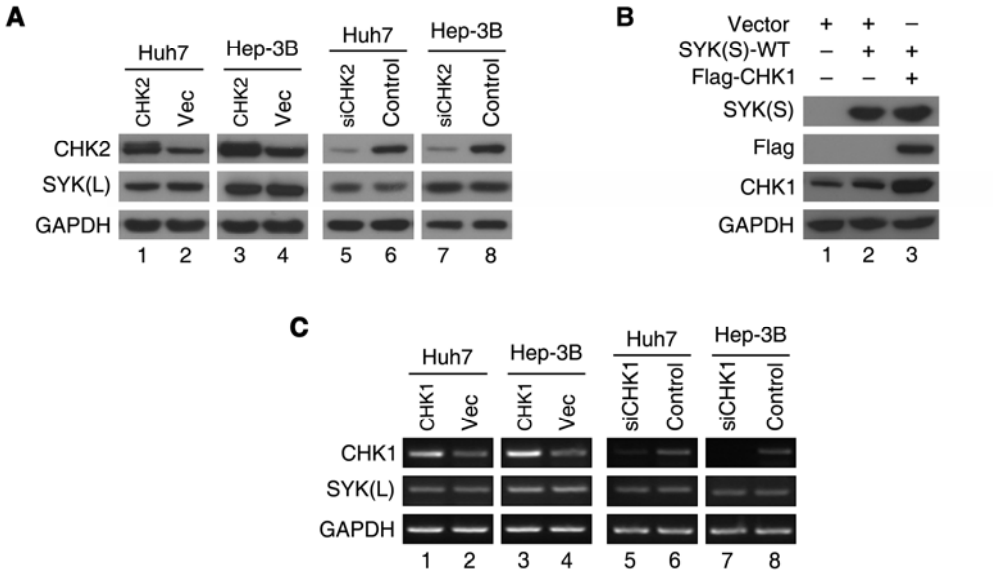
**Supplementary Table 1. PCR Primers**

| Gene | Forward primer<br>Reverse primer | Predicted size (bp) |
|---|---|---|
| SYK(L) | 5′ - CGAGGGAAAGAAGTTCGACACG - 3′ | 219 |
| | 5′ - CCAGGCTTTGGGAAGGAGTATG - 3′ | |
| CHK1 | 5′ - ATCACCATTGGCAATGAGCG - 3′ | 220 |
| | 5′ - TTGAAGGTAGTTTCGTGGAT - 3′ | |
| MMP2 | 5′ - GCTGGCTGCCTTAGAACCTTTC - 3′ | 170 |
| | 5′ - GAACCATCACTATGTGGGCTGAGA - 3′ | |
| MMP9 | 5′ - GCTACCACCTCGAACTTTGACAG - 3′ | 203 |
| | 5′ - TGCCGGATGCCATTCAC - 3′ | |
| Cyclin D1 | 5′ - CTGGAGCCCGTGAAAAAGAGC - 3′ | 434 |
| | 5′ - CTGGAGAGGAAGCGTGTGAGG - 3′ | |
| GAPDH | 5′ - AGAAG GCTGG GGCTC ATTTG - 3′ | 258 |
| | 5′ - AGGGG CCATC CACAG TCTTC - 3′ | |




**Supplementary Figure 1. There was a negative correlation between the protein levels of CHK1 and SYK(L) in HCC cell lines.**

Six different HCC cell lines were analyzed for the protein expression levels of both CHK1 and SYK(L) by Western blot (top panel). Protein intensities were normalized to GAPDH levels, and the correlation between SYK(L) and CHK1 protein levels in these six HCC cell lines is shown (bottom panel).

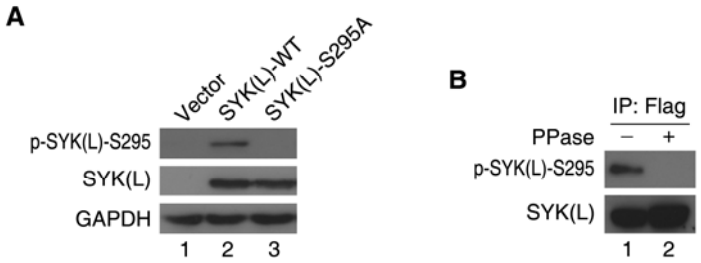



**Supplementary Figure 2. Effects of CHK1 or CHK2 on SYK(L) or SYK(S) in HCC cells.**

(**A, C**) The indicated cells were transfected with the indicated plasmids for 24 hrs (lanes 1-4) or siRNAs for 48 hrs (lanes 5-8) and then were analyzed by Western blot (**A**) or RT-PCR (**C**).

