A new role for the SRC family kinase HCK as a driver of SYK activation in MYD88 mutated lymphomas

Tracking no: ADV-2021-006147R1

Manit Munshi (Dana Farber Cancer Institute, United States) Xia Liu (Dana Farber Cancer Institute, United States) Amanda Kofides (Dana Farber Cancer Institute, United States) Nicholas Tsakmaklis (Dana Farber Cancer Institute, United States) Maria Luisa Guerrera (Dana Farber Cancer Institute, United States) Zachary Hunter (Dana Farber Cancer Institute, United States) Maria Lia Palomba (Memorial Sloan Kettering Cancer Center, United States) Kimon Argyropoulos (Memorial Sloan Kettering Cancer Center, United States) Christopher Patterson (Dana Farber Cancer Institute, United States) Alexa Canning (Dana Farber Cancer Institute, United States) Catherine Flynn (Dana Farber Cancer Institute, United States) Karen Branch (Massachusetts General Hospital, United States) Andrew Branagan (Massachusetts General Hospital, United States) Joshua Gustine (Boston University Medical Center, United States) Andrew Eriksson (Dana Farber Cancer Institute, United States) Josefa Pedraza (Dana Farber Cancer Institute, United States) Jinhua Wang (Dana Farber Cancer Institute, United States) Douglas Karp (Dana Farber Cancer Institute, United States) Kenneth Anderson (Dana Farber Cancer Institute, United States) Steven Treon (Dana Farber Cancer Institute, United States) Guang Yang (Blue Print Medicines, United States)

Abstract:
The SRC family kinase (SFK) HCK is transcriptionally upregulated and activated by mutated MYD88 (MYD88Mut), a key adaptor for Toll-receptor signaling. HCK activates BTK, AKT and ERK in MYD88Mut lymphoma cells. SYK, a BCR component is activated in MYD88Mut lymphoma cells. While the SFK LYN serves as a trigger for SYK activation in MYD88Mut ABC DLBCL cells, LYN activity is muted in MYD88Mut WM cells. We therefore investigated a role for HCK in mediating SYK activation. Over-expression of wild-type (HCKWT) or gatekeeper mutated (HCKThr333Met) HCK in MYD88Mut lymphoma cells triggered SYK activation. Conversely, HCK knockdown reduced p-SYK in MYD88Mut lymphoma cells. Co-immunoprecipitation experiments showed that HCK was complexed with p-SYK in MYD88Mut BCWM.1 and TMD8 cells, but not in MYD88 wild-type (WT) Ramos cells. Rescue experiments in MYD88Mut lymphoma cells expressing HCKThr333Met led to persistent HCK and SYK activation and resistance to the HCK inhibitor A419259. Treatment of primary MYD88Mut WM cells with A419259 reduced p-HCK and p-SYK expression. Taken together, our findings show that SYK is activated by HCK in MYD88Mut B-cell lymphomas cells; broaden the pro-survival signaling generated by aberrant HCK expression in response to MYD88Mut; and help define HCK as an important therapeutic target in MYD88Mut B-cell lymphomas.

Conflict of interest: COI declared - see note
COI notes: ST has received research funding, consulting fees, and/or honoraria from Pharmacyclics Inc., Janssen Oncology Inc., Beigene, X4 Pharmaceuticals, BMS, and Eli Lilly. JJC has received research funds and/or consulting fees from Abbvie, Beigene, Janssen, Pharmacyclics, Roche and TG Therapeutics. NSG is a founder, science advisory board member and equity holder in Syros, C4, Allorion, Jengu, B2S, Inception, EcCys, Larkspur (board member) and Soltego (board member). The Gray lab receives or has received research funding from Novartis, Takeda, Astellas, Taiho, Jansen, Kinogen, Arbella, Deerfield and Sanofi. JW is a consultant for Soltego. SJB is a member of the SAB of Adenoid Cystic Carcinoma Foundation. The Buhrlage lab has received research funding from AbbVie and Kinogen and in-kind resources from Novartis Institutes for Biomedical Research. ARB has received consulting fees from Adaptive Biotechnologies, Beigene, CSL Behring, Karyopharm, Pharmacyclics, Sanofi-Genzyme. ST, SB, JW, NSG and GY are named on patents owned by Dana Farber Cancer Institute for development of HCK targeted therapeutics, including KIN-8194. GY is currently employed by Blueprint. All work herein was performed before GY went to Blueprint. KCA is a consultant for Pfizer, Amgen, Astrazeneca, Janssen Oncology, Precision Biosciences, Mana, Window and a Founder and stock shareholder of C4 Therapeutics, Oncopep, Raqia, and NextRNA. NM is on the advisory boards and consultant to BMS, Janssen, Amgen, Takeda, AbbVie, Oncopep, Karyopharm, Adaptive Biotechnology, Legend, Raqia and Novartis, and holds equity ownership in Oncopep.

