**Supplemental Materials and Methods**

**Expression and Purification of NMT1**

A modified NMT1 gene containing a His6-tag at the N-terminus and the catalytic domain of NMT1 (excluding 108 amino acids of the N-terminus of the full length NMT1)was synthesized by GenScript (Fig. S6A-B). The gene was cloned in the pET-11a vector and transformed into Rosetta 2 competent cells by heat shock. Cells were grown at 37 oC in LB broth, supplemented with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol, to OD600 0.6-0.8. Cultures were induced with 1 mM IPTG and grown overnight at 18 oC. The cells were harvested by centrifugation at 5000 xg for 10 min. The cells were suspended in a buffer containing 20 mM Tris (pH 7.5), 500 mM NaCl, 10 mM imidazole, 1 mM MgCl2, and 0.1% (v/v) Triton X-100 and lysed by four 1 min rounds of sonication, 5 s on 5 s off, at 50% amplitude, on ice. The lysate was cleared by centrifugation at 48,000 xg for 20 min at 4 oC. Cleared lysate was applied to high-density nickel agarose beads (Gold Biotechnology, Olivette, MO) equilibrated with 20 mM Tris (pH 7.5), 500 mM NaCl and 10 mM imidazole. Protein was eluted with the equilibration buffer containing 250 mM imidazole. This elution was diluted 20 fold in Buffer A (20 mM Tris pH 8.9 and 1 mM DTT) and loaded onto a Mono Q anion-exchange column (GE Healthcare, Pittsburgh, PA). NMT1 was eluted in a 0-50% NaCl gradient over 20 column volumes of Buffer A and Buffer B (20 mM Tris pH 8.9, 1 mM DTT, and 1 M NaCl) (1). The His6-tag was cleaved using HRV 3C protease. The remaining free His-tag, His-tagged NMT1, and HRV 3C protease were separated from cleaved NMT1 by nickel affinity chromatography.

**Characterization of NMT1 kinetics**

Purified NMT1 catalyzes the incorporation of the myristoyl group of myristoyl-CoA into the N-terminus of glycine in the peptide of Gly-Ser-Asn-Lys-Ser-Lys-Pro-Lys (derived from the N-terminus of human pp60Src tyrosine kinase) releasing CoA. The amount of released CoA could be measured by 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin (CMP). The assay was performed in 96-well black microplates (Greiner Bio-One, Germany) and the reaction mixture contained 33.6 μL of 2X reaction buffer (100 mM HEPES and 1 mM EDTA), 4 μL of 5.6 μM of the purified hNMT1 stock, 30.4 μL of ddH2O, and 4 μL of 70, 100, 200, and 400 μM stock concentration of the above synthetic peptide, respectively. At each peptide concentration of the above mixture, 8 μL of the myristoyl-CoA stock solution was added to reach a final concentration of 0, 10, 20, 40, 80, 160 μM, respectively, and react time was 1, 2.5, 5, 10 minutes, respectively. Reactions were stopped by adding 80 μL of 30 μM of CMP, and incubated in the dark for 12 minutes. The fluorescence intensity was measured by a Flex Station 3, microplate reader (excitation at 390 nm; emission at 479 nm). The reciprocal values of the velocity and the myristoyl-CoA concentration were plotted on Lineweaver-Burk plots.

**LCL and GRU compounds and molecular docking**

LCL compounds were synthesized by the Synthetic Unit of Lipidomics Shared Resource at the Medical University of South Carolina as previously described (2-4). GRU compounds were synthesized by Dr. Iryna Lebedyeva’s lab. Molecular docking was performed using Autodock Vina against compound structures outlined in Figure 6 and S9 by using the binding pocket of the X-ray structure of NMT1 (4C2Y) (1).