(**B**) SMMC7721 cells were transfected with the indicated plasmids for 24 hrs, and then the cell lysates were analyzed by Western blot.

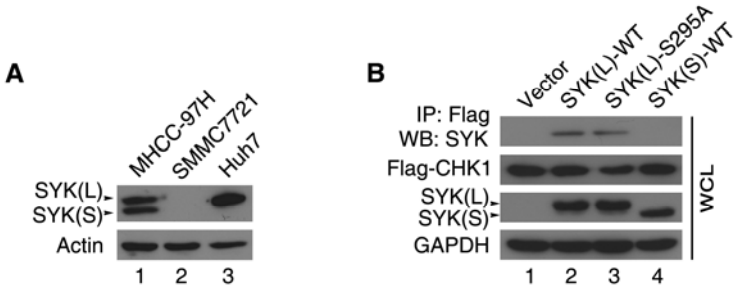
**Note**: CHK1, but not CHK2, regulates SYK(L), but not SYK(S), at the protein level.




**Supplementary Figure 3. Phosphorylation of SYK(L) on serine 295 is detectable in cells.**

(**A**) SMMC7721 cells lacking endogenous SYK(L) were transfected with SYK(L)-WT, SYK(L)-S295A or the vector control for 24 hrs. Subsequently, the cell lysates were analyzed by Western blot using the p-SYK(L)-S295 antibody that we generated. Note: the phospho-specific antibody p-SYK(L)-S295 was successfully generated.

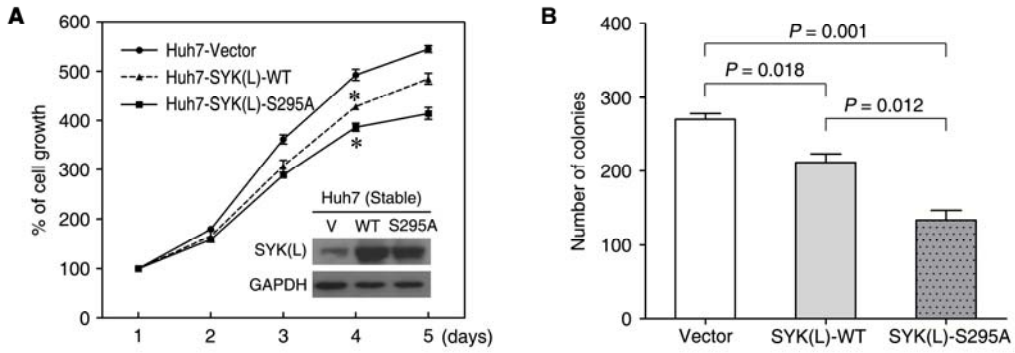
(**B**) The complexes immunoprecipitated using anti-SYK(L) were incubated with lambda phosphatase (PPase) and analyzed by immunoblotting.




**Supplementary Figure 4. Isoforms of SYK, SYK(L) and SYK(S) are detectable in HCC cell lines, and CHK1 binds SYK(L), but not SYK(S).**

(**A**) Immunoblot analysis of SYK expression in HCC cell lines using the anti-SYK mAb (N-19), which recognizes both isoforms of SYK. Note: MHCC-97H cells are positive for both SYK(L) and SYK(S), SMMC7721 cells are negative for both isoforms, and Huh7 cells are positive for SYK(L) and negative for SYK(S).