Preprint server: No;

Author contributions and disclosures: GY, MM and SPT conceived and designed the experiments and wrote the manuscript. GY and MM performed the data analysis; MM performed PhosFlow, immunoblotting and co-immunoprecipitation assays; XL maintained cell lines and performed lentiviral expression and knockdown studies. AK NT, MLG processed patient and healthy donor samples and performed CD19+ cell isolation. ZRH provided informatics support. MLP, KVA provided support of BCR signaling in WM. JW, SB, NSG provided medicinal chemistry input and drug development. JJC, CJP, KN, AK, ARB, CF, SS, JG, and SPT provided patient care, obtained consent and samples. NM and KCA provided data review and input into the writing of this manuscript.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Authors agree to share renewable materials, de-identified data sets and protocols for experiments upon request.

Clinical trial registration information (if any):
A new role for the SRC family kinase HCK as a driver of SYK activation in MYD88 mutated lymphomas

Manit Munshi,¹ Xia Liu,¹ Amanda Kofides,¹ Nickolas Tsakmaklis,¹ Maria Luisa Guerrera,¹ Zachary R. Hunter,¹,² M. Lia Palomba,³ Kimon V. Argyropoulos,³ Christopher J. Patterson,¹ Alexa G. Canning,¹ Kirsten Meid,¹ Joshua Gustine,¹ Andrew R. Branagan,⁴ Catherine A. Flynn,¹,² Shayna Sarosiek,¹,² Jorge J. Castillo,¹,² Jinhua Wang,⁵ Sara J. Buhrlage,⁵ Nathanael S. Gray,⁶ Nikhil C. Munshi,²,⁶ Kenneth C. Anderson,²,⁶ Steven P. Treon,¹,² and Guang Yang¹,²

¹Bing Center for Waldenstrom’s Macroglobulinemia, Dana Farber Cancer Institute and Harvard Medical School; ²Department of Medical Oncology, Dana Farber Cancer Institute and Harvard Medical School, Boston MA, USA; ³Lymphoma Service, Memorial Sloan Kettering Cancer Center, New York NY, USA; ⁴Division of Hematology and Oncology, Massachusetts General Hospital, Harvard Medical School, Boston MA, USA; ⁵Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston MA, USA; ⁶Jerome Lipper Multiple Myeloma Center, Dana Farber Cancer Institute, Boston MA, USA.

Corresponding author:
Steven P. Treon, M.D., Ph.D.
Bing Center for Waldenström’s Macroglobulinemia
Dana Farber Cancer Institute
M548, 450 Brookline Avenue, Boston, MA 02115 USA
Tel: (617) 632-2681 Fax: (617) 632-4862
Email: steven_treon@dfci.harvard.edu

Short Title: HCK activates SYK in MYD88 mutated B-cell lymphoma
Abstract Word Count: 200
Text Word Count: 1604
Tables: 0
Figures: 2
Suppl. Figures: 2
**Key Words:** MYD88, HCK, SYK, Waldenstrom Macroglobulinemia, ABC DLBCL.

**Key Points**

- HCK facilitates TLR/BCR crosstalk through activation of SYK in response to mutated MYD88.
- The HCK inhibitor A419259 selectively blocks SYK activation in MYD88 mutated cell lines and primary WM lymphoplasmacytic cells.