**Chemical synthesis of B13 and LCL204**

For the chemical synthesis of B13, (2*R*,3*R*)-2-amino-1-(4-nitrophenyl)-1,3-propanediol (0.1 mmol, 212 mg) was dissolved in dry THF (50 mL) and cooled to 4 oC, EDCI (156 mg, 1 mol) and HOBt (135 mg, 1 mmol) were added to the solution followed by the addition of tetradecanoic acid (228 mg, 1 mmol). The mixture was kept under N2 at room temperature overnight. After the reaction mixture was taken to dryness, the product was extracted with ethyl acetate and using sodium carbonate and 3M hydrochloric acid solutions for the extraction to give B13. Product B13(5) was then purified by column chromatography using ethyl acetate:hexane 1:10 as eluent.

For the chemical synthesis of LCL204, (2*R*,3*R*)-2-amino-1-(4-nitrophenyl)-1,3-propanediol (1 mmol, 212 mg) was reacted with tetradecanal (255 mg, 1.2 mmol) in methanol/0.05 N acetic acid, 9:1 for 15 min and sodium cyanoborohydride (NaCNBH3, 130 mg, 2 mmol) was added in portions during 1 h. The mixture was stirred overnight at room temperature, evaporated to dryness and the residue dissolved in DCM/MeOH, 2:1. The product was purified by recrystallization using DCM/Pentane 1:3 to give LCL204 (6) as white solid in 72 % yield.

**Quantitative RT-PCR analysis**

Total RNA was isolated from human prostate cell lines using an RNeasy kit (Qiagen). cDNA was prepared from 2 µg of total RNA using the High-Capacity cDNA Kit (Applied Biosystem). Each cDNA sample was diluted 10-fold, and a 5 μL aliquot was used in a 20 μL PCR reaction (PerfeCTa SYBR Green FastMix, Quantabio) containing primers at concentrations of 10 pM each. The primers are listed in Table S1. PCR reactions were run in triplicate and quantitated using StepOne Software v2.1 (Applied Biosystem). The results were expressed as a fold change of mRNA compared with control group. Expression data were normalized to GAPDH.

**Immunoprecipitation for the analysis of Src-AR interaction**

For the protein interactions between AR and Src(WT) or Src(WT/G2A), LNCaP and 22Rv1 cells were transduced with Src(WT) or Src(WT/G2A) genes by lentiviral infection. The transduced cells were grown in 100 mm dishes, and the protein lysates were extracted with the immunoprecipitation (IP)lysis buffer (25 mM Hepes pH 7.5, 50 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with protease inhibitor cocktail (Calbiochem) and phosphatase inhibitor cocktail 1 and 2 (P5726 and P2850, Sigma). 500 µg protein was incubated with specific antibodies for 16 h at 4 ℃. Protein A agarose beads were added, and the mixtures were incubated for 1 h at 4 ℃. After washing five times with IP lysis buffer, an equal volume of 2X SDS sample buffer was added to the immuoprecipitated proteins and boiled for 10 min. AR and Src proteins were analyzed by immunoblotting with specific antibodies.

**Immunohistochemistry**

Formalin-fixed/paraffin-embedded grafts and tissues were sectioned at 4 µm thickness and mounted on positively charged microscope slides. Sections were analyzed by hematoxylin and eosin (H&E), and immunohistochemistry (IHC) staining. The primary antibodies and dilutions used for detection of Src, Fyn, CK5, CK8, and AR expression were described previously (7). Phase or fluorescent images were taken under a fluorescence microscope.

**Antibodies**

Antibodies against Src, phospho-Src family (Tyr416), phospho-Erk1/2 (Thr202/Tyr204), Fak, phospho-Fak (Tyr925), Akt, phospho-Akt (Ser473), Fyn, GAPDH and Caveolin-1 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against Androgen receptor (AR) and Erk2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-NMT1 and γ-tubulin antibody and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Calbiochem (San Diego, CA). These antibodies were used for immunoblotting.