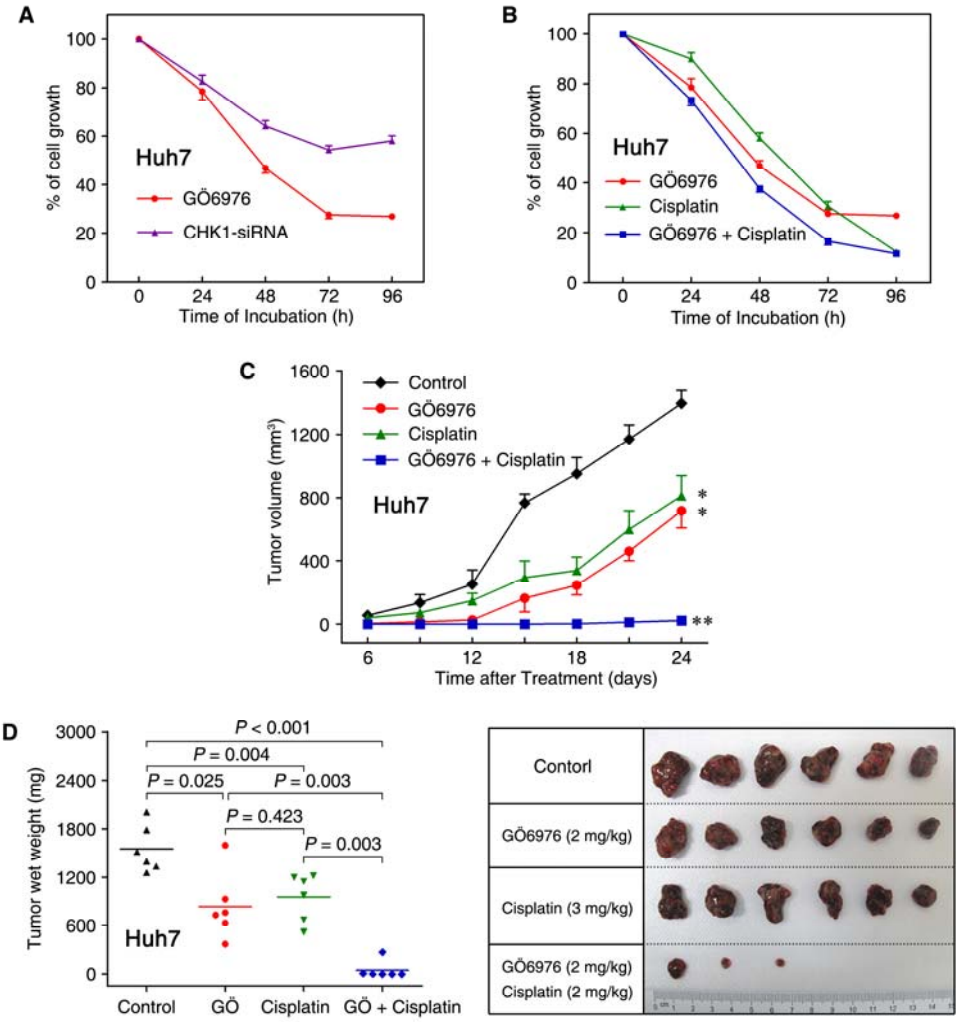
(**B**) SMMC7721 cells were transfected with Flag-CHK1 and the indicated plasmids for 24 hrs, and the whole cell lysates (WCLs) were resolved directly by SDS-PAGE or were first incubated with Flag-agarose and then analyzed by Western blot.




**Supplementary Figure 5. SYK(L) functions is negatively regulated by the CHK1-mediated phosphorylation of serine 295 in Huh7 cells.**

(**A**) Huh7 cells were stably transfected with the indicated plasmids, as shown by Western blot (insertion panel). Cellular proliferation were measured by MTT assay at the indicated times (n = 3). The dots represent the mean, while the bars indicate the SEM. * $P < 0.05$ and ** $P < 0.01$ using Student's t-test.

(**B**) The stable cell lines used in (**A**) were cultured for 14 days, and the colonies were counted and graphed (n = 3). The bars indicate the SEM. *P* values were obtained by Student's t-test.




**Supplementary Figure 6. Inhibition of CHK1 suppresses tumorigenicity through the CHK1/SYK(L) pathway in Huh7 cells in vitro and in vivo.**