**Abstract**

The SRC family kinase (SFK) HCK is transcriptionally upregulated and activated by mutated MYD88 (MYD88\textsuperscript{Mut}), a key adaptor for Toll-receptor signaling. HCK activates BTK, AKT and ERK in MYD88\textsuperscript{Mut} lymphomas. SYK, a BCR component is activated in MYD88\textsuperscript{Mut} lymphoma cells. While the SFK LYN serves as a trigger for SYK activation in MYD88\textsuperscript{Mut} ABC DLBCL cells, LYN activity is muted in MYD88\textsuperscript{Mut} WM cells. We therefore investigated a role for HCK in mediating SYK activation. Over-expression of wild-type (HCK\textsuperscript{WT}) or gatekeeper mutated (HCK\textsuperscript{Thr333Met}) HCK in MYD88\textsuperscript{Mut} lymphoma cells triggered SYK activation. Conversely, HCK knockdown reduced p-SYK in MYD88\textsuperscript{Mut} lymphoma cells. Co-immunoprecipitation experiments showed that HCK was complexed with p-SYK in MYD88\textsuperscript{Mut} BCWM.1 and TMD8 cells, but not in MYD88 wild-type (WT) Ramos cells. Rescue experiments in MYD88\textsuperscript{Mut} lymphoma cells expressing HCK\textsuperscript{Thr333Met} led to persistent HCK and SYK activation and resistance to the HCK inhibitor A419259. Treatment of primary MYD88\textsuperscript{Mut} WM cells with A419259 reduced p-HCK and p-SYK expression. Taken together, our findings show that SYK is activated by HCK in MYD88\textsuperscript{Mut} B-cell lymphomas cells; broaden the pro-survival signaling generated by aberrant HCK expression in response to MYD88\textsuperscript{Mut}, and help define HCK as an important therapeutic target in MYD88\textsuperscript{Mut} B-cell lymphomas.
Introduction

Activating MYD88 mutations are common in B-cell malignancies including Waldenstrom’s Macroglobulinemia (WM) and activated B-cell diffuse large B-cell lymphoma (ABC DLBCL). MYD88 is a component of the Toll-like receptor (TLR) pathway. Mutated MYD88 (MYD88\textsuperscript{Mut}) triggers assembly of a “Myddosome” complex leading to downstream pro-survival signaling that includes IRAK4/IRAK1 triggered NF-κB and HCK mediated BTK/NF-κB, PI3K/AKT, and MAPK/ERK signaling.\textsuperscript{1-5} HCK is upregulated in response to mutated MYD88 signaling that includes STAT3, NF-KB and the AP-1 complex component JunB in the presence of PAX5.\textsuperscript{6}

The activation of the B-cell receptor (BCR) signaling component SYK has also been observed in MYD88\textsuperscript{Mut} WM.\textsuperscript{7} Knockdown of MYD88 or a MYD88 signaling inhibitor abrogated SYK activation, while over-expression of mutated but not wild-type (WT) MYD88 amplified SYK activation in MYD88\textsuperscript{Mut} and MYD88\textsuperscript{WT} lymphoma cells.\textsuperscript{8} Importantly, knockdown of SYK or SYK inhibitors blocked p-STAT3 and p-AKT signaling and decreased viability of MYD88\textsuperscript{Mut} lymphoma cells.\textsuperscript{8} In ABC DLBCL, chronic active BCR signaling underlies SYK activation, and knockdown of SYK decreases cell viability, including those harboring MYD88 and CD79 mutations.\textsuperscript{9} While a role for the SFK LYN has been proposed to trigger BCR/SYK activation in ABC DLBCL, other findings suggest an inhibitory role through BCR down-modulation.\textsuperscript{9-11} Herein, we investigated if HCK, a SFK that is normally down-regulated in late-stage B-cell ontogeny, and transcriptionally upregulated and activated by MYD88\textsuperscript{Mut} could trigger SYK activation, and thereby facilitate TLR/BCR crosstalk.\textsuperscript{4,11,12}

Methods

Cell lines and drug treatment
**Patient samples and drug treatment**

Bone marrow mononuclear cells (BMMCs) were isolated as before and 2×10⁶ BMMCs treated for 1-2 hours with ibrutinib or A419259. PhosFlow analyses were performed on CD20⁺ lymphoplasmacytic lymphoma cells (LPCs), and western blotting on CD19⁺ LPCs as before. MYD88 genotyping was performed by AS-PCR. CXCR4 mutation status was determined by AS-PCR and Sanger sequencing. Healthy donor (HD) CD19⁺ B-cells from peripheral blood mononuclear cells (PBMCs) were used as controls for LYN and p-LYN assessments. Sample use was approved by DF/HCC IRB following written consent.

**Transcriptome and copy number analysis for LYN expression**

Gene expression for LYN was determined in 57 WM patients by validated next generation RNA sequencing as previously reported. MYD88 and CXCR4 mutation status for these patients was determined as described above. Findings were compared to LYN expression in healthy donor peripheral blood B-cells, memory B-cells and plasma cells. Copy number analysis for LYN was performed as before.

