Selective inhibition reveals the regulatory function of DYRK2 in protein synthesis and calcium entry

Tiantian Wei[1,2,3#], Jue Wang[4#], Ruqi Liang[1,2,4#], Wendong Chen[5#], Yilan Chen[6], Mingzhe Ma[4], An He[7], Yifei Du[4], Wenjing Zhou[8], Zhiying Zhang[1], Xin Zeng[1,4], Chu Wang[1,4], Jin Lu[9,10], Xing Guo[11], Xiaowei Chen[1,8], Youjun Wang[6], Ruijun Tian[5,6*], Junyu Xiao[1,2,3,12*], Xiaoguang Lei[1,2,4,13*]

[1]The State Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, Peking University, Beijing, China.

[2]Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China.

[3]Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100871, China.

[4]Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China.

[5]SUSTech Academy for Advanced Interdisciplinary Studies, Southern University of Science and Technology, Shenzhen 518055, China.

[6]Beijing Key Laboratory of Gene Resource and Molecular Development, Key Laboratory of Cell Proliferation and Regulation Biology, Ministry of Education, College of Life Sciences, Beijing Normal University, Beijing 100875, China

[7]Department of Chemistry, Southern University of Science and Technology, Shenzhen 518055, China.

[8]Institute of Molecular Medicine, Peking University, Beijing 100871, China.

23   [9]Peking University Institute of Hematology, People's Hospital, Beijing 100044, China.

24   [10]Collaborative Innovation Center of Hematology, Suzhou 215006, China.

25   [11]Life Sciences Institute, Zhejiang University, Hangzhou, 310058 China.

26   [12]Beijing Advanced Innovation Center for Genomics (ICG), Peking University, Beijing 100871,

27   China.

28   [13]Institute for Cancer Research, Shenzhen Bay Laboratory, Shenzhen, 518107, China

29   [#]These authors contributed equally

30   [*]Corresponding authors: Ruijun Tian (tianrj@sustech.edu.cn), Junyu Xiao

31   (junyuxiao@pku.edu.cn), Xiaoguang Lei (xglei@pku.edu.cn)

32
33

34 **Abstract:**

35   The dual-specificity tyrosine phosphorylation-regulated kinase DYRK2 has emerged

36 as a critical regulator of cellular processes. We took a chemical biology approach to gain

37 further insights into its function. We developed C17, a potent small-molecule DYRK2

38 inhibitor, through multiple rounds of structure-based optimization guided by several

39 co-crystallized structures. C17 displayed an effect on DYRK2 at a single-digit nanomolar

40 IC_{50} and showed outstanding selectivity for the human kinome containing 467 other

41 human kinases. Using C17 as a chemical probe, we further performed quantitative

42 phosphoproteomic assays and identified several novel DYRK2 targets, including

43 eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and stromal

44 interaction molecule 1 (STIM1). DYRK2 phosphorylated 4E-BP1 at multiple sites, and the

45 combined treatment of C17 with AKT and MEK inhibitors showed synergistic 4E-BP1

46 phosphorylation suppression. The phosphorylation of STIM1 by DYRK2 substantially

47 increased the interaction of STIM1 with the ORAI1 channel, and C17 impeded the

48 store-operated calcium entry process. These studies collectively further expand our

49 understanding of DYRK2 and provide a valuable tool to pinpoint its biological function.

50

51 **Keywords:** DYRK2 , Kinase inhibitor , Quantitative phosphoproteomics , 4E-binding

52 protein 1 , stromal interaction molecule 1

53

54   Dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs) belong to the

55   CMGC group of kinases together with other critical human kinases, such as

56   cyclin-dependent kinases (CDKs) and mitogen-activated protein kinases (MAPKs)

57   (Aranda et al., 2011; Becker and Joost, 1999; Manning et al., 2002). DYRKs uniquely

58   phosphorylate tyrosine residues within their activation loops *in cis* during biosynthesis,

59   although mature proteins display exclusive serine/threonine kinase activities (Lochhead et

60   al., 2005). There are five DYRKs in humans: DYRK1A, DYRK1B, DYRK2, DYRK3, and

61   DYRK4. DYRK1A has been extensively studied due to its potential function in the

62   pathogenesis of Down syndrome and neurodegenerative disorders (Becker and Sippl,

63   2011; Wegiel et al., 2011). DYRK3 has been shown to function as a central "dissolvase" to

64   regulate the formation of membrane-less organelles (Rai et al., 2018; Wippich et al.,

65   2013). On the other hand, DYRK2 is a crucial regulator of 26S proteasome activity (Guo

66   et al., 2016).

67   The 26S proteasome degrades the majority of proteins in human cells and plays a

68   central role in many cellular processes, including the regulation of gene expression and

69   cell division (Collins and Goldberg, 2017; Coux et al., 1996). Recent discoveries have

70   revealed that the 26S proteasome is subjected to intricate regulation by reversible

71   phosphorylation (Guo et al., 2017; Guo et al., 2016; Liu et al., 2020). DYRK2

72   phosphorylates the Rpt3 subunit in the regulatory particle of the proteasome at Thr25,

73   leading to the upregulation of proteasome activity (Guo et al., 2016). DYRK2 is

74   overexpressed in several tumors, including triple-negative breast cancer and multiple

75   myeloma, which are known to rely heavily on proteasome activity for progression, and

76   perturbation of DYRK2 activity impedes cancer cell proliferation and inhibits tumor growth

77   (Banerjee et al., 2018; Banerjee et al., 2019).

78   Our knowledge of the physiological functions of DYRK2 remains in its infancy, and

79   DYRK2 likely has cellular targets in addition to Rpt3. Substrates of many kinases,

80   especially Ser/Thr kinases, remain insufficiently identified. A major obstacle to discovering

81   physiologically relevant substrates of a kinase is the lack of highly specific chemical

82   probes that allow precise modulation of kinase function. Some DYRK2 inhibitors have

83   been reported; however, these compounds also inhibit other kinases, mostly other DYRK

84 family members, to various degrees (Chaikuad et al., 2016; Jouanne et al., 2017). We

85 have recently identified LDN192960 as a selective DYRK2 inhibitor and showed that

86 LDN192960 could alleviate multiple myeloma and triple-negative breast cancer

87 progression by inhibiting DYRK2-mediated proteasome phosphorylation (Banerjee et al.,

88 2019). To obtain even more potent and selective DYRK2 inhibitors, we applied a

89 structure-guided approach to further engineer chemical compounds based on the

90 LDN192960 scaffold. One of the best compounds we generated, compound C17 (C17),

91 displays an effect on DYRK2 at a single-digit nanomolar IC_{50} with moderate to excellent

92 selectivity against kinases closely related to DYRK2. Using this potent DYRK2 inhibitor as

93 a tool, we treated U266 cells with C17. We performed quantitative phosphoproteomic

94 analyses, which led to identifying several novel DYRK2 targets, including eukaryotic

95 translation initiation factor 4E-binding protein 1 (4E-BP1) and stromal interaction molecule

96 1 (STIM1). These results demonstrate that DYRK2 plays critical regulatory roles in

97 multiple cellular processes, including protein translation and store-operated calcium entry,

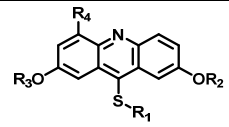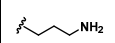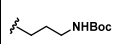98 and indicate that C17 can serve as a valuable probe for the study of DYRK2 function.
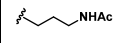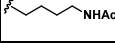
99

100 **Table 1**

101 **Table 1.** The Inhibitory activity and selectivity of acridine analogs of DYRK2.

102 **Table 1-source data 1**

103 Raw data of inhibitors against kinases for Table 1.

104

Cmpd.	R1	R2	R3	R4	IC50 at molecular level (nM)						Selectivity				
					DYRK2	DYRK1A	DYRK1B	DYRK3	Haspin	MARK3	DYRK2&DYRK1A	DYRK2&DYRK1B	DYRK2&DYRK3	DYRK2&Haspin	DYRK2&MARK3
LDN192960	~~~NH2	-CH3	-CH3	-H	53 ± 2	1859 ± 30	2900 ± 39	22 ± 4	18 ± 2	611 ± 19	35	55	~	~	12
1	~~~NHBoc	-CH3	-CH3	-H	38 ± 2	651 ± 29	1401 ± 91	115 ± 4	34 ± 3	36 ± 2	17	17	3	~	~
2	~~~NHAc	-CH3	-CH3	-H	31 ± 1	731 ± 36	1477 ± 128	94 ± 9	27 ± 3	27 ± 5	24	48	3	~	~
3	~~~NHAc	-CH3	-CH3	-H	41 ± 2	1018 ± 78	2495 ± 88	157 ± 18	24 ± 1	33 ± 7	25	61	4	~	~

No.	Structure														
4	(chain)-CN	-CH₃	-CH₃	-H	53 ± 2	964 ± 14	1386 ± 21	234 ± 10	30 ± 1	96 ± 3	18	26	4	~	2
5	piperidine	-CH₃	-CH₃	-H	89 ± 2	1026 ± 96	3488 ± 86	311 ± 22	53 ± 4	91 ± 5	12	39	3	~	1
6	piperidine	-CH₃	-CH₃	-H	20 ± 3	889 ± 131	697 ± 67	110 ± 11	45 ± 3	100 ± 4	44	35	6	2	5
7	piperidine	-CH₃	-CH₃	-CH₂OH	13 ± 1	2844 ± 49	2049 ± 116	26 ± 2	65 ± 5	107 ± 4	219	158	2	5	8
8	piperidine	-CH₃	-CH₃	-COOH	342 ± 77	7713 ± 1249	6311 ± 1380	8009 ± 130	308 ± 26	1613 ± 24	23	18	23	~	5
9	piperidine	-CH₃	-CH₃	-CH₂NH₂	797 ± 26	8774 ± 508	7799 ± 81	665 ± 28	716 ± 48	3390 ± 301	11	10	~	~	4
10	piperidine	-CH₃	-CH₃	-CF₂H	522 ± 210	53206 ± 16384	47964 ± 3582	402 ± 13	163 ± 21	460 ± 25	102	92	~	~	~
11	(chain)-NH₂	-Bn	-CH₃	-H	646 ± 164	139908 ± 677	4975 ± 328	2026 ± 600	1608 ± 52	555 ± 36	217	8	3	3	~
12	piperidine	-Bn	-CH₃	-H	427 ± 109	12504 ± 3260	8203 ± 674	539 ± 353	1085 ± 139	1062 ± 54	29	19	1	3	2
13	piperidine	-Bn	-CH₃	-H	124 ± 27	21608 ± 3431	2812 ± 543	1142 ± 129	1588 ± 40	359 ± 17	174	23	9	13	3
14	piperidine	-iPr	-CH₃	-H	85 ± 17	984 ± 127	3787 ± 234	93 ± 28	300 ± 21	215 ± 12	12	45	1	4	3
15	piperidine	-Bn	-Bn	-H	623 ± 18	19244 ± 1551	21110 ± 1388	496 ± 36	18643 ± 1365	1183 ± 127	31	34	~	30	2
16	(chain)-NH₂	-CH₃	-CH₃	-CH₂OH	25 ± 9	2243 ± 74	2257 ± 279	33 ± 6	90 ± 9	134 ± 8	90	90	1	4	5
17	pyrrolidine	-CH₃	-CH₃	-CH₂OH	9 ± 2	2145 ± 100	2272 ± 134	68 ± 5	26 ± 5	87 ± 7	240	252	8	3	10
18	pyrrolidine	-CH₃	-CH₃	-CH₂OH	18 ± 2	1250 ± 95	1222 ± 168	73 ± 13	16 ± 3	116 ± 13	69	68	4	~	6
19	azetidine	-CH₃	-CH₃	-CH₂OH	23 ± 3	1531 ± 52	3443 ± 294	108 ± 17	50 ± 1	210 ± 4	67	150	5	2	9
20	pyrrolidine	-CH₃	-CH₃	-CH₂NC(NH₂)₂	1498 ± 104	21535 ± 1910	25850 ± 1571	8477 ± 655	26509 ± 733	25535 ± 1385	14	16	6	18	17
21	(fused ring structure)				159 ± 7	3014 ± 137	3514 ± 511		69 ± 6	1564 ± 252	1315 ± 87	19	22	~	10	8
22	(fused ring structure)				3761 ± 202	24733 ± 1669	25948 ± 540	2426 ± 257	9750 ± 127	16770 ± 1788	7	7	~	3	4

105

106 **Results**

Structure-based optimization of DYRK2 inhibitors

107   **Structure-based optimization of DYRK2 inhibitors**

108   LDN192960 was identified as a DYRK2 inhibitor (Banerjee et al., 2019; Cuny et al.,

109   2010; Cuny et al., 2012). It occupies the ATP-binding pocket of DYRK2 and mediates

110   extensive hydrophobic and hydrogen bond interactions (Banerjee et al., 2019).