**Supplemental References**

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**Supplemental figures**

**Figure S1. Knockdown of NMT1 by shRNA-NMT1 inhibits Src myristoylation and proliferation of prostate cancer cells.**

1. Diagram of the lentiviral vector expressing the shRNA targeting NMT1. **(B)** shRNA-NMT1 inhibited the expression of NMT1 in PC-3 cells. PC-3 cells were transfected with control vector or three different shRNAs (#1-3) by lentiviral infection. shNMT1#1 showed the highest efficacy and was used for further studies. **(C)** SYF1 (Src-/-Yes-/-Fyn-/-) cells expressing Src(WT) were transduced with shRNA-NMT1 (MOI of 0, 1, 5, 10, and 20) by lentiviral infection. The infected cells were grown with 60 μM myristic acid azide for 24 h. The total myristoylated proteins or myristoyl-Src were detected by streptavidin-HRP via Click chemistry. The protein expression levels of NMT1, Src, and the tubulin loading control were detected by Western blot analysis. **D)** NMT1 was knocked down by two independent shRNA-NMT1 (#1 and #3) in PNT2, LNCaP, 22Rv1, PC-3, and DU145 cells by lentiviral infection. The growth curve was measured by the MTT assay from day 0 to day 5. Also see Figure 1.

**Figure S2. Knockdown of Src and Fyn kinase inhibits the growth of prostate cancer cells. (A-B)** PC-3 cancer cells were transduced with shRNA-control or two independent shRNA-Src. **(C-D)** PC-3 or LNCaP were transduced with shRNA-control or shRNA-Fyn by lentiviral infection. The proliferation of the transduced cells was measured by the MTT assay. The efficiency of knockdown was confirmed by Western blot analysis (inserted panel). \**P*<0.05; \*\**P*<0.01.

**Figure S3. The contribution of myristoylation and palmitoylation to the activity of SFKs. (A)** SYF1 (Src-/-Fyn-/-Yes-/-) cells were transfected with Src(Y529F) (constitutively active mutant), Src(Y529F/S3C/S6C) (gain of palmitoylation sites mutant), Src(Y529F/G2A) (loss of myristoylation site mutant), or Src(Y529F/K298M) (kinase dead mutant) by lentiviral infection. The levels of total Src, pSrc(Y416), total Erk, pErk, total Fak, pFak(Y925), and tubulin were determined by immunoblotting. **(B)** SYF1 cells were transfected with Fyn(Y528F) (constitutively active mutant), Fyn(Y528F/C3S/C6S) (loss of palmitoylation sites mutant), Fyn(Y528F/G2A) (loss of myristoylation site mutant), or Fyn(Y528F/K298M) (kinase dead mutant) by lentiviral infection. The levels of total Fyn, pSrc(Y416) (which detects the activation of Fyn), total Erk, pErk1/2, total Fak, and pFak(Y925) were determined by immunoblotting. **(C)** The Src kinase mutants mediated transformation potential. The SYF1 cells and SYF1 cells stably expressing Src(WT), Src(Y529F), Src(Y529F/S3C/S6C), Src(Y529F/G2A), or Src(Y529F/K298M) were subjected to the soft agar assay. The number of colonies is reported as the mean ± SEM. The expression levels of Src kinase were confirmed before the assay (not shown). **(D)** The SYF1 cells and SYF1 cells stably expressing Fyn(WT), Fyn(Y528F), Fyn(Y528F/C3S/C6S), Fyn(Y528F/G2A), or Fyn(Y528F/K298M) were subjected to the soft agar assay. The number of colonies is reported as the mean ± SEM. Representative phase and RFP images of colonies generated from the soft agar assay of the above were displayed. \**P*<0.05, \*\**P*<0.01.

**Figure S4. Fyn kinase has no synergistic effect with androgen receptor (AR). (A)** Schematic for examining the synergy of Fyn and AR in prostate tumorigenesis. Primary prostate epithelial cells (from Bl6 mice) were transduced with AR (GFP marker), Fyn(WT) (RFP marker), or co-transduced with Fyn(WT) and AR by lentiviral infection. The infected cells were combined with UGSM, and implanted under the renal capsule of SCID mice. Regenerated prostate tissue was isolated after 8 weeks. **(B)** H&E, RFP/GFP, and IHC staining of AR in regenerated tissue. Scale bar, 100 μm. No pathological phenotype was observed in Fyn(WT)+AR regenerated tissues, suggesting no synergistic effect of Fyn(WT) and AR in tumor progression.