**HCK knockdown and overexpression studies**

HCK knockdown or overexpression of wild-type (HCKWT) or gatekeeper mutant of HCK (HCKThr³³³Met) was performed using lentiviral expression vectors as previously described.
**Signaling studies**

Cells were treated for 1-2 hours prior to PhosFlow and western blot analysis. Alexa Fluor® 647-conjugated p-SYK(Tyr525/Tyr526), APC-Cy7-conjugated CD20 (BD Biosciences, San Jose, CA), and p-HCK(Tyr410) combined with DyLight® 650-conjugated goat F(ab’)2 anti-rabbit IgG antibody (Abcam, Cambridge, MA) were used for PhosFlow analysis. Western blotting was performed using antibodies to p-SYK(Tyr525/Tyr526) (R&D Systems), p-LYN(Tyr396) (GeneTex Inc.), SYK, LYN, p-AKT(Ser473), AKT, p-ERK1/2(Thr202/Tyr204), ERK1/2, and HCK (Cell Signaling Technologies, MA).

**Co-immunoprecipitation studies**

Co-immunoprecipitation (co-IP) studies were performed as previously described using an HCK antibody (Cell Signaling Technologies).

**Statistical Analysis**

Pairwise comparisons using Wilcoxon rank sum exact test was performed for gene expression from transcriptome analysis.

**Results and Discussion**

**p-LYN expression is downregulated in MYD88 WM cells**

In previous studies, the SFK LYN was shown to be a trigger for SYK activation, and in response to chronic BCR activation in ABC DLBCL. By next generation sequencing, LYN shows variable expression in WM patients, and was significantly lower in LPCs from MYD88Mut patients who are CXCR4WT when compared to expression levels in healthy donor peripheral and memory B-cells (Supplemental Figure 1A). Conversely,
no difference in LYN expression was observed between LPCs from MYD88\textsuperscript{Mut} and CXCR4\textsuperscript{Mut} patients and healthy donor peripheral and memory B-cells.

To clarify the activation state of LYN in MYD88 mutated lymphoma cells, we examined p-LYN expression. MYD88\textsuperscript{Leu265Pro} mutated TMD8, HBL-1 and OCI-Ly3 ABC DLBCL cells showed strong expression of p-LYN, a finding previously attributed to chronic BCR signaling.\textsuperscript{9} Conversely, p-LYN expression was low in MYD88\textsuperscript{Leu265Pro} mutated BCWM.1 and MWCL-1 WM cells, and SU-DHL-2 ABC DLBCL cells that carry an MYD88\textsuperscript{Ser222Arg} mutation (Figure 1A). Moreover, p-LYN expression was absent or low in CD19-selected BM primary LPCs from 6 MYD88\textsuperscript{Leu265Pro} WM patients, including three of whom amply expressed LYN protein. Copy number analysis showed no remarkable alterations in LYN expression (Supplemental Figure 1B). By comparison, LYN was uniformly expressed in CD19-selected peripheral blood B-cells from 6 healthy donors and showed robust expression of p-LYN (Figure 1B). Taken together, the above findings show variable expression for LYN in MYD88 mutated WM patients, though CXCR4 mutation status impacted LYN expression levels. While copy number loss of LYN is common in WM, such loss is typically subclonal and not impacted by CXCR4 mutation status.\textsuperscript{16} Our copy number findings for LYN are consistent with these prior observations. Expression differences in LYN may therefore be related to epigenomic (methylation) changes that accompany CXCR4 mutation status and tumor differentiation.\textsuperscript{17,18} Indeed, lower expression levels of LYN were observed in healthy donor plasma cells (Supplemental Figure 1A) consistent with the known down-regulation of BCR pathway that accompanies B-cell to plasma cell differentiation. As MYD88\textsuperscript{MUT}CXCR4\textsuperscript{WT} versus MYD88\textsuperscript{MUT}CXCR4\textsuperscript{MUT} WM cells show more advanced plasmacytic differentiation, the finding of lower LYN expression in this subgroup is not surprising.\textsuperscript{15,18} Importantly, the lack of SFK LYN activity observed in primary MYD88\textsuperscript{MUT} WM samples (including one that was CXCR4\textsuperscript{MUT}) suggests that LYN is unlikely to mediate SYK activation in MYD88\textsuperscript{Leu265Pro} WM but may be its driver in MYD88\textsuperscript{Leu265Pro} DLBCL cells due to chronic active BCR signaling.\textsuperscript{9}

**HCK modulates SYK phosphorylation in MYD88 mutated WM cells.**
We next investigated a direct role for the SFK HCK in mediating SYK activation. We over-expressed HCK in MYD88Leu265Pro BCWM.1 and MWCL-1 WM cell lines, and TMD8 ABC DLBCL cells. In all three cell lines, over-expression of HCK triggered a robust increase in phosphorylation of SYK^{Tyr525/Tyr526} versus vector-only transduced cells (Figure 1C). Moreover, using an inducible vector system, HCK knockdown markedly reduced SYK^{Tyr525/Tyr526} phosphorylation in BCWM.1 WM and TMD8 ABC DLBCL cells (Figure 1D). In both experiments, total SYK levels remained unchanged (Figures 1C, D).