111   Nevertheless, LDN192960 also inhibits other DYRK2-related kinases, especially Haspin

112   and DYRK3 (Banerjee et al., 2019). To generate DYRK2 inhibitors with better selectivity,

113   we synthesized a series of new compounds based on the same acridine core structure

114   (Table 1). The amine side chain was first changed to a protected amine (compounds 1-3),

115   a cyano group (compound 4), or a cyclic amine (compounds 5-6) (Figure 1—figure

116   supplement 1A, Table 1). Among these candidates, compound 6 exhibited the most potent

117   inhibitory effect towards DYRK2, with an *in vitro* IC$_{50}$ of 17 nM. In comparison,

118   LDN192960 showed an IC$_{50}$ of 53 nM when the same protocol was used (Table

119   1)—treating HEK293T cells transiently expressing DYRK2 with increasing concentrations

120   of compound 6 efficiently inhibited Rpt3-Thr25 phosphorylation, with the maximal effect

121   observed at an inhibitor concentration of less than 3 μM (Figure 1—figure supplement 1B).

122   Notably, compound 6 also displays good selectivity towards DYRK2 than other kinases,

123   including DRYK1A, DRYK1B, DYRK3, Haspin, and MARK3 (IC$_{50}$ values of 889, 697,

124   121305, 45, and 100 nM, respectively; Table 1). Therefore, compound 6 was chosen as

125   the lead compound for further chemical modification.

126   We subsequently crystallized DYRK2 in complex with compound 6 and determined

127   the structure at a resolution of 2.2 Å (Figure 1A, Figure 1—figure supplement 1C). Not

128   surprisingly, compound 6 binds the ATP-binding site of DYRK2 like LDN192960. A water

129   molecule is located deep inside the binding pocket acting as a bridge in the interactions

130   between LDN192960 and the protein. The newly added amino side chain displays

131   apparent densities and adopts an extended conformation. An in-depth analysis of the

132   crystal structure revealed several additional sites for chemical expansion that may further

133   strengthen its interaction with DYRK2 (Figure 1—figure supplement 2A-B). First, a

134   hydrophilic group can be introduced into the acridine core to functionally replace the

135   aforementioned water molecule and maintain constant contact with DYRK2. Second, a

136   bulky functional group can replace the methoxy groups to mediate other interactions with

137   DYRK2. Finally, the amine side chain can be altered to stabilize its conformation (Figure

138   1—figure supplement 2A-B). To this end, we synthesized 9 new compounds (compounds

139   7-15) and evaluated their inhibitory effects on DYRK2 and related kinases (Figure

140   1—figure supplement 2C-D). We also determined the co-crystallized structures of several

141   of these compounds with DYRK2 to visualize their detailed interactions (Figure 1, Figure

142   1—figure supplement 3). Compound 7, introducing a hydroxymethyl group to the acridine

143   core, inhibits DRYK2 efficiently as compound 6 while displaying better selectivity against

144   other DRYK family members (Table 1). The co-crystallized structure shows that the

145   hydroxymethyl group directly contacts the main chain amide group of Ile367 and indirectly

146   coordinates Glu266 and Phe369 via a water molecule (Figure 1D). Compared to

147   compound 7, compounds 8-10, which contain a carboxyl, aminomethyl, and fluoromethyl

148   group, respectively, instead of a hydroxymethyl group, display reduced inhibition towards

149   DYRK2. Compounds 11-15, designed to replace the methoxy group with a bulkier side

150   chain, showed significantly decreased activity and selectivity and were not further pursued

151   (Table 1).

152   Further chemical modification was carried out based on compound 7. By changing

153   the 6-membered ring to a straight-chain or smaller ring, we synthesized compounds 16-19

154   (Table 1, Figure 1—figure supplement 2E). Among these compounds, C17 with an

155   (S)-3-methylpyrrolidine side-chain exhibited the best potency and selectivity among all the

156   analogs (Table 1). Interestingly, we noticed that compound 18 containing an

157   (R)-3-methylpyrrolidine side chain was not as good as C17, indicating that the chirality of

158   the 3-methylpyrrolidine motif plays an essential role in both potency and selectivity.

159   Further modification of compound 17 (leading to compound 20) to promote further

160   hydrogen bond interactions with DYRK2 failed to improve the inhibitory effect. We also

161   wondered whether acridine was the best aromatic core structure and synthesized two new

162   compounds (compounds 21 and 22) by changing one side of the benzene group to a

163   sulfur-containing thiazole structure (Figure 1—figure supplement 2F), which we thought

164   might facilitate hydrophobic interactions with DYRK2 within the ATP-binding pocket;

165   however, they did not have as effective an inhibitory effect as compound 17 (Table 1).

166

167   **Figure 1**

168   **Figure 1.** Crystal structures of DYRK2 bound to novel inhibitors. (**A**) Overall structure of

169   DYRK2 (grey) bound to 6 (green), 7 (pink), C17 (orange), and 18 (blue). (**B**) Composite

170   omit maps are contoured at 1.5σ and shown as grey meshes to reveal the presence of

171   compounds 6, 7, 17, and 18 in the respective crystal structures. (**C-F**) Close-up view of

172   the DYRK2 binding pocket with compounds 6, 7, 17, and 18. Hydrogen bonds are shown

173   as dashed lines. Water molecules are indicated with red spheres.

174   **Figure 1—source data 1**

175   Data collection and refinement statistics of crystal structures of DYRK2 with different

176   inhibitors.

177

178   **C17 is a potent and selective DYRK2 inhibitor**

179   We set to comprehensively characterize the inhibitory function of compound 17

180   (Figure 2A), referred to as C17 hereafter. *In vitro*, C17 displays an effect on DYRK2 at a

181   single-digit nanomolar IC_{50} value (9 nM) (Figure 2B, Figure 2—figure supplement 1A). To

182   further evaluate the selectivity of C17, we performed kinome profiling analyses. Among

183   the 468 human kinases tested, C17 targeted only DYRK2, Haspin, and MARK3 at a

184   concentration of 500 nM (Figure 2C). Nonetheless, the *in vitro* IC_{50} values of C17 for

185   Haspin and MARK3 (26 nM and 87 nM, respectively) were 3-10-fold higher than that for

186   DYRK2 (Figure 2B, Figure 2—figure supplement 1B-F). Similarly, C17 also inhibited

187   DYRK3 to a lesser extent (IC_{50} of 68 nM). In contrast, LDN192960 inhibited DYRK3 and

188   Haspin more than it inhibited DYRK2 (Banerjee et al., 2019; Cuny et al., 2010).

189   Significantly, C17 also efficiently suppressed DYRK2 activity in the cell and abolished

190   Rpt3-Thr25 phosphorylation at an inhibitor concentration of less than 1 μM (Figure 2D).

191   Taken together, these data demonstrate that C17 is a highly potent and selective DYRK2

192   inhibitor both *in vitro* and *in vivo*.

193   Acridine derivatives have traditionally been used as antibacterial, antiparasitic, and

194   anticancer agents since these compounds usually show strong DNA intercalating effects

195   (Chaikuad et al., 2016; Jouanne et al., 2017). Considering the potential toxicity of C17 due

196   to its possible DNA-binding capacity, we also assessed the DNA-binding effect of C17

197 (Figure 2—figure supplement 3). Isothermal titration calorimetry revealed that C17

198 (Kd=22.9 µM) binds to DNA with significantly lower affinity than LDN192960 binds to DNA

199 (Kd=198 nM), possibly because of the introduction of hydroxymethyl group on the acridine

200 core, which is not present in LDN192960.

201

202 **Figure 2**

203 **Figure 2.** C17 is a potent and selective inhibitor of DYRK2. (**A**) Chemical structure of C17.

204 (**B**) IC_{50} values of C17 against DYRK1A, DYRKIB, DYRK3, Haspin and MARK3. (**C**)

205 Kinome profiling of C17 at 500 nM was carried out using 468 human kinases

206 (https://www.discoverx.com/). (**D**) C17 inhibits Rpt3-Thr25 phosphorylation. HEK293T

207 cells stably expressing FLAG-DYRK2 were treated with the indicated concentrations of

208 C17 for 1 h. The cells were lysed, and immunoblotting was carried out with the indicated

209 antibodies.

210 **Figure 2—source data 1**
211 Raw data of C17 Kinome profiling list for Figure 2C.
212 **Figure 2—source data 2**
213 Raw data of Western blot for Figure 2D.

214

215 **DYRK2 substrate profiling by quantitative phosphoproteomic analyses**

216 Quantitative phosphoproteomic approaches have significantly expanded the scope of

217 phosphorylation analysis, enabling the quantification of changes in thousands of

218 phosphorylation sites simultaneously (Alvarez-Salamero et al., 2017). To obtain a

219 comprehensive list of potential DYRK2 targets, we treated the myeloma U266 cells with

220 C17 and carried out quantitative phosphoproteomic analyses (Chen et al., 2018; Hogrebe

221 et al., 2018). We prepared lysates of U266 cells treated with C17 or the DMSO control and

222 trypsinized them. Phosphorylated peptides were then enriched using Ti^{4+}-immobilized

223 metal ion affinity chromatography (IMAC) tips and analyzed by LC-MS/MS (Figure 3A). A

224 total of 15,755 phosphosites were identified, among which 12,818 (81%) were serine, and

225 2,798 (18%) were threonine. A total of 10,647 (68%) phosphosites were Class I

226 (localization probability >0.75), 2,557 (16%) were Class II (0.5< localization probability

227 ≤0.75), and 2,401 (16%) were Class III (0.25< localization probability ≤0.5) (Figure 3B).

228   This is a very comprehensive phosphoproteomic dataset prepared for DYRK2 substrate

229   profiling by treating the U266 cells with 10 µM of C17. A good Pearson correlation

230   coefficient of 0.9 was obtained for the phosphosite intensities among the treatment and

231   control samples (Figure 3—figure supplement 1), and the coefficient of variance of the

232   intensities of the majority of the phosphosites was lower than 20% (Figure 3—figure

233   supplement 2), demonstrating the high quantification precision of our label-free

234   phosphoproteomic analysis. Remarkably, C17 treatment led to significant downregulation

235   of 373 phosphosites (Figure 3C), including pThr37 of the eukaryotic translation initiation

236   factor 4E-binding protein 1 (4E-BP1), as well as pSer519 and pSer521 in the stromal

237   interaction molecule 1 (STIM1) (Figure 3D, Figure 3—figure supplement 3). Interestingly,

238   another 445 phosphosites were upregulated (Figure 3C), suggesting that DYRK2 likely

239   inhibited some downstream kinases or activated phosphatases, and suppressing its

240   activity reversed these effects. Together, these data demonstrate that DYRK2 is involved

241   in a network of phosphorylation events and can directly or indirectly regulate the

242   phosphorylation status of many proteins. The top pathways with which DYRK2 may

243   participate were revealed by a global analysis of the significantly up-and down-regulated

244   phosphoproteins (Figure 3E).

245

246   **Figure 3**

247   **Figure 3.** Quantitative phosphoproteomic analysis of U266 cells treated with C17. (**A**)

248   Workflow of the phosphoproteomic approach. Triplicate samples treated with/without 10

249   µM C17 for 1 h were separately lysed and digested, and the phosphorylated peptides

250   were enriched by the Ti^{4+}-IMAC tip and analyzed by LC-MS/MS. (**B**) Distribution of the

251   assigned amino acid residues and their localization probabilities (Class I >0.75, Class

252   II >0.5 and ≤0.75, Class III >0.25 and ≤0.5) for all identified phosphorylation sites. (**C**)

253   Volcano plot (FDR < 0.05 and S0 = 2) shows the significantly up-and downregulated

254   phosphosites after C17 treatment. (**D**) MS/MS spectra of the phosphosites of two potential

255   DYRK2 substrates, pT37 of 4E-BP1 and pS519 and pS521 of STIM1. (**E**) Global

256   canonical pathway analysis of the significantly up-and downregulated phosphoproteins. –

257   Log_{10} adjusted p-values associated with a pathway are presented.

Figure 3—source data 1

Raw data of the significantly up- and down-regulated phosphosites after U266 cells

treated with C17 for Figure 3C.