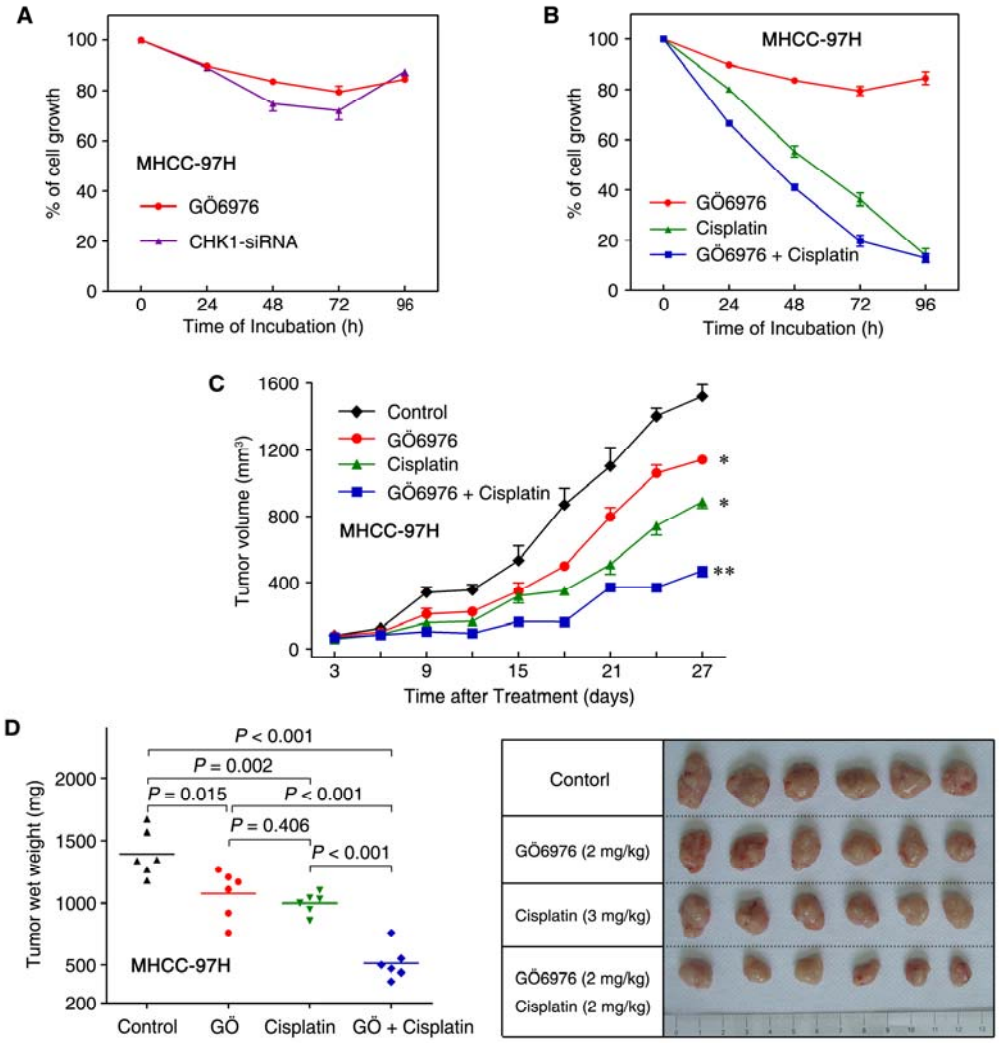
(**A**) Huh7 cells positive for SYK(L) and negative for SYK(S) were transfected with CHK1 siRNA, or scramble siRNA with or without GÖ6976 (100 nM) for different amounts of time, as indicated, and then were subjected to an MTT assay (n = 3). The dots represent the mean, while the bars indicate the SEM.

(**B**) Huh7 cells were incubated with GÖ6976 (100 nM), cisplatin (6.7 μM) or both drugs, as indicated, for different times and were subjected to an MTT assay (n = 3).

The dots represent the mean, while the bars indicate the SEM.

(**C**) Mice bearing xenografts composed of Huh7 cells were generated as described in the Experimental Procedures section and were treated with DMSO control, GÖ6976, cisplatin or both drugs. The tumor volumes were measured and recorded every three days after injection for 6 days, and tumor growth curves were created for each group (n = 6). The dots represent the mean, while the bars indicate the SEM. * $P < 0.05$ and ** $P < 0.001$ using Student's t-test.

(**D**) The xenografts were excised from mice and weighed after 24 days, as shown in the right panel. Each dot represented a tumor weight, the mean tumor weights in each group were indicated by solid lines (left panel; n = 6), and *P* values were obtained using the Student's t-test.