**Activated SYK is complexed with HCK in MYD88 mutated B-cell lymphoma cells.**

To clarify if HCK and activated SYK were present in the same signaling complex, we performed co-immunoprecipitation (Co-IP) experiments using an HCK antibody in MYD88Mut BCWM.1, TMD8 and MYD88WT Ramos cells. The HCK antibody effectively pulled down p-SYK in MYD88Mut BCWM.1 and TMD8 cells, but not in MYD88WT Ramos cells (Figure 1E).

**HCK kinase activity is responsible for SYK activation in MYD88 B-cell lymphoma cells.**

In previous studies we showed that A419259, a potent toolbox inhibitor of HCK, shows selective killing of MYD88 mutated lymphoma cells. To confirm whether HCK kinase activity triggered SYK activation, we performed rescue experiments with A419259 in MYD88 mutated BCWM.1 and MWCL-1 WM and TMD8 ABC DLBCL cells expressing either HCK^{WT} or HCK gatekeeper mutated HCK^{Thr333Met} that abrogates A419259 binding. BCWM.1 and MWCL-1 cells transduced to express HCK^{Thr333Met} protein showed a >2 log-fold increase in resistance to A419259 versus those transduced with either vector alone or HCK^{WT} protein. By PhosFlow analysis, expression of HCK^{Thr333Met} but not HCK^{WT} led to persistent activation of HCK and SYK in the presence of A419259 in BCWM.1 (Figures 2A, 2B) and MWCL-1 (Figures 2C, 2D) WM cells, and TMD8.
(Figures 2E, 2F) ABC DLBCL cells. Consistent with these observations, treatment of primary MYD88\textsuperscript{Mut} WM LPCs cells with A419259 also abrogated both HCK and SYK phosphorylation (Figure 2G).

Taken together, the above studies support the activation of SYK by the SFK HCK in MYD88\textsuperscript{Mut} B-cell lymphomas cells. The paucity of LYN activation in MYD88\textsuperscript{Leu2655Pro} WM cells may suggest that in WM, the primary functional trigger for SYK activation may involve the SFK HCK whereas in MYD88\textsuperscript{Leu265Pro} DLBCL both SFKs (LYN and HCK) may contribute to SYK activation. Our findings are consistent with those of Phelan et al,\textsuperscript{19} who identified a MYD88-TLR9-BCR super complex as a driver of BCR signaling in ABC DLBCL, and potentially extend those observations by identifying mutated MYD88 directed HCK as an enabler of SYK activation. In deference to ABC DLBCL wherein chronic active BCR signaling is known to trigger SYK through activation of LYN, we did not find evidence for LYN activation in MYD88\textsuperscript{Mut} WM cells.\textsuperscript{9,10} This previously unrecognized finding may also argue against chronic active BCR signaling in WM, though further studies are needed to clarify this point. Our findings further broaden the role played by aberrant HCK expression in promoting MYD88\textsuperscript{Mut} pro-survival signaling, that previously included BTK, ERK and AKT (Supplemental Figure 1). The downstream consequences of HCK may also be relevant in non-MYD88\textsuperscript{Mut} driven diseases such as mantle cell lymphoma, wherein HCK is activated.\textsuperscript{20}

The recognition that HCK underlies SYK activation in MYD88\textsuperscript{Mut} B-cell lymphomas may also be therapeutically relevant. A novel HCK inhibitor KIN-8194 with greater kinome selectivity and better tolerance over A419259 was more active versus ibrutinib in MYD88\textsuperscript{Mut} lymphoma xenograft models.\textsuperscript{21} KIN-8194 also blocked SYK, suggesting that a broader shut down of MYD88 pro-survival signaling may be achieved with HCK inhibitors (Supplemental Figure 1). In summary, our findings show that SYK can be activated by HCK; broaden the pro-survival signaling generated by aberrant HCK expression in response to MYD88\textsuperscript{Mut}; and help further establish the SFK HCK as a relevant therapeutic target in MYD88\textsuperscript{Mut} B-cell lymphomas.
Data Sharing Statement

Authors agree to share renewable materials, de-indentified data sets and protocols for experiments upon request (steven_treon@dfci.harvard.edu).