4E-BP1 is a direct cellular target of DYRK2

We set out to determine whether some of the 337 downregulated phosphosites are

genuine DYRK2 targets. We first examined 4E-BP1 for several reasons. First, C17

treatment decreased the pThr37 level in U266 cells (Figure 3C). Second, a previous study

showed that Ser65 and Ser101 in 4E-BP1 can be phosphorylated by DYRK2 *in vitro*,

indicating that 4E-BP1 is a potential DYRK2 substrate (Wang et al., 2003). And lastly,

several phosphosite-specific antibodies for 4E-BP1 are commercially available. 4E-BP1 is

a master regulator of protein synthesis. It has been well established that its

phosphorylation by other kinases such as mTORC1 leads to its dissociation from

eukaryotic initiation factor 4E (eIF4E), allowing mRNA translation (Laplante and Sabatini,

2012; Ma et al., 2009).

Using an antibody that detects 4E-BP1 only when it is phosphorylated at Thr37 and

Thr46, we found that C17 treatment significantly reduced the level of pThr37/pThr46 of

endogenous 4E-BP1 in HEK293T cells (Figure 4A), consistent with our mass spec

analyses in U266 cells. Further investigations using two other 4E-BP1

phosphosite-specific antibodies showed that C17 also decreased the phosphorylation of

Ser65 in endogenous 4E-BP1 (Figure 4A) by a previous study (Wang et al., 2003); as well

as Thr70. Knockdown of endogenous DYRK2 using a short hairpin RNA (shRNA) also

significantly reduced the phosphorylation of these sites (Figure 4B). Successful

knockdown is demonstrated by quantitative RT-PCR analysis (Figure 4—figure

supplement 1). Similarly, C17 suppressed DYRK2-mediated phosphorylation of 4E-BP1

when overexpressed in the HEK293 cells (Figure 4C). To ascertain whether DYRK2 can

directly phosphate 4E-BP1, we performed an *in vitro* kinase assay using purified DYRK2

and 4E-BP1 proteins. DYRK2 efficiently phosphorylated 4E-BP1 at multiple sites,

including Thr37/Thr46, Ser65, and Thr70, whereas the kinase-deficient DYRK2 mutant

(D275N) displayed no activity (Figure 4D). C17 suppressed the phosphorylation of these

288   sites in a dose-dependent manner (Figure 4E). These results demonstrate that DYRK2

289   effectively phosphorylated 4E-BP1 on multiple sites *in vivo* and *in vitro*.

290   4E-BP1 is targeted by multiple kinases (Qin et al., 2016). Indeed, C17 or DYRK2

291   shRNA decreased but did not abolish the phosphorylation of 4E-BP1 (Figure 4A, 4B). A

292   previous study showed that combined inhibition of AKT and MEK kinases suppressed

293   4E-BP1 phosphorylation and tumor growth (She et al., 2010). We observed similar results

294   when we treated the HEK293A cells with AKTi (an AKT1/AKT2 inhibitor) and PD0325901

295   (a MEK inhibitor). Significantly, knockdown of DYRK2 in the presence of these

296   compounds further markedly diminished 4E-BP1 phosphorylation (Figure 4B). To assess

297   whether C17 can also elicit a synergistic effect with these kinase inhibitors, we treated

298   HEK293A, HCT116, and U266 cells with these molecules, either alone or in combination,

299   and examined the phosphorylation status of endogenous 4E-BP1 (Figure 4F-H). The

300   presence of C17 potentiated the inhibitory effect of AKTi and PD0325901 in all these cells.

301   Together, these results confirm that 4E-BP1 is a direct cellular target of DYRK2 and

302   suggest the potential use of DYRK2 inhibitors in combination with other kinase inhibitors

303   for cancer therapy.

304

305   **Figure 4**

306   **Figure 4.** 4E-BP1 is a substrate of DYRK2. (**A**) C17 treatment for 1 h reduced the

307   phosphorylation of endogenous 4E-BP1 in HEK293T cells. The phosphorylation status of

308   4E-BP1 was analyzed by immunoblotting cell lysates using indicated antibodies. (**B**)

309   DYRK2 knockdown decreases the phosphorylation of endogenous 4E-BP1 in HEK293T

310   cells. (**C**) HEK293A cells stably expressing HA-DYRK2 and FLAG-4E-BP1 were treated

311   with indicated concentrations of C17 for 1 h. The cells were lysed, and immunoblotting

312   was carried out with indicated antibodies. (**D**) DYRK2 directly phosphorylated 4E-BP1 at

313   multiple sites. (**E**) C17 inhibited DYRK2-mediated 4E-BP1 phosphorylation in a

314   concentration-dependent manner. (**F-H**) C17 displayed a synergistic effect with AKT and

315   MEK inhibitors to suppress 4E-BP1 phosphorylation in HEK293A (**F**), HCT116 (**G**), and

316   U266 cells. (**H**) The cells were treated with indicated concentrations of PD032590,

317   AKTi-1/2, and C17 alone or in combination for 1 h. Cell lysates were immunoblotted with

318   indicated antibodies.

319   **Figure 4—source data 1**
320   Raw data of Western blot for Figure 4A and B.
321   **Figure 4—source data 2**
322   Raw data of Western blot for Figure 4C and D.
323   **Figure 4—source data 3**
324   Raw data of Western blot for Figure 4E and F.
325   **Figure 4—source data 4**
326   Raw data of Western blot for Figure 4G and H.
327
328

329   **DYRK2 promotes STIM1-ORAI1 interaction to modulate SOCE**

330   In addition to 4E-BP1, another potential target of DYRK2 is STIM1, as the

331   phosphorylation levels of both Ser519 and Ser521 in endogenous STIM1 were

332   significantly reduced upon DYRK2 inhibition in our mass spectrometry analyses (Figure

333   3C). STIM1 is a single-pass transmembrane protein residing in the endoplasmic reticulum

334   (ER) and plays a vital role in the store-operated calcium entry (SOCE) process (Collins et

335   al., 2013). The luminal domain of STIM1 senses calcium depletion in the ER and induces

336   protein oligomerization and puncta formation (Liou et al., 2005; Prakriya and Lewis, 2015;

337   Zheng et al., 2018b). Oligomerized STIM1 then travels to the ER-plasma membrane

338   contact site and activates the ORAI1 calcium channel. The cytosolic region of STIM1

339   contains multiple phosphorylation sites, and it has been shown that the function of STIM1

340   is regulated by several kinases, including ERK1/2 (Pozo-Guisado et al., 2013;

341   Pozo-Guisado and Martin-Romero, 2013).

342   Purified wild-type DYRK2, but not the kinase-dead mutant D275N, induced mobility

343   changes of the cytosolic region of STIM1 (STIM1$^{235-END}$) in SDS-PAGE gel (Figure 5A). As

344   increasing amounts of DYRK2 lead to greater shifts of STIM1$^{235-END}$, there are likely

345   multiple DYRK2 phosphorylation sites in STIM1. Consistently, DYRK2 induced a mobility

346   shift of STIM1 when they were co-expressed in the HEK293A cells (Figure 5B). To further

347   pinpoint DYRK2-specific phosphorylation sites, we co-expressed DYRK2, Orai1, and

348   STIM1 in HEK293A cells, treated the cells with C17, isolated STIM1 using Anti-FLAG

349   agarose, and then subjected it to label-free quantitative mass spectrometry analyses. The

350   phosphorylation levels of at least eight phosphosites on four peptides of STIM1 were

351 significantly reduced upon treatment with C17 compared with the untreated sample

352 (Figure 5—figure supplement 1A-B), including Ser519 and Ser521 that were identified in

353 the U266 phosphoproteome analysis (Figure 3C). In a separate mass spec experiment,

354 phosphorylation of Ser608 and Ser616 were also reduced by C17. Together, these results

355 demonstrate that DYRK2 can phosphorylate multiple sites in the cytosolic region of

356 STIM1.

357 STIM1 puncta formation indicates its oligomerization and activation (Liou et al., 2005;

358 Prakriya and Lewis, 2015; Zheng et al., 2018b). To assess the functional outcome of

359 STIM1 phosphorylation by DYRK2, we co-expressed STIM1 and DYRK2 in an

360 Orai-deficient (Orai-KO) cell line, which has all three Orai genes genetically ablated

361 (Zheng et al., 2018a). DYRK2 induced the appearance of STIM1 puncta under resting

362 conditions, indicating that DYRK2 promotes STIM1 oligomerization (Figure 5C). In

363 contrast, the STIM1 puncta were not observed in the presence of C17. DYRK2 also failed

364 to promote the punctate formation of STIM1-10M, a STIM1 variant with all ten potential

365 DYRK2 phosphorylation sites mutated to Ala (Figure 5C, Figure 5—figure supplement

366 1C).

367 To further understand the importance of STIM1 phosphorylation, we examined the

368 interaction between STIM1 and Orai1 using co-immunoprecipitation. Expression of WT

369 DYRK2 significantly increased the interaction between STIM1 and Orai1, whereas

370 expression of DYRK2-KD exerted no such effect (Figure 5D). Treating cells with C17

371 effectively abolished the DYRK2-dependent STIM1-Orai1 interaction. Notably, both

372 STIM1-1-491, a C-terminal truncated STIM1 (Figure 5C, Figure 5—figure supplement 1C),

373 and STIM1-10M displayed significantly reduced interaction with Orai1 even in the

374 presence of WT DYRK2 (Figure 5E), suggesting that DYRK2-mediated phosphorylation is

375 essential to promote the binding between STIM1 and Orai1. C17 also decreased the

376 interaction between STIM1 and Orai1 without exogenously expressing DYRK2 (Figure

377 5F).

378 We examined fluorescence resonance energy transfer (FRET) between STIM1-YFP

379 and CFP-Orai1 to validate the regulatory function of DYRK2 on STIM1-Orai1 interaction.

380 The FRET signals between STIM1-YFP and CFP-Orai1 were significantly increased in

381   HEK293 cells in the presence of WT DYRK2 (Figure 5G). To exclude the influence of

382   endogenous STIM1, we performed further analyses in a STIM1-STIM2 DKO cell line

383   (Zheng et al., 2018a). The FRET signals between STIM1-1-491 and Orai1 were unaltered

384   by DYRK2 (Figure 5H), indicating that the effect of DYRK2 is dependent on the C-terminal

385   region of STIM1. Furthermore, the FRET signals between STIM1-10M and Orai1 were

386   unaffected by DYRK2 (Figure 5I). These results are consistent with the

387   co-immunoprecipitation results and demonstrate that DYRK2 can promote the

388   STIM1-Orai1 interaction via STIM1 phosphorylation.

389   Lastly, to examine the physiological relevance of the STIM1-Orai1 interaction

390   regulated by DYRK2, we performed SOCE analyses in HEK293A cells expressing

391   GCaMP6f, a genetically encoded calcium sensor (Nakai et al., 2001). Treating cells grown

392   in a calcium-free medium containing thapsigargin resulted in a transient increase in

393   GCaMP6f fluorescence due to calcium release from the ER to the cytosol (Figure 5J,

394   black line). Subsequent addition of calcium to the cell culture medium resulted in a

395   marked increase in GCaMP6f signaling, indicating calcium entry into the cells, further

396   augmented by STIM1 overexpression (Figure 5J, blue line). Pre-treating cells with C17 for

397   1 h substantially reduced SOCE in cells with either endogenous (Figure 5J, green line) or

398   overexpressed STIM1 (Figure 5J, red line). Quantifications of these results are present in

399   Figure 5K. Taken together, our results strongly suggest that DRYK2 can directly enhance

400   SOCE by phosphorylating STIM1 and promoting its interaction with ORAI1, which can all

401   be effectively inhibited by C17.