**Figure S5. Loss of myristoylation in Src kinase inhibits its interaction with androgen receptor (AR) and activation of AR down-stream signaling. (A and D)** 22Rv1 and LNCaP prostate cancer cells were transduced with Src(WT), Src(G2A), or control by lentiviral infection. The expression of total Src, pSrc(Y416), and AR in 22Rv1 (A) and LNCaP (D) cells were confirmed by Western blot analysis. **(B-C and E-F)** Protein-protein interactions between endogenous AR and overexpressed Src(WT) or Src(G2A). Src and AR were detected by immunoprecipitation (IP) from 22Rv1 cells (B and C) and LNCaP (E and F) in the presence or absence of the AR agonist R1881 (5 nM). **(G)** The mRNA levels of PSA, KLK2, and TMPRSS2 genes by synergy of exogenously expressed Src(WT)/Src(G2A) in LNCaP cells. The addition of R1881 on the regulation of PSA in LNCaP cells was a positive control. \**P*<0.05, \*\**P*<0.01.

**Figure S6. Expression and purification of NMT1. (A)** The human NMT1 DNA sequence (with an exclusion of the 108 amino acids DNA sequence in the N-terminus) was optimized for bacterial expression and synthesized by GenScript. **(B)** Comparison of protein sequences between the full-length NMT1 protein (upper panel) and the purified NMT1 (lower panel). The N-terminus of NMT1 possesses an inhibitory function of the NMT1 enzymatic activity (8). To increase the enzymatic activity of NMT1 for inhibitor screening, the un-highlighted N-terminal region in NMT1 protein was excluded and an N-terminal His6-tag was added for the protein purification (Lower panel). **(C)** The NMT1 gene was expressed in *Escherichia coli*. The protein was purified by Ni-NTA affinity chromatography (Lane 1). The purified protein was further purified by anion exchange chromatography (Lane 2). The His6-tag was removed by HRV 3C protease by enzymatic digestion (Lane 3). The processed protein was used for enzymatic assays and crystallization. Protein was detected by Coomassie blue staining (i), Western blot analysis using anti-His6-tag (ii), and human NMT1 antibody (iii).

**Figure S7. Kinetic analysis of purified NMT1**. **(A)** NMT1 catalyzes the attachment of the myristoyl- group from myristoyl-CoA to the N-terminal Gly of a peptide or protein, and releases HSCoA. The reaction of HSCoA with 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin (CMP), is detected by fluorescence (excitation 390 nm and emission at 479 nm). Inhibitors were examined for their ability to reduce fluorescence. **(B)** Kinetic characterization of the purified NMT1. Lineweaver–Burk plots of hNMT1 with different concentrations of myristoyl-CoA (1, 2, 4, or 8 μM) in the presence of 1.25, 2.5, 5, or 10 μM of the peptide. Km and Vmax are reported and the Km/Vmax remained the same.

**Figure S8. Identifying compounds inhibiting Src myristoylation by Click chemistry. (A)** Schematic of the experimental design to examine the inhibitory effect of a compound on the myristoylation of Src kinase at the cellular level. SYF1 cells expressing Src(WT) or Src(G2A), a positive control of un-myristoylated-Src were cultured in a DMEM medium with 2% BSA and 30 μM of a compound for 24 h,followed by addition of myristic acid-azide to the medium. Cells were harvested for the detection of myristoylated-Src by Click chemistry. **(B)** Schematic of the Click reaction to detect the myristoylation of Src kinase. The incorporation of myristic acid-azide to Src kinase is catalyzed by NMT enzymes, which will be reduced by an inhibitor. The myristoylated-Src in protein lysate is labeled by biotin-alkyne via the Click chemistry reaction. The labeled myristoylated-Src is detected by streptavidin-HRP in Western analysis. **(C)** The expression of myristoylated Src was detected by streptavidin-HRP, and the expression of total Src and GAPDH under treatment of LCL and GRU compounds was also measured by Western blot analysis.

**Figure S9. Structures and/or IC50 values of LCL and GRU compounds.** The LCL compounds were previously synthesized by Dr. Bielawska Alicja’s lab. The compounds GRU1-20 were synthesized by Dr. Iryna Lebedyeva’s lab. The IC50 of LCL compounds were measured by the fluorescence-based assay as described in the Figure S7A. GRU compounds were not further studied due to no activity in inhibition of Src myristoylation shown in Figure S8C.