Acknowledgments

The authors gratefully acknowledge the generous support of Peter Bing M.D., the International Waldenstrom’s Macroglobulinemia Foundation, the Leukemia and Lymphoma Society (Grant: R6507-18), the NIH SPORE in Multiple Myeloma (Grant: 2P50CA100707-16A1), the Edward and Linda Nelson Fund for WM Research, the Kerry Robertson Fund for WM Research, the Bauman Family Trust, the Siegel Family Fund for WM, and the WM patients who provided samples for these studies.

Authorship

GY, MM and SPT conceived and designed the experiments and wrote the manuscript. GY and MM performed the data analysis; MM performed PhosFLow, immunoblotting and co-immunoprecipitation assays; XL maintained cell lines and performed lentiviral expression and knockdown studies. AK NT, MLG processed patient and healthy donor samples and performed CD19+ cell isolation. ZRH provided informatics support. MLP, KVA provided support of BCR signaling in WM. JW, SB, NSG provided medicinal chemistry input and drug development. JJC, CJP, KM, AK, ARB, CF, SS, JG, and SPT provided patient care, obtained consent and samples. NM and KCA provided data review and input into the writing of this manuscript.

Disclosure of Conflicts of Interest

ST has received research funding, consulting fees, and/or honoraria from Pharmacyclics Inc., Janssen Oncology Inc., Beigene, X4 Pharmaceuticals, BMS, and
Eli Lilly. JJC has received research funds and/or consulting fees from Abbvie, Beigene, Janssen, Pharmaciescics, Roche and TG Therapeutics. NSG is a founder, science advisory board member and equity holder in Syros, C4, Allorion, Jengu, B2S, Inception, EoCys, Larkspur (board member) and Soltego (board member). The Gray lab receives or has received research funding from Novartis, Takeda, Astellas, Taiho, Jansen, Kinogen, Arbella, Deerfield and Sanofi. JW is a consultant for Soltego. SJB is a member of the SAB of Adenoid Cystic Carcinoma Foundation. The Buhrlage lab has received research funding from AbbVie and Kinogen and in-kind resources from Novartis Institutes for Biomedical Research. ARB has received consulting fees from Adaptive Biotechnologies, Beigene, CSL Behring, Karyopharm, Pharmaciescics, Sanofi-Genzyme. ST, SB, JW, NSG and GY are named on patents owned by Dana Farber Cancer Institute for development of HCK targeted therapeutics, including KIN-8194. GY is currently employed by Blueprint. All work herein was performed before GY went to Blueprint. KCA is a consultant for Pfizer, Amgen, Astrazeneca, Janssen Oncology, Precision Biosciences, Mana, Window and a Founder and stock shareholder of C4 Therapeutics, Oncopep, Raqia, and NextRNA. NM is on the advisory boards and consultant to BMS, Janssen, Amgen, Takeda, AbbVie, Oncopep, Karyopharm, Adaptive Biotechnology, Legend, Raqia and Novartis, and holds equity ownership in Oncopep.
References