402

403   **Figure 5**

404   **Figure 5.** Phosphorylation of STIM1 by DYRK2 modulates SOCE. (**A**) DYRK2 directly

405   phosphorylated STIM1. GST-STIM1$^{235\text{-}END}$ was incubated with wild-type or

406   kinase-deficient DYRK2 in the presence of Mn-ATP for 30 minutes. Phosphorylation of

407   GST-STIM1$^{235\text{-}END}$ was indicated by the mobility change of STIM1 in SDS-PAGE gel. (**B**)

408   DYRK2 phosphorylated STIM1 *in vivo*. HEK293A cells were co-transfected with

409   FLAG-STIM1 and DYRK2 for 36 h, then states immunoblotted with the indicated

410   antibodies. (**C**) Typical confocal microscopy images showing the effects of

411 mCherry-DYRK2 and/or C17 (1 μM) on the puncta formation of STIM1 in the HEK293

412 Orai1/Orai2/Orai3-TKO cells. The scale bar is 10 μm. The experiments were repeated, six

413 cells were examined each time. (**D**) DYRK2 promoted the interaction between STIM1 and

414 Orail1. HEK293A cells were co-transfected with FLAG-STIM1, GFP-Orai1, and DYRK2

415 for 36 h. STIM1 was immunoprecipitated with FLAG agarose, and the associated proteins

416 were analysed using the indicated antibodies. (**E**) Phosphosites mutations in STIM1

417 disrupt the interaction with Orai1. (**F**) C17 inhibits the interaction between FLAG-STIM1

418 and GFP-Orai1 without exogenously expressing DYRK2. (**G-I**) Effects of DYRK2 on the

419 FRET signals between STIM1-YFP and CFP-Orai1. Upper panel, typical traces; lower

420 panel, statistics. (**G**) HEK293 cells stably expressing STIM1-YFP and CFP-Orai1. (n=3,

421 ****, P<0.0001. unpaired Student's t-test). (H) HEK293 STIM1-STIM2 DKO cells stably

422 expressing Orai1-CFP cells transiently expressing STIM1-1-491-YFP (n=3, unpaired

423 Student's t-test). (**I**) HEK STIM1-STIM2 DKO cells transiently expressing STIM1-YFP (red)

424 or STIM1-10M (blue). (n=3, ****, P<0.0001, unpaired Student's t-test). (**J**) C17 inhibited

425 SOCE in HEK293A cells. HEK293A cells were transfected with GCAMP6f or GCAMP6f

426 plus STIM1 for 24 h and then treated with 1 μM C17 for 1 h. Before thapsigargin treatment,

427 the cell culture medium was switched to a Ca^{2+}-free medium containing thapsigargin

428 (1 μM, solid lines) or DMSO (dashed lines) was added to the cells, and 2 mM Ca^{2+} was

429 added 12 minutes later. The red and green lines correspond to C17-treated cells. Blue

430 and black lines represent untreated cells. GCAMP6f fluorescence was monitored by a

431 Zeiss LSM 700 laser scanning confocal microscope. (**K**) Quantification of **J**. The following

432 number of cells were monitored: STIM1, 45 cells on 3 coverslips (blue solid line); STIM1 +

433 C17 (1 μM), 48 cells on 3 coverslips (red solid line); endogenous, 47 cells on 3 coverslips

434 (black solid line); endogenous + C17 (1 μM), 42 cells on 3 coverslips (green solid line).

435 STIM1(-Tg), 43 cells on 3 coverslips (blue dashed line). STIM1 + C17 (1 μM) (-Tg), 43

436 cells on 3 coverslips (red dashed line); endogenous (-Tg), 43 cells on 3 coverslips (black

437 dashed line); and endogenous + C17 (1 μM) (-Tg), 43 cells on 3 coverslips (green dashed

438 line). Error bars represent the means ± SEM. (**L**) A hypothetic model depicts

439 DYRK2-mediated STIM1 activation.

440 **Figure 5—source data 1**

441   Raw data of Coomassie Blue Staining for Figure 5A.

442   **Figure 5—source data 2**

443   Raw data of Western blot and Coomassie Blue Staining for Figure 5B.

444   **Figure 5—source data 3**

445   Raw data of Western blot for Figure 5D and E.

446   **Figure 5—source data 4**

447   Raw data of Western blot for Figure 5F.

448   **Figure 5—source data 5**

449   Raw data of FRET responses between STIM1-YFP and CFP-Orai1 for Figure 5G, FRET

450   responses between STIM1-1-491-YFP and CFP-Orai1 for Figure 5H, FRET responses

451   between STIM1-YFP, STIM1-10M-YFP and CFP-Orai1 for Figure 5I.

452   **Figure 5—source data 6**

453   Raw data of Store-operated Ca^{2+} entry (SOCE) analyses for Figure 5J.

454

455   **Discussion**

456   We used a structure-based approach to design, synthesize and evaluate a series of

457   new analogs based on the acridine core structure and eventually identified C17 as a

458   potent and selective DYRK2 inhibitor. We showed that C17 affects DYRK2 at a single-digit

459   nanomolar IC50 and inhibits DYRK2 more potently than closely related kinases such as

460   DYRK3, Haspin, and MARK3. The crystal structure of DYRK2 bound to C17 revealed

461   critical interactions that explain its high selectivity, including a hydrogen bond between the

462   (S)-3-methylpyrrolidine ring and Glu352 in DYRK2.

463   C17 provided us with a unique tool to interrogate the physiological functions of

464   DYRK2. We treated U266 cells with C17 and performed quantitative phosphoproteomic

465   analyses. We found that the cellular phosphorylation pattern is significantly altered by C17,

466   suggesting that DYRK2 likely has multiple cellular targets and is involved in a network of

467   biological processes. We then identified several leading phosphosites that are

468   downregulated and demonstrated that 4E-BP1 and STIM1 are bona fide substrates of

469   DYRK2. We showed that DYRK2 efficiently phosphorylated 4E-BP1 at multiple sites,

470   including Thr37, and combined treatment of C17 with AKT and MEK inhibitors resulted in

471   marked suppression of 4E-BP1 phosphorylation. Therefore, DYRK2 likely functions

472   synergistically with other kinases to regulate protein synthesis.

473   For the first time, we also discovered that DYRK2 could efficiently phosphorylate
474   STIM1, and phosphorylation of STIM1 by DYRK2 substantially increased the interaction
475   between STIM1 and ORAI1. Treating cells with C17 suppressed SOCE, validating the
476   critical role of DYRK2 in regulating calcium entry into cells. These data allow us to present
477   a hypothetical model showing how DYRK2 triggers the activation of STIM1 (Figure 5L).
478   Under resting conditions, the cytosolic portion of STIM1 likely adopts an inactive
479   conformation. DYRK2 can phosphorylate STIM1 and induce its oligomerization, which
480   then interacts with the Orai1 channel and leads to its opening. One inadequacy of our
481   study is the lack of further insight into the regulation mechanism of this process. In
482   particular, what is the upstream signal that triggers DYRK2 activation? Nevertheless, our
483   data offer a valuable model that allows further investigation of the relationship between
484   DYRK2 and SOCE.

485   Recently, Mehnert et al. developed a multilayered proteomic workflow and
486   determined how different pathological-related DYRK2 mutations altered protein
487   conformation, substrates modification, and biological function (Qin et al., 2016). DYRK2 is
488   implicated in regulating multiple cellular processes, and the selective DYRK2 inhibitor we
489   developed here will serve as a valuable tool in dissecting its complex downstream
490   pathways.

491

Materials and methods

Antibodies and Reagents

Antibodies used in this study were: anti-4E-PB1 (Cell Signaling Technology, RRID: AB_2097841), anti-phosphorylated 4E-BP1 (Thr37/46) (Cell Signaling Technology, RRID: AB_560835), anti-phosphorylated 4E-BP1 (Ser65) (Cell Signaling Technology, RRID: AB_330947), anti-phosphorylated 4E-BP1 (Thr70) (Cell Signaling Technology, RRID: AB_2798206), anti-HA (Cell Signaling Technology, RRID: AB_1549585), anti-Flag (Sigma, RRID: AB_259529), anti-Flag (Abcam, #ab205606), anti-GFP (Proteintech, RRID: AB_11182611), Anti-GFP (Abcam, #ab183734), anti-RPT3 (Thermo Fisher Scientific, RRID: AB_2781512), anti-GAPDH (TransGen Biotech, #HC301-01). Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (H+L) or HRP-conjugated anti-mouse IgG (H+L) purchased from Transgene Biotechnology (#HC101-01, #HC201-01). Rabbit anti-pThr25 polyclonal antibody was generated using the following phospho-peptide as immunogen: LSVSRPQ(pT)GLSFLGP as reported previously (Guo et al., 2016). Reagents used in this study were: AKTi-1/2 (Selleck, #S80837), PD0325901 (Aladdin, #P125494), Thapsigargin (Aladdin, #T135258). Inhibitors were dissolved in dimethyl sulfoxide. All chemical reagents were used as supplied by Sigma-Aldrich, J&K Scientific, Alfa Aesar Chemicals, Energy Chemicals and Bide Pharmatech. DCM, DMF, DMSO were distilled from calcium hydride; tetrahydrofuran was distilled from sodium/benzophenone ketyl prior to use.

Cloning

The GCaMP6f, pEGFP-Orai1, and mCherry-STIM1 plasmids were kindly gifted from the Xiaowei Chen Lab (Peking University, China). The GFP-tagged human DYRK1A, 1B, 2, 3, 4,

514   pLL3.7-DYRK2-shRNA, psPAX2, and pMD2.G plasmids were kindly gifted from the Xing Guo

515   Lab (Zhejiang University, China). DYRK2$^{208-552}$ was subcloned into the pQlinkHx vector

516   (Clontech) with an engineered N-terminal His tag. STIM1$^{235-END}$ and full-length 4EBP1 were

517   subcloned into the pQlinkGx vector (Clontech) with an engineered N-terminal GST tag.

518   Full-length STIM1 was subcloned into a pCCF vector (Clontech) with an engineered

519   N-terminal FLAG tag. The HA-mcherry-DYRK2 and HA-mcherry-DYRK2-D275N plasmids

520   were generated by modification of pEGFP-DYRK2 and pEGFP-DYRK2-D275N plasmids.

521   HA-mcherry was PCR amplified from pmCherry-N1 plasmid and replaced EGFP by

522   homologous recombination. All plasmids were verified by DNA sequencing.

523   **Cell culture, transfection and infection**

524   Mammalian cells were all grown in a humidified incubator with 5% CO_2 at 37 °C. HEK293T

525   (RRID:CVCL_0063), HEK293A (Thermo Fisher, R70507), and HEK293 (RRID:CVCL_0045)

526   cells were grown in Dulbecco's Modified Eagle Media (DMEM, Gibco) supplemented with 10%

527   FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco). U266

528   (RRID:CVCL_0566) cells were grown in RPMI 1640 (Gibco) supplemented with 10% FBS, 4

529   mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco). HCT116 cells

530   (China Infrastructure of Cell Line Resources, 1101HUM-PUMC000158) were grown in Iscove's

531   Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 10% FBS, 4 mM L-glutamine,

532   100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco). All cell lines were confirmed by STR

533   (short tandem repeat) profiling and tested negative for mycoplasma contamination. All cell

534   lines are not in the list of commonly misidentified cell lines maintained by the International Cell

535   Line Authentication Committee (version 11). Transient transfection of HEK293T, HEK293A

536 cells were carried out using Lipofectamine 2000 (Thermo Fisher Scientific) or X-tremeGENE 9

537 DNA Transfection reagent (Roche) as recommended by the manufacturer, and transfected

538 cells were used in experiments 24–48 h later. In Lipofectamine transfection, the cells were

539 cultured to ~70-80% confluency in 10-cm dishes, followed by transfection with 10–12 μg

540 plasmid. The cells were changed with fresh DMEM after 12 h and incubated for 36 h before

541 further experiments. In X-tremeGENE 9 DNA transfection, the cells were cultured to ~50

542 confluency in 35-mm glass bottom dishes coated with poly-D-lysine, followed by transfection

543 with 1-3 μg plasmid. The cells were changed with fresh DMEM after 6 h and incubated for

544 24-36 h before further experiments. Lentiviruses were produced using the psPAX2 and

545 pMD2.G packaging vectors. Viral media were passed through a pre-wetted 0.45 μm filter and

546 mixed with 10 μg mL^{-1} Polybrene (Sigma) before being added to recipient cells. Infected cells

547 were selected with puromycin (1-2 μg mL^{-1}, Life Technologies) to generate stable populations.