**Figure S10.** **Loss of myristoylation inhibits the localization of Src kinase at the cytoplasmic membrane. (A-D)** Expression levels of Src kinase in the cytosol (Cyt) and total membrane (TM) fractions were determined by immunoblotting. SYF1 (Src-/-Yes-/-Fyn-/-) cells stably expressed **(A)** Src(WT)/Src(G2A), **(B)** Src(Y529F)/Src(Y529F/G2A), control vector (-/-), **(C)** Fyn(WT)/Fyn (G2A), or **(D)** Fyn(Y528F)/Fyn(Y528F/G2A). Cell lysates were fractionated into cytosolic and membrane fractions. Caveolin-1 and GAPDH were used as markers for TM and Cyt fractions, respectively.The ratio of Src or Fyn in the membrane versus the cytosol fraction was much higher in Src(WT)/Src(Y529F) compared with Src(G2A)/Src(Y529F/G2A), or in Fyn(WT)/Fyn(Y528F) compared with Fyn(G2A)/Fyn(Y528F/G2A). **(E)** Localization of Src(Y529F) and Src(Y529F/G2A) in lipid rafts by sucrose gradient centrifugation and immunoblotting. Following centrifugation, twelve fractions were collected starting from the top (#4) to bottom (#12). Src kinase, Fak, and Caveolin-1 (lipid raft marker) were detected in equal aliquots of each fraction by immunoblotting. Src kinase could not be detected in fractions 1-3 (not shown).

**Figure S11. The myristoyl-CoA analog, B13 has limited effect in PNT2 normal prostate cells and 293T cells. (A)** PNT2 cells were cultured in the medium with 0, 1, 5, 10, or 15 μM B13. The media with compound were replaced daily and cell viability was determined by the MTT assay. **(B)** PNT2 cells were cultured in the medium with 0 or 15 μM B13 for 3 days. The cells were collected and stained with propidium iodide for cell cycle analysis. The percentage of cells in G0/G1, S, and G2/M phases were recorded by flow cytometry. **(C)** PNT2 cells treated with 0, 1, 5, 10, or 15 μM B13 were analyzed for the expression of CDK2, CDK6, cyclin D1, p27, and tubulin. **(D)** 293T cells were cultured with 0, 1, 5, 10, 15 μM B13 for 5 days. Cell viability was determined by the MTT assay. B13 showed limited inhibition on 293T cells. N.S. no significant; \*: p<0.05; \*\*:p<0.01.

**Figure S12. The additive effect of B13 with knockdown of Src kinase in regulating proliferation of 22Rv1 and PC-3 cells.** 22Rv1 andPC-3 cancer cells were transduced with control or shRNA-Src, and treated with or without B13. Proliferation was measured by the MTT assay. **(A)** The inhibition of the proliferation of PC-3 cells in B13+shRNA-Src group were lower than shRNA-Src or B13 group, suggesting B13 had additive effect with shRNA-Src, but was largely over-lapped. **(B)** The inhibition on the proliferation of 22Rv1 cells in B13+shRNA-Src group had no significant difference in comparison with shRNA-Src group, suggesting B13 had no additive effect with shRNA-Src in 22Rv1 cells. Collectively, consistent with genetic knockout presented in Figure 2E, inhibition by B13 on NMT activity largely overlapped with shRNA-Src. \*\*: p<0.01 (each treatment group was compared with the control group). #: p<0.05; ##:p<0.01 (the compared groups were indicated in the figure).

**Figure S13. Effects of B13 in host mice. (A-B)** PC-3 prostate cancer cells were subcutaneously injected into both flank sides of SCID mice (3 months-old, n=6 per group). Vehicle or B13 (75mg/kg/mouse) was administered intravenously twice a week for 6 weeks. The body weight before and after B13 treatment (A) and the weight of major organs (the liver, heart, lung, and kidney) with/without B13 treatment (B) were measured as mean + SEM. N.S.: no significant difference.