1. Ngo VN, Young RM, Schmitz R, et al. Oncogenically active MYD88 mutations in human lymphoma. Nature. 2011;470(7332):115-119.
2. Treon SP, Xu L, Yang G, et al. MYD88 L265P somatic mutation in Waldenstrom's macroglobulinemia. N Engl J Med. 2012;367(9):826-833.
3. Yang G, Zhou Y, Liu X, et al. A mutation in MYD88 (L265P) supports the survival of lymphoplasmacytic cells by activation of Bruton tyrosine kinase in Waldenstrom macroglobulinemia. Blood. 2013;122(7):1222-1232.
4. Yang G, Buhrlage SJ, Tan L, et al. HCK is a survival determinant transactivated by mutated MYD88, and a direct target of ibrutinib. Blood. 2016;127(25):3237-3252.
5. Chen JG, Liu X, Munshi M, et al. BTK(Cys481Ser) drives ibrutinib resistance via ERK1/2 and protects BTK(wild-type) MYD88-mutated cells by a paracrine mechanism. Blood. 2018;131(18):2047-2059.
6. Liu X, Chen JG, Munshi M, et al. Expression of the prosurvival kinase HCK requires PAX5 and mutated MYD88 signaling in MYD88-driven B-cell lymphomas. Blood Adv. 2020;4(1):141-153.
7. Argyropoulos KV, Vogel R, Ziegler C, et al. Clonal B cells in Waldenstrom's macroglobulinemia exhibit functional features of chronic active B-cell receptor signaling. Leukemia. 2016;30(5):1116-1125.
8. Munshi M, Liu X, Chen JG, et al. SYK is activated by mutated MYD88 and drives pro-survival signaling in MYD88 driven B-cell lymphomas. Blood Cancer J. 2020;10(1):12.
9. Davis RE, Ngo VN, Lenz G, et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature. 2010;463(7277):88-92.
10. Kurosaki T, Takata M, Yamanashi Y, et al. Syk activation by the Src-family tyrosine kinase in the B cell receptor signaling. J. Exp. Med. 1994;179(5):1725-9.
11. Mansueto MS, Reens A, Rakhlina L, Chi A, Pan BS, Miller JR. A reevaluation of the spleen tyrosine kinase (SYK) activation mechanism. J Biol Chem. 2019;294(19):7658-7668.
12. Taguchi T, Kiyokawa N, Sato N, et al. Characteristic expression of Hck in human B-cell precursors. Exp. Hematol. 2000; 28:55-64.
13. Xu L, Hunter ZR, Yang G, et al. MYD88 L265P in Waldenstrom macroglobulinemia, immunoglobulin M monoclonal gammopathy, and other B-cell lymphoproliferative
disorders using conventional and quantitative allele-specific polymerase chain reaction. Blood. 2013;121(11):2051-2058.
14. Xu L, Hunter ZR, Tsakmaklis N, et al. Clonal architecture of CXCR4 WHIM-like mutations in Waldenström Macroglobulinaemia. Br J Haematol 2016; 172:735–44.
15. Hunter ZR, Xu L, Yang G, et al. Transcriptome sequencing reveals a profile that corresponds to genomic variants in Waldenstrom Macroglobulinemia. Blood 2016; 128:827-38.
16. Hunter ZR, Xu L, Yang G, et al. The genomic landscape of Waldenstrom macroglobulinemia is characterized by highly recurring MYD88 and WHIM-like CXCR4 mutations, and small somatic deletions associated with B-cell lymphomagensis. Blood 2014; 123(11):1637-46.
17. Roos-Weil D, Giacopelli B, Arman M, et al. Identification of 2 DNA methylation subtypes of Waldenström macroglobulinemia with plasma and memory B-cell features. Blood 2020;136(5):585-595.
18. Hunter ZR, Treon SP. Epigenomics in Waldenstrom Macroglobulinemia.Blood 2020; 136(5): 527–529.
19. Phelan JD, Young RM, Webster DE, et al. A multiprotein supercomplex controlling oncogenic signalling in lymphoma. Nature 2018; 560:387-391.
20. Lantermans HC, Minderman M, Kuil A, et al. Identification of the SRC-family tyrosine kinase HCK as a therapeutic target in mantle cell lymphoma. Leukemia 2021; 35(3):881-86.
21. Yang G, Wang J, Tan L, et al. The HCK/BTK inhibitor KIN-8194 is active in MYD88 driven lymphomas and overcomes mutated BTKCys481 ibrutinib resistance. Blood 2021; doi: 10.1182/blood.2021011405. Online ahead of print.
22. Schaeffer M, Schneiderbauer M, Weideler S, et al. Signaling through a novel domain of gp130 mediates cell proliferation and activation of Hck and Erk kinases. Mol Cell Biol 2001; 21(23):8068-81.
23. Podar K, Mostoslavsky G, Sattler M, et al. Critical Role for Hematopoietic Cell Kinase (Hck)-mediated Phosphorylation of Gab1 and Gab2 Docking Proteins in Interleukin 6-induced Proliferation and Survival of Multiple Myeloma Cells. J. Biol. Chem. 279(20):21658-65.
24. Hodge LS, Ziesmer SC, Yang ZZ, et al. Constitutive activation of STAT5A and STAT5B regulates IgM secretion in Waldenstrom’s macroglobulinemia. Blood 2014; 123(7):1055-8.
Figure Legends