548 **DYRK2 protein purification and co-crystallization**

549 DYRK2$^{208-552}$ with an N-terminal 6×His affinity tag and TEV protease cleavage site which

550 expressed in *E. coli* BL21 (DE3). Bacterial cultures were grown at 37 °C in LB medium to an

551 OD600 of 0.6-0.8 before induced with 0.5 mM IPTG overnight at 18 °C. Cells were collected by

552 centrifugation and frozen at -80 °C. For protein purification, the cells were suspended in the

553 lysis buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 20 mM imidazole, 5% glycerol, 5 mM

554 β-mercaptoethanol, and 1 mM phenylmethanesulfonylfluoride) and disrupted by sonication.

555 The insoluble debris was removed by centrifugation. The supernatant was applied to a Ni-NTA

556 column (GE Healthcare). The column was washed extensively with the wash buffer (50 mM

557 HEPES, pH 7.5, 500 mM NaCl, 50 mM imidazole, 5% glycerol, and 5 mM β-mercaptoethanol)

558   and bound DYRK2 protein was eluted using the elution buffer (50 mM HEPES, pH 7.5, 500

559   mM NaCl, 500 mM imidazole, 5% glycerol, and 5 mM β -mercaptoethanol). After cleavage with

560   TEV protease, the protein sample was passed through a second Ni-NTA column to separate

561   untagged DYRK2 from the uncut protein and the protease. Final purification was performed

562   using a Superdex 200 gel filtration column (GE Healthcare), and the protein was eluted using

563   the final buffer (25 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM DTT, and 5% glycerol). Purify the

564   DYRK2-D275N using the same method as shown above. Purified DYRK2 and DYRK2-D275N

565   were concentrated to 10 mg mL^{-1} and flash-frozen with liquid nitrogen.

566   DYRK2$^{208-552}$ was incubated with 200 μM compounds on ice before crystallization. The

567   protein-compounds mixture was then mixed in a 1:1 ratio with the crystallization solution (0.36

568   M-0.5 M sodium citrate tribasic dihydrate, 0.01 M sodium borate, pH 7.5-9.5) in a final drop

569   size of 2 μl. The DYRK2-compounds crystals were grown at 18 °C by the sitting-drop vapor

570   diffusion method. Cuboid-shaped crystals appeared after 4-7 days. Crystals were

571   cryoprotected in the crystallization solution supplemented with 35% glycerol before frozen in

572   liquid nitrogen.

573   The X-ray diffraction data were collected at Shanghai Synchrotron Radiation Facility (SSRF)

574   beamline BL17U. The diffraction data were indexed, integrated, and scaled using HKL-2000

575   (HKL Research). The structure was determined by molecular replacement using the published

576   DYRK2 structure (PDB ID: 3K2L) (Soundararajan et al., 2013) as the search model using the

577   Phaser program (McCoy et al., 2007). Chembiodraw (version 13.0) was used to generated

578   the .cif files for compounds, and then compounds were fitted using the LigandFit program in

579   Phenix (Adams et al., 2010). The structural model was further adjusted in Coot (Emsley et al.,

580   2010) and refined using Phenix. The quality of the structural model was checked using the

581   MolProbity program in Phenix. The crystallographic data and refinement statistics are

582   summarized in Figure 1-source data 1.

583   **IC$_{50}$ determination**

584   IC$_{50}$ determination was carried out using the ADP-Glo^{TM} kinase assay system (Promega,

585   Madison, WI). Active DYRK1A, DYRK1B, DYRK2, DYRK3, Haspin and MARK3 were purified

586   as reported previously. C17 IC$_{50}$ measurements were carried out against the kinases with final

587   concentrations between 0.01 nM to 100 µM *in vitro* (C17 was added to the kinase reaction

588   prior to ATP master mix). The values were expressed as a percentage of the DMSO control.

589   DYRK isoforms (1 ng/µL diluted in 50 mM Tris-HCl pH7.5, 2 mM DTT) were assayed against

590   Woodtide (KKISGRLSPIMTEQ) in a final volume of 5 µL containing 50 mM Tris pH 7.5, 150

591   µM substrate peptide, 5 mM MgCl$_2$ and 10-50 µM ATP (10 µM for DYRK2 and DYRK3, 25 µM

592   for DYRK1A and 50 µM for DYRK1B) and incubated for 60 min at room temperature. Haspin

593   (0.2 ng/µL diluted in 50 mM Tris-HCl pH7.5, 2 mM DTT) was assayed against a substrate

594   peptide H3(1-21) (ARTKQTARKSTGGKAPRKQLA) in a final volume of 5 µL containing 50 mM

595   Tris pH 7.5, 200 µM substrate peptide, 5 mM MgCl$_2$ and 200 µM ATP and incubated for 120

596   min at room temperature. MARK3 (1 ng/µL diluted in 50 mM Tris-HCl pH7.5, 2 mM DTT) was

597   assayed against Cdc25C peptide (KKKVSRSGLYRSPSMPENLNRPR) in a final volume of 5

598   µL 50 mM Tris pH 7.5, 200 µM substrate peptide, 10 mM MgCl$_2$ and 5 µM ATP and incubated

599   for 120 min at room temperature. After incubation, the ADP-Glo^{TM} kinase assay system was

600   used to determine kinase activity following the manufacturer's protocol. IC$_{50}$ curves were

601   developed as % of DMSO control and IC_{50} values were calculated using GraphPad Prism

602   8.4.0 software. Results are means ± SD for triplicate reactions with similar results obtained in

603   at least one other experiment.

604   **KINOMEscan® kinase profiling**

605   The KINOMEscan® kinase profiling assay was carried out at The Largest Kinase Assay Panel

606   in the world for Protein Kinase Profiling (https://www.discoverx.com). C17 kinase selectivity

607   was determined against a panel of 468 protein kinases. Results are presented as a

608   percentage of kinase activity in DMSO control reactions. Protein kinases were assayed *in vitro*

609   with 500 nM final concentration of C17 and the results are presented as an average of

610   triplicate reactions ± SD or in the form of comparative histograms.

611   **Quantitative phosphoproteomic analysis**

612   Triplicate U266 cells treated with/without C17 were lysed by the lysis buffer containing 1% (v/v)

613   Triton X-100, 7M Urea, 50 mM Tris-HCl, pH 8.5, 1 mM pervanadate, protease inhibitor mixture

614   (Roche), and phosphatase inhibitor mixtures (Roche). The cell lysates were firstly digested

615   with trypsin (Promega, USA) by the in-solution digestion method (Chen et al., 2018). After

616   desalting, the Ti^{4+}-IMAC tip was used to purify the phosphopeptides. The phosphopeptides

617   were desalted by the C18 StageTip prior to the LC MS/MS analysis(Chen et al., 2018). An

618   Easy-nLC 1200 system coupled with the Q-Exactive HF-X mass spectrometer (Thermo Fisher

619   Scientific, USA) was used to analyze the phosphopeptide samples with 1 h LC gradient. The

620   raw files were searched against Human fasta database (71772 protein entries, downloaded

621   from Uniprot on March 27, 2018) by MaxQuant (version 1.5.5.1). The oxidation (M),

622   deamidation (NQ), and Phospho (STY) were selected as the variable modifications for the

623   phosphopeptide identification, while the carbamidomethyl was set as the fixed modification.

624   The false discovery rate (FDR) was set to 0.01 on PTM site, peptide, and protein level.

625   Label-free quantification (LFQ) and match between runs were set for the triplicate analysis

626   data. The MaxQuant searching file "Phospho (STY)Sites.txt" was loaded into the Perseus

627   software (version 1.5.5.3) to make volcano plots using student's t-test and cutoff of "FDR<

628   0.05 and S0=2". The pathway analysis was performed using the Kyoto Encyclopedia of Genes

629   and Genomes (KEGG) database with cutoff of adjusted p-value < 0.05.

630   **Quantitative RT-PCR**

631   Total RNA from cells was extracted using the RNeasy Mini Kit (Qiagen) and

632   reverse-transcribed with the PrimeScript Real Time reagent Kit (with genomic DNA Eraser,

633   TAKARA). The product of reverse transcription was diluted five times then subjected to

634   quantitative rtPCR reaction in Applied Biosystems ViATM7 Real-Time PCR System (Applied

635   Biosvstems). The 20 μl quantitative rtPCR reaction contained 2 μl of the reverse-transcription

636   reaction mixture, 2× Hieff quantitative rtPCR SYBR Green Master Mix (Yeasen), 0.2 μM

637   quantitative rtPCR forward primer, 0.2 μM quantitative rtPCR reverse primer (Figure 4—figure

638   supplement 2) and ddH$_2$O. The quantitative rtPCR reaction condition was as follows: 95 °C, 5

639   min; (95 °C. 10 s; 60 °C, 30 s) ×40 cycles; 95 °C, 15 s; 60 °C, 1 min; 95 °C. 15 s (collect

640   fluorescence at a ramping rate of 0.05 °C s-1); 4 °C, hold. Data analysis was performed by

641   QuantStudioTM Real-Time PCR Software v.1.3.

642   **STIM1 and 4EBP1 protein purification**

643   The cytosolic domain of STIM1 (bases 235-END) with an N-terminal GST-tag and TEV

644   protease cleavage site which expressed in E. coli BL21 (DE3). Bacterial cultures were grown

645   at 37 °C in LB medium to an OD600 of 0.6-0.8 before induced with 0.5 mM IPTG overnight at

646   18 °C. Cells were collected by centrifugation and frozen at -80 °C. For protein purification, the

647   cells were suspended in the lysis buffer 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5 mM

648   β-mercaptoethanol, and 1 mM phenylmethanesulfonylfluoride and disrupted by sonication.

649   The insoluble debris was removed by centrifugation. The supernatant was applied to a

650   glutathione-Sepharose column (GE Healthcare) and eluted in lysis buffer containing 20 mM

651   glutathione. Purify the GST-4EBP1 using the same method as shown above. Purified STIM1

652   and 4EBP1 were flash-frozen with liquid nitrogen.

653   ***In vitro* kinase assays**

654   DYRK2 kinase assays were performed in 50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MnCl_2$,

655   10 mM ATP using STIM1 or 4EBP1 as substrate. The kinase reactions were initiated by the

656   addition of DYRK2 with indicated concentration. Assays (25 µl volume) were carried out at

657   30 °C for 30 minutes, and terminated by addition of SDS-PAGE buffer containing 20 mM EDTA

658   and then boiled. The reaction mixtures were then separated by SDS-PAGE and visualized by

659   Coomassie Blue staining or analyzed by immuno-blot using primary antibodies as indicated

660   throughout.

661   **Co-immunoprecipitation and western blotting**

662   HEK293A cells were cultured and transfected as described above. After transfection, the cells

663   were washed three times with Ca^{2+}-free buffer containing 10 mM HEPES, 10 mM D-glucose,

664 150 mM NaCl, 4 mM KCl, 3 mM MgCl$_2$ and 0.1 mM EGTA (pH 7.4). Treatment of DMEM

665 containing 1 μM of C17 at 37℃ were used for DYRK2 inhibition. Ca^{2+}-store depletion was

666 triggered by incubating cells with 2 μM thapsigargin for 20 min. The cells were then lysed with

667 lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% (v/v) Nonidet

668 P40 (substitute), 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium

669 pyrophosphate, 0.27 M sucrose, 2 mM dithiothreitol (DTT), 1 mM benzamidine, 0.1 mM PMSF

670 (added before lysis), 1% (v/v) protease inhibitor cocktail (Roche) and 1% (v/v) Phosphatase

671 Inhibitor Cocktail (Roche). Protein concentrations were determined with the BCA protein assay

672 kit Pierce (Thermo-Pierce). For immunoprecipitations, lysates containing equal protein

673 amounts were incubated with FLAG–beads 2 h at 4°C. FLAG–beads were washed three times

674 with lysis buffer containing 0.15 M NaCl. Proteins were eluted from the FLAG–beads by

675 addition of 300 ug FLAG peptides (Smart Lifesciences). Eluted proteins were reduced by

676 addition of loading buffer with 4 mM DTT followed by heating at 95 °C for 10 minutes. For

677 western blotting, samples were electrophoresed in 10% or 12% gels and transferred to PVDF

678 membranes. All antibody dilutions and washes were carried out in Tris-buffered saline (TBS;

679 137 mM NaCl, 19 mM Tris HCl and 2.7 mM KCl, at pH 7.4) containing 0.1% Tween-20 (TBS-T).