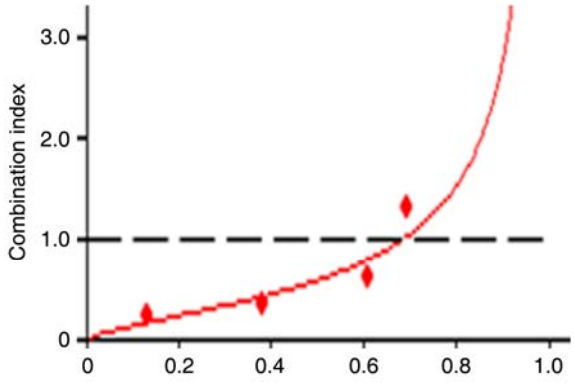

**Supplementary Figure 7. Inhibition of CHK1 suppresses tumorigenicity through the CHK1/SYK(L) pathway in MHCC-97H cells in vitro and in vivo.**

(**A**) MHCC-97H cells positive for both SYK(L) and SYK(S) were transfected with CHK1 siRNA, or scramble siRNA with or without GÖ6976 (100 nM) for different amounts of time, as indicated, and then were subjected to an MTT assay (n = 3). The dots represent the mean, while the bars indicate the SEM.

(**B**) MHCC-97H cells were incubated with GÖ6976 (100 nM), cisplatin (6.7 μM) or both drugs, as indicated, for different times and were subjected to an MTT assay (n = 3). The dots represent the mean, while the bars indicate the SEM.

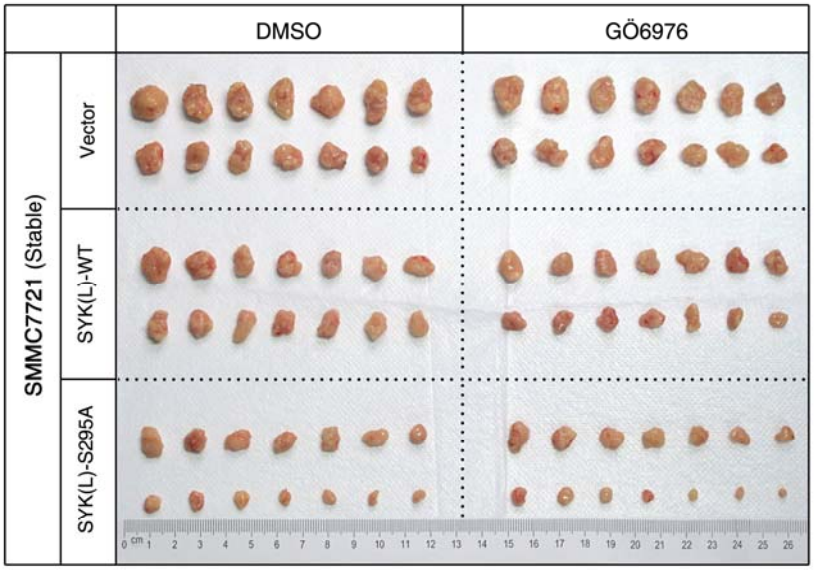
(**C**) Mice bearing xenografts composed of MHCC-97H cells were treated with DMSO control, GÖ6976, cisplatin or both drugs. The tumor volumes were measured and recorded every three days after injection for 3 days, and tumor growth curves were created for each group (n = 6). The dots represent the mean, while the bars indicate the SEM. * $P < 0.05$ and ** $P < 0.001$ using Student's t-test.

(**D**) The xenografts were excised from mice and weighed after 27 days, as shown in the right panel. Each dot represented a tumor weight, the mean tumor weights in each group were indicated by solid lines (left panel; n = 6), and *P* values were obtained using the Student's t-test.