Figure 1. The SRC family kinase HCK activates SYK in MYD88 mutated lymphomas cells. (A) Expression of p-LYN\textsuperscript{Tyr396} levels by western blot analysis in MYD88\textsuperscript{L265P} BCWM.1, MWCL-1 WM cells, TMD8, HBL-1, OCI-Ly3; MYD88\textsuperscript{Ser222Arg} SU-DHL-2 ABC DLBCL cells; and MYD88\textsuperscript{WT} OCI-Ly7, OCI-Ly19 GCB DLBCL cells, Ramos Burkitt’s lymphoma cells, RPMI-8226 and MM.1S multiple myeloma cells. The expression levels of total LYN in these cells as well as protein loading control GAPDH are also shown. (B) p-LYN\textsuperscript{Tyr396} levels by western blot analysis in CD19-selected bone marrow lymphoplasmacytic cells (LPC) from 6 MYD88\textsuperscript{Leu265Pro} WM patients of whom WM1 was also CXCR4\textsuperscript{Mut} and WM2-6 were CXCR4\textsuperscript{WT}, and CD19-selected peripheral blood (PB) B-cells from 6 healthy donors; lysates from OCI-Ly7 GCB DLBCL cells were used for p-LYN and protein loading control. The expression of total LYN is also shown. (C) Expression of p-SYK\textsuperscript{Tyr525/Tyr526} levels by western blot analysis in vector-only, HCK\textsuperscript{WT} or HCK\textsuperscript{Thr333Met} transduced BCWM.1, MWCL-1 WM cells and TMD8 ABC DLBCL cells. Expression levels of total HCK, SYK in these cells as well as GAPDH for protein loading control are shown. (D) Changes in p-SYK\textsuperscript{Tyr525/Tyr526} and p-ERK1/2\textsuperscript{Thr202/Tyr204} levels following HCK knockdown with doxycycline inducible shRNA1 and shRNA2 or scrambled control vector in BCWM.1 WM and TMD8 ABC DLBCL cells. p-SYK\textsuperscript{Tyr525/Tyr526} and p-ERK1/2\textsuperscript{Thr202/Tyr204} levels were detected at day 9 following 1.0 µg/ml doxycycline induction. p-ERK1/2\textsuperscript{Thr202/Tyr204} a known downstream signaling component of HCK served as a positive control for these experiments. Expression levels of total HCK, SYK and ERK1/2 as well as GAPDH for protein loading control are also shown. (E) p-
SYK\textsuperscript{Y525/526} protein levels by western blot analysis following a co-immunoprecipitation (co-IP) with HCK protein in MYD88\textsuperscript{Mut} BCWM.1 and TMD8 cells, and MYD88\textsuperscript{WT} Ramos cell lysates. Magnetic beads only and rabbit IgG were used as co-IP experimental controls. HCK total protein was also shown as an indication of the co-IP efficiency in these cells. IgG heavy chain was shown as an indication of the quantity of antibodies used in the co-IP experiments. Above experiments were performed at least twice with representative results shown.

Figure 2. SYK activation is driven by the SRC family kinase HCK in MYD88 mutated lymphoma cells. (A) Relative p-HCK\textsuperscript{Tyr410} levels by PhosFlow analysis following the treatment with DMSO or the HCK inhibitor A419259 at the indicated concentrations for 1.0 hour in vector-only, HCK\textsuperscript{WT} or HCK\textsuperscript{Thr333Met} transduced BCWM.1 cells. (B) Relative p-SYK\textsuperscript{Y525/526} levels by PhosFlow analysis following treatment with DMSO or A419259 at indicated concentrations for 1.0 hour in vector-only, HCK\textsuperscript{WT} or HCK\textsuperscript{Thr333Met} transduced BCWM.1 cells. (C) Relative p-HCK\textsuperscript{Tyr410} levels by PhosFlow analysis following the treatment with DMSO or A419259 at indicated concentrations for 1.0 hour in vector-only, HCK\textsuperscript{WT} or HCK\textsuperscript{Thr333Met} transduced MWCL-1 cells. (D) Changes in p-SYK\textsuperscript{Tyr525/Tyr526} levels following the treatment with DMSO or A419259 at indicated concentrations for 1.0 hour in vector-only, HCK\textsuperscript{WT} or HCK\textsuperscript{Thr333Met} transduced MWCL-1 cells. The expression levels of total SYK in these cells as well as protein loading control GAPDH are also shown. (E) Relative p-HCK\textsuperscript{Tyr410} levels by PhosFlow analysis following the treatment with DMSO or A419259 at indicated concentrations for 1.0 hour in vector only, HCK\textsuperscript{WT} or HCK\textsuperscript{Thr333Met} transduced TMD8 cells. (F) Changes in p-SYK levels following the treatment with DMSO or A419259 at indicated concentrations for 1.0 hour in vector-only, HCK\textsuperscript{WT} or HCK\textsuperscript{Thr333Met} transduced TMD8 cells. The expression levels of total SYK in these cells as well as protein loading control GAPDH are also shown. (G) p-HCK\textsuperscript{Tyr410} levels and p-SYK\textsuperscript{Tyr525/Tyr526} levels by PhosFlow analysis following the treatment with DMSO or A419259 at the indicated concentrations for 1.0
hour in CD20⁺ gated WM patient bone marrow lymphoplasmacytic cells. Above experiments were performed at least twice with representative results shown.