680 Membranes were blocked in 5% non-fat milk solution in TBS-T for 1 h at room temperature,

681 incubated with indicated primary antibodies overnight at 4 °C, and incubated with secondary

682 antibodies (horseradish peroxidase-linked anti-mouse or anti-rabbit) for 1 h at room

683 temperature. Blots were developed with AMERSHAM ImageQuant 800 (GE Healthcare) and

684 exposed to film.

685 **Quantitative analysis of phosphorylation sites on STIM1**

686   Triplicate HEK293A cells co-transfected with GFP-ORAI1, FLAG-DRYK2 and FLAG-STIM1 for

687   36 h was treated with 1 μM and 10 μM C17 respectively for 1 h. After collected, cells were

688   washed with the Ca^{2+}-free buffer to remove excess Ca^{2+} and then lysed by the lysis buffer. For

689   immunoprecipitations, lysates containing equal protein amounts were incubated with FLAG–

690   beads for 1 h at 4 °C, which were washed three times with the lysis buffer afterwards. The

691   proteins were eluted from the FLAG–beads by addition of 500 ug FLAG peptides (Smart

692   Lifesciences). Then the eluted proteins were digested with trypsin by the FASP digestion

693   method(Wisniewski et al., 2009). The peptides were analyzed on a Q Exactive Plus mass

694   spectrometer (Thermo Fisher Scientific) with 1 h LC gradient. The raw files were searched

695   against Human fasta database (downloaded from Uniprot) by MaxQuant (version 1.6.3.4). The

696   oxidation (M), deamidation (NQ), and Phospho (STY) were selected as the variable

697   modifications for the phosphopeptide identification, while the carbamidomethyl was set as the

698   fixed modification. The false discovery rate (FDR) was set to 0.01 on PTM site, peptide, and

699   protein level. Label-free quantification (LFQ) and match between runs were set for the

700   triplicate analysis data.

701   **Confocal microscopy**

702   Confocal imaging were carried out with a ZEISS LSM880 imaging system equipped with 65×

703   oil objective (NA=1.45, Zeiss), 488- and 543-nm laser, controlled by Zen 2.3 SP1 software.

704   YFP (505±35) and mCherry (640±50) emission were collected with CaAsP PMT (Optical

705   section, 1.1 μm). Image analysis was performed using Image J Fiji (NIH) (Zheng et al., 2018a).

706   Each repeat contains data from at least 6 cells.

707 **Fluorescence imaging**

708 Fluorescence signals were recorded using a ZEISS obersever-7 microscope equipped with an

709 X-Cite 120-Q (Lumen dynamics), brightline filter sets (Semrock Inc.), a 40×oil objective (NA =

710 1.30), and a Prime 95B Scientific CMOS (sCMOS) camera (Teledyne Imaging). This system

711 was controlled by Slide book6 software (3i). For fluorescence resonance energy transfer

712 (FRET) measurements, three-channel-corrected FRET include cyan fluorescent protein (CFP),

713 yellow fluorescent protein(YFP) and FRET raw were collected with corresponding filters, F_{CFP}

714 ($438 \pm 12 E_x$/ $483 \pm 16 E_m$), F_{YFP} ($510 \pm 10 E_x$/$542 \pm 13.5 E_m$) and $FRET_{raw}$ ($438 \pm 12 E_x$/$542 \pm 13.5 E_m$),

715 every 10 sec. Calibration of bleed through from FRET donor or acceptor to FRET channel

716 (0.20, and 0.36, correspondingly), as well as the system-dependent factor, G (2.473) were

717 done as described earlier (Ma et al., 2015). These parameters were then used to generate

718 calculate FRET efficiency (Eapp) values from raw fluorescent signals, similar to those

719 previously described (Ma et al., 2017). At least 16 cells were collected for each repeat.

720 Corresponding results were calculated with Matlab 2014a software and plotted with GraphPad

721 Prism 8.4.0 software. Representative traces of at least three independent experiments are

722 shown as mean ± SEM.

723 **Confocal imaging and intracellular Ca^{2+} measurement**

724 Intracellular Ca^{2+} measurement was performed on a Zeiss LSM 700 laser scanning confocal

725 microscope equipped with a 63× oil immersion objective lens (N.A. = 1.4) controlled by ZEN

726 software. GCaMP6f fluorescence was excited using a 488-nm line of solid-state laser and

727 fluorescence emission was collected with a 490- to 555-nm band-pass filter; mCherry

728　fluorescence was excited using a 555-nm line of solid-state laser and fluorescence emission

729　was collected with a 580-nm long-pass filter. Two high-sensitivity PMTs were used for

730　detection. Cells were imaged at 10 s intervals for up to 20 mins. All live cell imaging

731　experiments were performed at room temperature. Data were processed and analyzed using

732　Zen and ImageJ software.

733　For intracellular Ca^{2+} measurement, HEK293A cells were plated on glass-bottom 35-mm

734　dishes and transfected as described above. Cells were washed with Ca^{2+} free buffer 3 times

735　24 h after transfection. For DYRK2 inhibition, cells were treated with DMEM containing 1 μM of

736　C17 at 37℃ for 1 h before Ca^{2+} free buffer rinse. Depletion of Ca^{2+}-stores was triggered by

737　incubating cells with 1 μM thapsigargin in Ca^{2+}-free buffer, and Store-operated Ca^{2+} entry

738　(SOCE) was induced by addition of 2 mM $CaCl_2$ to thapsigargin containing buffer. 1 μM C17

739　was added for DYRK2 inhibition assay. The intracellular free calcium concentration was

740　measured by monitoring the fold change of GCaMP6f fluorescence, the data were shown as

741　the mean ± SEM.

742　**Statistics and data presentation**

743　Most experiments were repeated 3 times with multiple technical replicates to be eligible for the

744　indicated statistical analyses, and representative image has been shown. All results are

745　presented as mean ± SD unless otherwise mentioned. Data were analysed using Graphpad

746　Prism 8.4.0 statistical package.

747　**Data availability**

748   The structural coordinates of DYRK2 in complex with compounds 5, 6, 7, 8, 10, 13, 14, 17, 18,

749   19, and 20 have been deposited in the Protein Data Bank with accession codes 7DH3, 7DG4,

750   7DH9, 7DHV, 7DHC, 7DHK, 7DHO, 7DJO, 7DL6, 7DHH, and 7DHN, respectively.

751   All the raw mass spectrometry data as well as the identified and significantly regulated

752   phosphosites tables have been deposited in the public proteomics repository MassIVE and are

753   accessible at ftp://massive.ucsd.edu/MSV000087106/

754
755

756　**Figure 1—figure supplement 1.** Chemical compounds derived from LDN192960. (**A**)

757　Structure of amino side chain change analogues 1-6 based on LDN192960. (**B**) HEK293T

758　cells stably expressing FLAG-DYRK2 were treated with the indicated concentrations of

759　compound 6 in 1h. Cells were lysed and immunoblotting was carried out with the indicated

760　antibodies. (**C**) Structure of DYRK2 in complex with compound 6. DYRK2 is shown as ribbons

761　and colored in blue white. The 2Fo-Fc difference electron density map (1.5 σ which reveals the

762　presence of 6 and water is shown as a gray mesh. The 6 and water are omitted to calculate

763　the map).

764　**Figure 1—figure supplement 1-source data 1.**

765　Raw data of Western blot for Figure 1—figure supplement 1B.

766

767　**Figure 1—figure supplement 2.** Structure-guided engineering of DYRK2 inhibitors based on

768　compound 6. (**A**) The possible sites for further expansion based on the co-crystal structure of

769　6 and DYRK2. (**B**) Overview of modification of compound 6. (**C**) Modifications for inner space 1.

770　(**D**) Modifications for cavity around ATP-binding pocket. (**E**) Modifications of amine side chain

771　based on compound 7. (**F**) Modifications based on compound 17.

772

773　**Figure 1—figure supplement 3.** The 2Fo-Fc composite omit maps (1.5 σ surrounding

774　compounds 5, 10, 13, 14, 19 and 20 are shown in the co-crystal structures with DRYK2

775　respectively).

776

777　**Figure 2—figure supplement 1.** IC_{50} of C17 on DYRK2 and its main off targets. (**A-E**) IC_{50} of

778　C17 on DYRK2, DYRK1A, DYRK1B, DYRK3, Haspin and MARK3. The IC_{50} graph was plotted

779   using GraphPad Prism 8.4.0 software. The results are presented as the percentage of kinase

780   activity relative to the DMSO-treated control. Results are means ± SD for triplicate reactions

781   with similar results obtained in at least one other experiment.

782

783   **Figure 2—figure supplement 2.** Binding strength of LDN192960 and C17 with calf thymus

784   DNA. (**A**) Binding strength of LDN192960 with calf thymus DNA tested by Isothermal titration

785   calorimetry. (**B**) Binding strength of C17 with calf thymus DNA tested by Isothermal titration

786   calorimetry.

787

788   **Figure 3—figure supplement 1.** Correlation of the intensities of phosphosites between any

789   two samples in phosphoproteomic analysis of U266 cells treated with/without C17.

790   **Figure 3—figure supplement 1-source data 1.**

791   Raw data of the intensities of phosphosites in phosphoproteomic analysis of U266 cells

792   treated with/without C17.

793

794   **Figure 3—figure supplement 2.** Coefficient of variance of the intensities of phosphosites in

795   phosphoproteomic analysis of U266 cells treated with/without C17.

796   **Figure 3—figure supplement 2-source data 1.**

797   Raw data of the intensities of phosphosites in phosphoproteomic analysis of U266 cells

798   treated with/without C17.

799

800 **Figure 3—figure supplement 3.** The intensities for pT37 phosphosite of EIF4E-BP1 and

801 pS519, pS521 phosphosites of STIM1. Data was presented as mean values ± SD (error bars).

802 **Figure 3—figure supplement 3-source data 1.**

803 Raw data of the intensities for pT37 phosphosite of 4E-BP1 and pS519, pS521 phosphosites

804 of STIM1 for Figure 3—figure supplement 1-source data 1.

805

806 **Figure 4—figure supplement 1.** Knockdown efficiency of DYRK2-expression in wild-type

807 HEK293T stably expressed DYRK2 shRNA was measured by qPCR. GAPDH was used as an

808 internal standard, and fold change was calculated by comparing expression levels relative to

809 those of pLL3.7-shRNA-scramble (negative control). Data are presented as the means ± SD

810 (n=3 biological replicates per condition, [***], P=0.0001, unpaired Student's t-test).

811 **Figure 4—figure supplement 1-source data 1.**

812 Raw qPCR data of knockdown efficiency of DYRK2-expression in wild-type HEK293T for

813 Figure 4—figure supplement 1-source data 1.

814

815 **Figure 4—figure supplement 2.** All primer sequences for qRT-PCR, shRNA targeting

816 sequences are listed.

817

818 **Figure 5—figure supplement 1.** (**A**) Quantitative analysis of phosphorylation sites on STIM1.

819 Workflow for the identification of phosphosites influenced by C17 on STIM1. Triplicate

820 HEK293A cells co-transfected with FLAG-STIM1 were treated with 10 µM C17 for 1 h,

821 enriched by FLAG-beads, digested by FASP (Filter-Aided Sample Preparation) and quantified

822 by label-free proteomics. (**B**) The changed phosphorylation levels on peptides of STIM1. The

823 phosphorylation of eight phosphosites (shown in red) on four peptides of STIM1 was

824 significantly reduced upon treatment with C17 compared with the control group. (**C**) STIM1

825 constructs used.

826 **Figure 5—figure supplement 1-source data 1.**

827 Raw data of quantitative analysis of phosphorylation sites on STIM1 by DYRK2 upon C17

828 treatment for Figure 5—figure supplement 1-source data 1.