**Supplementary Figure 8. The combination of cisplatin with GÖ6976 on cell proliferation is synergistic.**

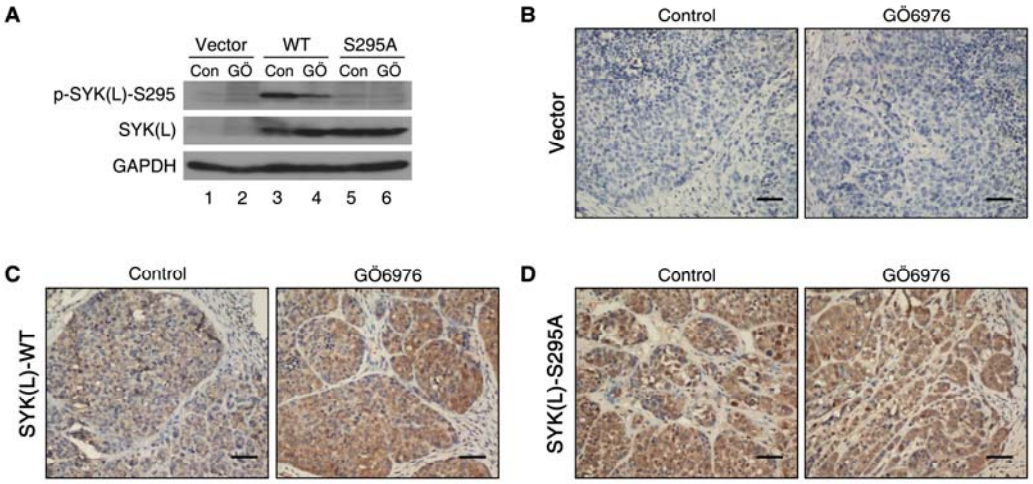
SMMC7721 cells were exposed to various concentrations of cisplatin and GÖ6976 alone or two drug-combination at a fixed ratio. After treated for 72 hours, cell viability was measured by MTS assay. CI was calculated by using the Calcusyn software. n = 2.




| Group / Treatment | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Mean | *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|
| Vector | DMSO | 1.136 | 0.805 | 0.701 | 0.694 | 0.693 | 0.653 | 0.648 | 0.589 | 0.362 |
| | | 0.515 | 0.500 | 0.451 | 0.424 | 0.408 | 0.395 | 0.225 | | |
| | GÖ6976 | 0.974 | 0.759 | 0.653 | 0.625 | 0.578 | 0.512 | 0.496 | 0.516 | |
| | | 0.460 | 0.446 | 0.418 | 0.393 | 0.344 | 0.315 | 0.253 | | |
| SYK(L)-WT | DMSO | 0.680 | 0.525 | 0.401 | 0.386 | 0.351 | 0.346 | 0.336 | 0.366 | 0.009 |
| | | 0.331 | 0.324 | 0.315 | 0.312 | 0.287 | 0.271 | 0.256 | | |
| | GÖ6976 | 0.433 | 0.345 | 0.342 | 0.335 | 0.319 | 0.303 | 0.274 | 0.256 | |
| | | 0.226 | 0.210 | 0.205 | 0.181 | 0.168 | 0.147 | 0.102 | | |
| SYK(L)-S295A | DMSO | 0.301 | 0.264 | 0.261 | 0.235 | 0.216 | 0.173 | 0.165 | 0.152 | 0.734 |
| | | 0.125 | 0.113 | 0.092 | 0.056 | 0.052 | 0.038 | 0.033 | | |
| | GÖ6976 | 0.343 | 0.249 | 0.233 | 0.211 | 0.187 | 0.166 | 0.137 | 0.139 | |
| | | 0.112 | 0.102 | 0.075 | 0.059 | 0.033 | 0.027 | 0.018 | | |

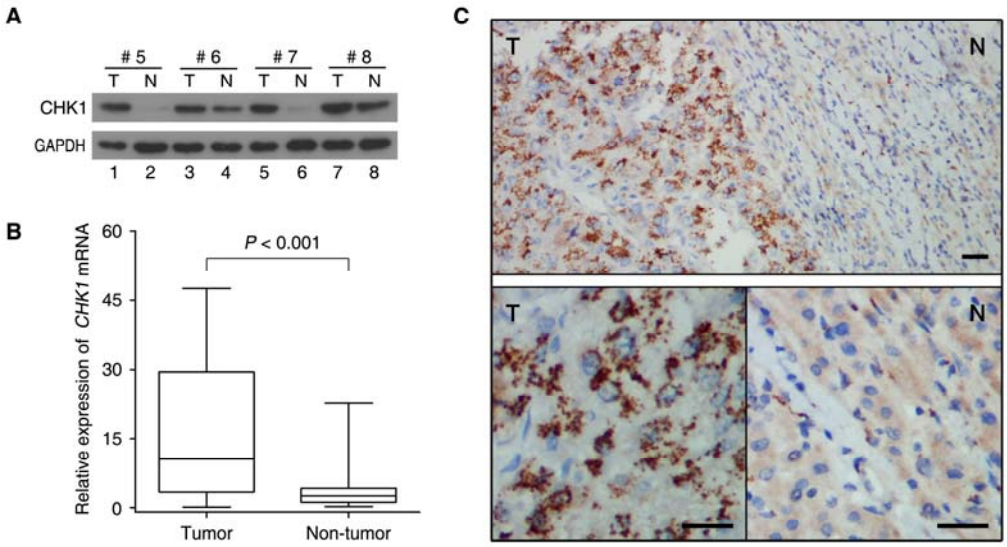
**Supplementary Figure 9. Inhibition of CHK1 suppresses tumorigenicity through the CHK1/SYK(L) pathway in HCC in vivo**

On day 27, the xenografts as described in Figure 5 were excised from mice and weighed, as shown in the upper panel. The detailed tumor wet weight was presented in the lower panel. *P* values were obtained using the Student's t-test. n = 14.




**Supplementary Figure 10. Inhibition of CHK1 suppresses tumorigenicity through the CHK1/SYK(L) pathway in HCC in vivo**

(**A, B, C, D**) On day 27, the xenografts as described in Figure 5 were excised from mice and weighed, and then were collected for western blots (**A**) or immunohistochemical staining of SYK(L) (**B, C, D**). Representative examples illustrating the total protein levels of SYK(L) of their immunostaining from different groups as indicated. The scale bar was set at 50 μm.

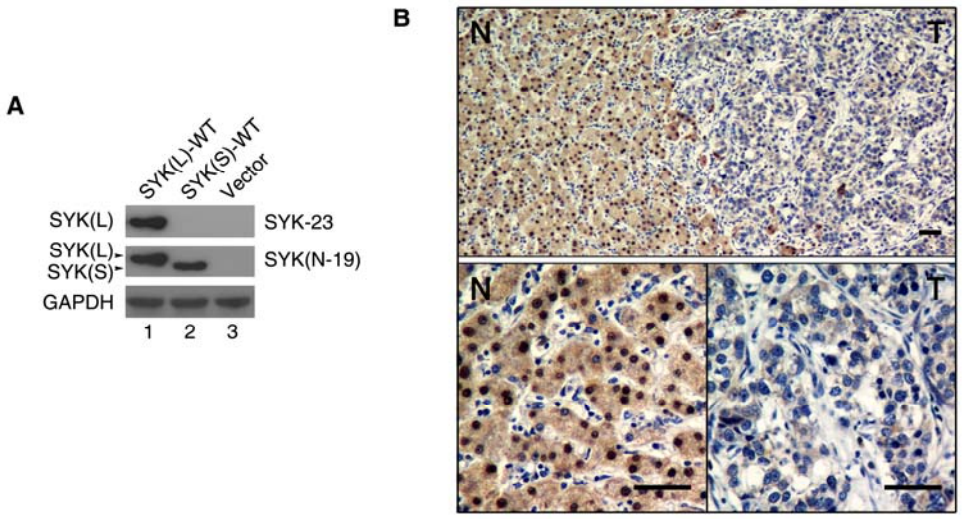



**Supplementary Figure 11. CHK1 is overexpressed in HCC tissues.**

(**A**) Representative HCC tissues (T) or their non-tumor counterparts (N) were extracted and tested using a Western blot.

(**B**) Eighty pairs of HCC tissues (i.e., tumor tissues) and their non-tumor counterparts (i.e., non-tumor tissues) were extracted and exposed to qRT-PCR to determine CHK1 mRNA levels, which were normalized to the internal GAPDH control and graphed. *P* < 0.001 using Student's t-test.

(**C**) Representative images of CHK1 expression in both non-tumor (N) and matched HCCtissues (T), as detected by immunohistochemistry. The scale bar was set to 50 μ m.