829

830 **References:**
831 Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J.,
832 Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W.*, et al.* (2010). PHENIX: a comprehensive
833 Python-based system for macromolecular structure solution. Acta crystallographica Section D,
834 Biological crystallography *66*, 213-221.
835 Alvarez-Salamero, C., Castillo-Gonzalez, R., and Navarro, M.N. (2017). Lighting Up T
836 Lymphocyte Signaling with Quantitative Phosphoproteomics. Front Immunol *8*, 938.
837 Aranda, S., Laguna, A., and de la Luna, S. (2011). DYRK family of protein kinases:
838 evolutionary relationships, biochemical properties, and functional roles. FASEB J *25*, 449-462.
839 Banerjee, S., Ji, C., Mayfield, J.E., Goel, A., Xiao, J., Dixon, J.E., and Guo, X. (2018). Ancient
840 drug curcumin impedes 26S proteasome activity by direct inhibition of dual-specificity
841 tyrosine-regulated kinase 2. Proc Natl Acad Sci U S A *115*, 8155-8160.
842 Banerjee, S., Wei, T., Wang, J., Lee, J.J., Gutierrez, H.L., Chapman, O., Wiley, S.E., Mayfield,
843 J.E., Tandon, V., Juarez, E.F.*, et al.* (2019). Inhibition of dual-specificity tyrosine
844 phosphorylation-regulated kinase 2 perturbs 26S proteasome-addicted neoplastic progression.
845 Proc Natl Acad Sci U S A *116*, 24881-24891.
846 Becker, W., and Joost, H.G. (1999). Structural and functional characteristics of Dyrk, a novel
847 subfamily of protein kinases with dual specificity. Prog Nucleic Acid Res Mol Biol *62*, 1-17.
848 Becker, W., and Sippl, W. (2011). Activation, regulation, and inhibition of DYRK1A. FEBS J *278*,
849 246-256.
850 Chaikuad, A., Diharce, J., Schroder, M., Foucourt, A., Leblond, B., Casagrande, A.S., Desire,
851 L., Bonnet, P., Knapp, S., and Besson, T. (2016). An Unusual Binding Model of the Methyl
852 9-Anilinothiazolo[5,4-f]quinazoline-2-carbimidates (EHT 1610 and EHT 5372) Confers High
853 Selectivity for Dual-Specificity Tyrosine Phosphorylation-Regulated Kinases. Journal of
854 Medicinal Chemistry *59*, 10315-10321.
855 Chen, W., Chen, L., and Tian, R. (2018). An integrated strategy for highly sensitive
856 phosphoproteome analysis from low micrograms of protein samples. Analyst *143*, 3693-3701.
857 Collins, G.A., and Goldberg, A.L. (2017). The Logic of the 26S Proteasome. Cell *169*, 792-806.
858 Collins, H.E., Zhu-Mauldin, X., Marchase, R.B., and Chatham, J.C. (2013).
859 STIM1/Orai1-mediated SOCE: current perspectives and potential roles in cardiac function and

860   pathology. Am J Physiol-Heart C *305*, H446-H458.

861   Coux, O., Tanaka, K., and Goldberg, A.L. (1996). Structure and functions of the 20S and 26S
862   proteasomes. Annu Rev Biochem *65*, 801-847.

863   Cuny, G.D., Robin, M., Ulyanova, N.P., Patnaik, D., Pique, V., Casano, G., Liu, J.F., Lin, X.J.,
864   Xian, J., Glicksman, M.A.*, et al.* (2010). Structure-activity relationship study of acridine analogs
865   as haspin and DYRK2 kinase inhibitors. Bioorganic & Medicinal Chemistry Letters *20*,
866   3491-3494.

867   Cuny, G.D., Ulyanova, N.P., Patnaik, D., Liu, J.F., Lin, X.J., Auerbach, K., Ray, S.S., Xian, J.,
868   Glicksman, M.A., Stein, R.L.*, et al.* (2012). Structure-activity relationship study of
869   beta-carboline derivatives as haspin kinase inhibitors. Bioorganic & Medicinal Chemistry
870   Letters *22*, 2015-2019.

871   Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of
872   Coot. Acta crystallographica Section D, Biological crystallography *66*, 486-501.

873   Guo, X., Huang, X., and Chen, M.J. (2017). Reversible phosphorylation of the 26S
874   proteasome. Protein Cell *8*, 255-272.

875   Guo, X., Wang, X., Wang, Z., Banerjee, S., Yang, J., Huang, L., and Dixon, J.E. (2016).
876   Site-specific proteasome phosphorylation controls cell proliferation and tumorigenesis. Nature
877   cell biology *18*, 202-212.

878   Hogrebe, A., von Stechow, L., Bekker-Jensen, D.B., Weinert, B.T., Kelstrup, C.D., and Olsen,
879   J.V.   (2018).   Benchmarking   common   quantification   strategies   for   large-scale
880   phosphoproteomics. Nat Commun *9*, 1045.

881   Jouanne, M., Rault, S., and Voisin-Chiret, A.S. (2017). Tau protein aggregation in Alzheimer's
882   disease: An attractive target for the development of novel therapeutic agents. European
883   Journal of Medicinal Chemistry *139*, 153-167.

884   Laplante, M., and Sabatini, D.M. (2012). mTOR signaling in growth control and disease. Cell
885   *149*, 274-293.

886   Liou, J., Kim, M.L., Heo, W.D., Jones, J.T., Myers, J.W., Ferrell, J.E., Jr., and Meyer, T. (2005).
887   STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. Curr Biol *15*,
888   1235-1241.

889   Liu, X., Xiao, W., Zhang, Y., Wiley, S.E., Zuo, T., Zheng, Y., Chen, N., Chen, L., Wang, X.,
890   Zheng, Y.*, et al.* (2020). Reversible phosphorylation of Rpn1 regulates 26S proteasome
891   assembly and function. Proc Natl Acad Sci U S A *117*, 328-336.

892   Lochhead, P.A., Sibbet, G., Morrice, N., and Cleghon, V. (2005). Activation-loop
893   autophosphorylation is mediated by a novel transitional intermediate form of DYRKs. Cell *121*,
894   925-936.

895   Ma, G., Wei, M., He, L., Liu, C., Wu, B., Zhang, S.L., Jing, J., Liang, X., Senes, A., Tan, P.*, et al.*
896   (2015). Inside-out Ca(2+) signalling prompted by STIM1 conformational switch. Nat Commun
897   *6*, 7826.

898   Ma, G., Zheng, S., Ke, Y., Zhou, L., He, L., Huang, Y., Wang, Y., and Zhou, Y. (2017).
899   Molecular Determinants for STIM1 Activation During Store- Operated Ca2+ Entry. Curr Mol
900   Med *17*, 60-69.

901   Ma, S., Bhattacharjee, R.B., and Bag, J. (2009). Expression of poly(A)-binding protein is
902   upregulated during recovery from heat shock in HeLa cells. FEBS J *276*, 552-570.

903   Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002). The protein

904   kinase complement of the human genome. Science *298*, 1912-1934.

905   McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., and Read, R.J.

906   (2007). Phaser crystallographic software. Journal of applied crystallography *40*, 658-674.

907   Nakai, J., Ohkura, M., and Imoto, K. (2001). A high signal-to-noise Ca(2+) probe composed of

908   a single green fluorescent protein. Nat Biotechnol *19*, 137-141.

909   Pozo-Guisado, E., Casas-Rua, V., Tomas-Martin, P., Lopez-Guerrero, A.M.,

910   Alvarez-Barrientos, A., and Martin-Romero, F.J. (2013). Phosphorylation of STIM1 at ERK1/2

911   target sites regulates interaction with the microtubule plus-end binding protein EB1. J Cell Sci

912   *126*, 3170-3180.

913   Pozo-Guisado, E., and Martin-Romero, F.J. (2013). The regulation of STIM1 by

914   phosphorylation. Commun Integr Biol *6*, e26283.

915   Prakriya, M., and Lewis, R.S. (2015). Store-Operated Calcium Channels. Physiol Rev *95*,

916   1383-1436.

917   Qin, X., Jiang, B., and Zhang, Y. (2016). 4E-BP1, a multifactor regulated multifunctional protein.

918   Cell Cycle *15*, 781-786.

919   Rai, A.K., Chen, J.X., Selbach, M., and Pelkmans, L. (2018). Kinase-controlled phase

920   transition of membraneless organelles in mitosis. Nature *559*, 211-216.

921   She, Q.B., Halilovic, E., Ye, Q., Zhen, W., Shirasawa, S., Sasazuki, T., Solit, D.B., and Rosen,

922   N. (2010). 4E-BP1 is a key effector of the oncogenic activation of the AKT and ERK signaling

923   pathways that integrates their function in tumors. Cancer Cell *18*, 39-51.

924   Soundararajan, M., Roos, A.K., Savitsky, P., Filippakopoulos, P., Kettenbach, A.N., Olsen, J.V.,

925   Gerber, S.A., Eswaran, J., Knapp, S., and Elkins, J.M. (2013). Structures of Down syndrome

926   kinases, DYRKs, reveal mechanisms of kinase activation and substrate recognition. Structure

927   (London, England : 1993) *21*, 986-996.

928   Wang, X., Li, W., Parra, J.L., Beugnet, A., and Proud, C.G. (2003). The C terminus of initiation

929   factor 4E-binding protein 1 contains multiple regulatory features that influence its function and

930   phosphorylation. Mol Cell Biol *23*, 1546-1557.

931   Wegiel, J., Gong, C.X., and Hwang, Y.W. (2011). The role of DYRK1A in neurodegenerative

932   diseases. FEBS J *278*, 236-245.

933   Wippich, F., Bodenmiller, B., Trajkovska, M.G., Wanka, S., Aebersold, R., and Pelkmans, L.

934   (2013). Dual specificity kinase DYRK3 couples stress granule condensation/dissolution to

935   mTORC1 signaling. Cell *152*, 791-805.

936   Wisniewski, J.R., Zougman, A., Nagaraj, N., and Mann, M. (2009). Universal sample

937   preparation method for proteome analysis. Nat Methods *6*, 359-362.

938   Zheng, S., Zhou, L., Ma, G., Zhang, T., Liu, J., Li, J., Nguyen, N.T., Zhang, X., Li, W.,

939   Nwokonko, R*., et al.* (2018a). Calcium store refilling and STIM activation in STIM- and

940   Orai-deficient cell lines. Pflugers Arch *470*, 1555-1567.

941   Zheng, S., Zhou, L., Ma, G., Zhang, T., Liu, J., Li, J., Nguyen, N.T., Zhang, X., Li, W.,

942   Nwokonko, R*., et al.* (2018b). Calcium store refilling and STIM activation in STIM- and

943   Orai-deficient cell lines. Pflugers Arch *470*, 1555-1567.

944   Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch,

945   S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for

946   biological-image analysis. Nat Methods 9, 676-682.

947

948

Appendix 1

General information for chemical synthesis

3   NMR spectra were recorded on a Varian 400 MHz spectrometer, Bruker 400 MHz NMR
4   spectrometer (ARX400), Bruker 400 MHz NMR spectrometer (AVANCE III), Bruker-500M Hz
5   NMR spectrometer (500M) and Bruker-600M Hz NMR spectrometer (600M) at ambient
6   temperature with $CDCl_3$ as the solvent unless otherwise stated. Chemical shifts are reported in
7   parts per million relative to $CDCl_3$ (1H, δ 7.26; 13C, δ 77.16) and MeOD (1H, δ 3.31; 13C, δ
8   49.00). Data for 1H NMR are reported as follows: chemical shift, integration, multiplicity (s =
9   singlet, d = doublet, t = triplet, q = quartet, quint = quintet, sixt = sixtet, m = multiplet) and
10   coupling constants. High-resolution mass spectra were obtained at Peking University Mass
11   Spectrometry Laboratory using a Bruker APEX Flash chromatography. The samples were
12   analyzed by UPLC/MS on a Waters Auto Purification LC/MS system (Waters C18 5 μm 150 X
13   4.6 mm SunFire separation column) or prepared by HPLC/MS on a Waters Auto Purification
14   LC/MS system (ACQUITY UPLC ® BEH C18 17 μm 2.1X50 mm column). Analytical thin layer
15   chromatography was performed using 0.25 mm silica gel 60-F plates, using 250 nm UV light
16   as the visualizing agent and a solution of phosphomolybdic acid and heat as developing
17   agents. Flash chromatography was performed using 200-400 mesh silica gel. Yields refer to
18   chromatographically pure materials, unless otherwise stated. All reactions were carried out in
19   oven-dried glassware under an argon atmosphere unless otherwise noted.