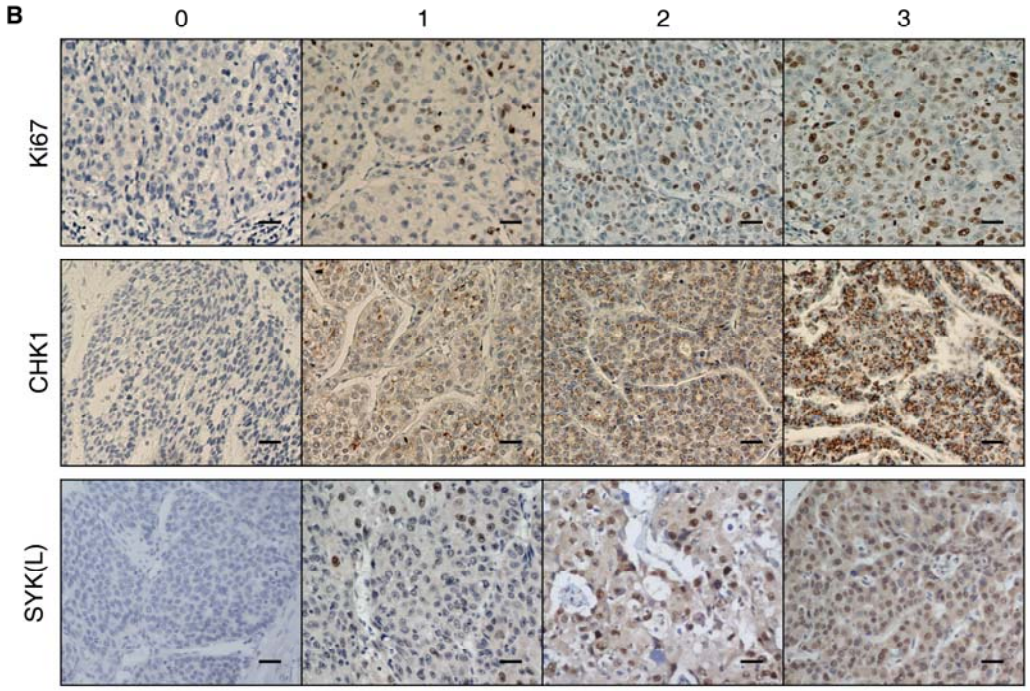



**Supplementary Figure 12. Anti-SYK-23 specifically recognizes SYK(L), but not SYK(S), in cells and HCC tissues.**

(**A**) SMMC7721 cells negative for both SYK(L) and SYK(S) were transfected with the indicated plasmids for 24 hours. The cell lysates were analyzed by Western blot. (**B**) Representative images of CHK1 expression in both non-tumor (N) and matched HCC tissues (T) detected by immunohistochemistry using the anti-SYK-23 antibody. The scale bar was set to 50 μm.

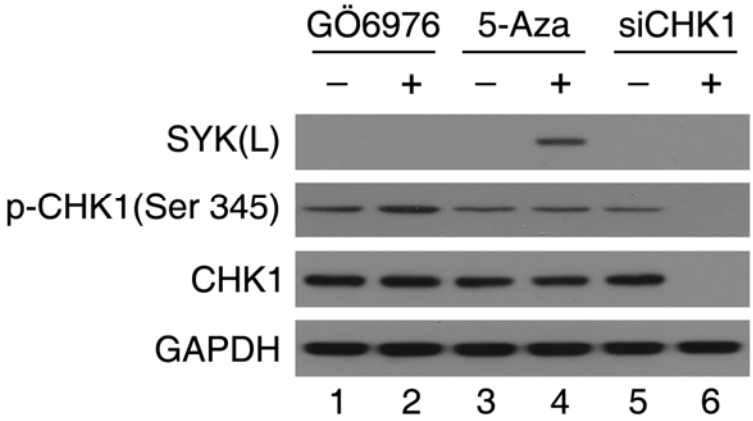
A

| | Ki67 expression | | r | *P*-value |
|---|---|---|---|---|
| | Low (n = 49) | High (n = 113) | | |
| **CHK1** expression | | | | |
| Low (n = 30) | 25 | 5 | | |
| High (n = 132) | 24 | 108 | 0.586 | **< 0.001** |
| **SYK(L)** expression | | | | |
| Low (n = 127) | 35 | 92 | | |
| High (n = 35) | 14 | 21 | -0.111 | 0.158 |




**Supplementary Figure 13. There was an correlation between CHK1 and ki67 protein level, but not between SYK(L) and ki67 in HCC tissues.**

(**A, B**) Immunohistochemical staining of ki67, CHK1 and SYK(L) was performed in 162 HCC tissues with anti-ki67, anti-CHK1 or anti-SYK-23. Representative examples illustrating the ranges of intensities of their immunostaining from 0 to 3 are shown in (**B**), and a summary of the correlations are shown in (**A**). The scale bar was set at 50 μ m.




**Supplementary Figure 14. Endogenous CHK1 is silenced by its promoter methylation in SMMC7721 cells.**

SMMC7721 cells were transfected with CHK1 siRNA, or scramble siRNA with or without GÖ6976 (100 nM) for 48 hours, or with or without DNA methyltransferase inhibitor 5-Aza (2 μM) for 5 days, as indicated, and then were subjected to Western blot.