20

21   **Synthetic Procedures:**

22   **Appendix 1-scheme 1.** Synthesis route of C1-C4.

23   **5-methoxy-2-((4-methoxyphenyl)amino) benzoic acid (1a).** 2-bromo-5-methoxybenzoic
24   acid (9.26 g, 40 mmol), 4-methoxyaniline (6.90 g, 56 mmol), copper (0.73 g, 11 mmol), cuprous
25   oxide (0.82 g, 5.7 mmol) and potassium carbonate (7.74 g, 56 mmol) were added to 100 mL
26   DMF, the mixture was stirred at 80 °C overnight. The resulting slurry was cooled to room
27   temperature, and 2M HCl was added into the mixture until the system became acidic and a
28   large amount of solid was precipitated. After filtration, the precipitate was washed with water
29   and dried to give compound **1a** (4.02 g, 37%) as a dark green solid. **1a** was used in next step
30   without further purification.

31   **9-chloro-2,7-dimethoxyacridine (1b).** Compound **1a** (2.58 g, 9.45 mmol) was added in a
32   sealed tube, and 30 mL of phosphorus oxychloride was added under argon atmosphere. The
33   reaction was heated at 130 °C for 8 h. The resulting slurry was poured onto ice with vigorous
34   stirring, and a large amount of a yellow solid was precipitated. After filtration, the precipitate

35   was washed with water and dried to give compound **1b** (2.58 g, quant.) as an orange solid. **1b**

36   was used in next step without further purification.

37   ***tert*-butyl (3-((2,7-dimethoxyacridin-9-yl)thio)propyl)carbamate (1).** To a solution of **1b** (50

38   mg, 0.183 mmol) in 5 mL of anhydrous DMF, sodium hydrosulfide hydrate powder (67% 22.9

39   mg, 0.274 mmol) was added under argon atmosphere, and the reaction was stirred at 50 °C

40   for 2 h until full conversion of **1b**. *N*-Boc-3-aminopropyl bromide (60.9 mg, 0.274 mmol) and

41   potassium carbonate (50.5 mg, 0.365 mmol) were added into the slurry and the reaction was

42   allowed to react at room temperature overnight. The solvent was then evaporated and the

43   residue was dissolved with dichloromethane and washed with water. The combined organic

44   extracts were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was

45   purified by chromatography on a silica gel column to give compound **1** as a light yellow solid

46   (47.4 mg, 61%). ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.10 (d, $J = 9.3$ Hz, 2H), 7.94 (s, 2H), 7.42 (d, $J =$

47   9.3 Hz, 2H), 4.04 (s, 6H), 3.20 (d, $J = 5.9$ Hz, 2H), 2.95 (t, $J = 7.2$ Hz, 2H), 1.70 - 1.61 (m, 2H),

48   1.39 (s, 9H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 158.5, 156.0, 144.3, 136.0, 132.1, 130.7, 124.1,

49   102.2, 79.5, 55.8, 39.6, 33.6, 30.8, 28.5. HRMS(ESI) [M + H]$^+$ calculated for $C_{23}H_{29}N_2O_4S$:

50   429.1843, found: 429.1831.

51

52   **Compounds 2-4**. By employment of the above-described procedure, starting from **1b** and

53   using suitable bromides, compounds 2-4 were prepared.

54   ***N*-(3-((2,7-dimethoxyacridin-9-yl)thio)propyl)acetamide (2). Yield 58%.** ^{1}H NMR (400 MHz,

55   CDCl$_3$) δ 8.10 (d, $J = 9.4$ Hz, 2H), 7.94 (d, $J = 2.7$ Hz, 2H), 7.42 (dd, $J = 9.4$, 2.8 Hz, 2H), 4.04

56   (s, 6H), 3.30 (dd, $J = 13.2$, 6.7 Hz, 2H), 2.96 (t, $J = 7.3$ Hz, 2H), 1.88 (s, 3H), 1.69 - 1.59 (m,

57   2H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 170.2, 158.6, 144.3, 135.8, 132.2, 130.7, 124.1, 102.2, 55.8,

58   38.7, 33.6, 30.4, 23.4. HRMS(ESI) [M + H]$^+$ calculated for $C_{20}H_{23}N_2O_3S$: 371.1424, found:

59   371.1428.

60   ***N*-(4-((2,7-dimethoxyacridin-9-yl)thio)butyl)acetamide (3). Yield 57%.** ^{1}H NMR (400 MHz,

61   CDCl$_3$) δ 8.10 (d, $J = 9.3$ Hz, 2H), 7.95 (d, $J = 2.7$ Hz, 2H), 7.42 (dd, $J = 9.3$, 2.7 Hz, 2H), 4.04

62   (s, 6H), 3.15 (dd, $J = 13.1$, 6.7 Hz, 2H), 2.94 (t, $J = 7.1$ Hz, 2H), 1.86 (s, 3H), 1.59 (m, 2H), 1.49

63   (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 170.1, 158.4, 144.2, 136.2, 132.0, 130.7, 124.0, 102.2,

64   55.8, 39.0, 35.9, 28.9, 27.5, 23.3. HRMS(ESI) [M + H]$^+$ calculated for $C_{21}H_{25}N_2O_3S$: 385.1580,

65   found: 385.1572.

66   **4-((2,7-dimethoxyacridin-9-yl)thio)butanenitrile (4). Yield 52%.** ^{1}H NMR (400 MHz, CDCl$_3$)

67   δ 8.11 (d, $J = 9.4$ Hz, 2H), 7.89 (d, $J = 2.7$ Hz, 2H), 7.43 (dd, $J = 9.4$, 2.8 Hz, 2H), 4.04 (s, 6H),

68   3.07 (t, $J = 7.0$ Hz, 2H), 2.47 (t, $J = 7.0$ Hz, 2H), 1.82 - 1.72 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$)

69 δ 158.7, 144.3, 134.4, 132.3, 130.6, 124.2, 118.8, 101.7, 55.8, 34.4, 25.8, 16.4. HRMS(ESI) [M

70 + H]$^+$ calculated for $C_{19}H_{19}N_2O_2S$: 339.1162, found: 339.1159.

71

72 **Appendix 1-scheme 2.** Synthesis route of C5-C6.

73

74 **tert-butyl 4-((2,7-dimethoxyacridin-9-yl)thio)piperidine-1-carboxylate (5a).** To a solution of

75 **1b** (50 mg, 0.183 mmol) in 5 mL of anhydrous DMF, sodium hydrosulfide hydrate powder (67%

76 22.9 mg, 0.274 mmol) was added under argon atmosphere, and the reaction was stirred at

77 50 °C for 2 h until full conversion of **1b**. 4-bromopiperidine-1-carboxylic acid tert-butyl ester

78 (72.4mg，0.274mmol) and potassium carbonate (50.5 mg, 0.365 mmol) were added into the

79 slurry and the reaction was allowed to react at room temperature overnight. The solvent was

80 then evaporated and the residue was dissolved with dichloromethane and washed with water.

81 The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*.

82 The residue was purified by chromatography on a silica gel column to give compound **5a** as a

83 light yellow solid (45.4mg, 55%). ^{1}H NMR: (400 MHz, CDCl$_3$) δ 8.08 (d, $J = 9.3$ Hz, 2H), 7.93 (d,

84 $J = 2.7$ Hz, 2H), 7.40 (dd, $J = 9.3$, 2.8 Hz, 2H), 4.01 (s, 6H), 3.98-3.84 (m, 2H), 3.25 - 3.17 (m,

85 1H), 2.81 (ddd, $J = 13.5$, 10.5, 3.0 Hz, 2H), 1.86-1.72 (m, 2H), 1.72-1.58 (m, 2H), 1.42 (s, 9H).

86 ^{13}C NMR (101 MHz, CDCl$_3$) δ 158.4, 154.7, 144.3, 134.8, 132.0, 131.1, 124.0, 102.4, 79.8,

87 55.7, 46.9, 43.2, 33.1, 28.5. HRMS(ESI) [M + H]$^+$ calculated for $C_{25}H_{31}N_2O_4S$: 455.1999, found:

88 455.1995.

89 **Compounds 6a**. By employment of the above-described procedure, starting from **1b** and

90 using suitable bromide, compound **6a** were prepared.

91 **tert-butyl 4-(((2,7-dimethoxyacridin-9-yl)thio)methyl)piperidine-1-carboxylate (6a). Yield**

92 **79%.** ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.10 (d, $J = 9.4$ Hz, 2H), 7.94 (d, $J = 2.7$ Hz, 2H), 7.42 (dd, J

93 $= 9.4$, 2.8 Hz, 2H), 4.03 (d, $J = 48.7$ Hz, 6H), 4.15 - 3.98 (overlapped, m, 2H), 2.82 (d, $J = 6.8$

94 Hz, 2H), 2.59 (t, $J = 12.1$ Hz, 2H), 1.86 (br s, 2H), 1.53 - 1.46 (m, 1H), 1.43 (s, 9H), 1.23-1.14

95 (m, $J = 10.9$ Hz, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 158.4, 154.8, 144.2, 136.7, 132.0, 130.4,

96 123.9, 102.0, 79.5, 55.6, 43.6, 42.9, 36.8, 28.5. HRMS(ESI) [M + H]$^+$ calculated for

97 $C_{26}H_{36}N_2O_4S$: 469.2156, found: 469.2153.

98 **2,7-dimethoxy-9-(piperidin-4-ylthio)acridine (5).** Compound **5a** (45.4mg, 0.100 mmol) was

99 dissolved in a 5% trifluoroacetic acid dichloromethane solution and the mixture was allowed to

100 react at room temperature for 2h. The solvent was then evaporated and the residue was

101 dissolved with methanol, then purified by HPLC/MS on a Waters Auto Purification LC/MS

102 system (ACQUITY UPLC ® BEH C18 17 μm 2.1X50 mm column) to afford **5** as a dark red

103 solid (35.2mg, 75%). ^{1}H NMR (400 MHz, MeOD) δ 8.05 (d, $J = 9.4$ Hz, 2H), 8.00 (d, $J = 2.7$ Hz,

104 2H), 7.49 (dd, $J = 9.4$, 2.8 Hz, 2H), 4.05 (s, 6H), 3.36-3.33 (m, 1H), 2.99 (d, $J = 6.8$ Hz, 2H),

105 2.85 (td, $J = 12.8$, 2.9 Hz, 2H), 2.13 (d, $J = 16.5$ Hz, 2H), 1.70-1.65 (m, 2H). ^{13}C NMR (151

106 MHz, MeOD) δ 160.9, 144.0, 138.6, 132.9, 129.7, 126.7, 104.0, 56.0, 45.5, 44.4, 30.9.

107 HRMS(ESI) [M + H]$^+$ calculated for $C_{20}H_{23}N_2O_2S$: 355.1475, found: 355.1467.

108 **Compounds 6**. By employment of the above-described procedure, starting from **6a**,

109 compounds **6** was prepared.

110 **2,7-dimethoxy-9-((piperidin-4-ylmethyl)thio)acridine (6). Yield 56%.** ^{1}H NMR (400 MHz,

111 MeOD) δ 8.24 (d, $J = 9.4$ Hz, 2H), 8.17 (d, $J = 2.3$ Hz, 2H), 7.85 (dd, $J = 9.4$, 2.4 Hz, 2H), 4.14

112 (s, 6H), 3.75-3.68 (m, 1H), 3.39 (d, $J = 13.3$ Hz, 2H), 3.01 (t, $J = 10.9$ Hz, 2H), 2.18 - 2.09 (m,

113 2H), 2.05 - 2.01 (m, 2H), 1.37 - 1.32 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 61.0, 151.0, 135.6,

114 131.8, 131.1, 124.0, 104.0, 56.8, 44.7, 44.2, 35.9, 29.1. HRMS(ESI) [M + H]$^+$ calculated for

115 $C_{21}H_{25}N_2O_2S$: 369.1631, found: 369.1638.

116

117 **Appendix 1-scheme 3.** General procedure of C7, C16-C19 synthesis.

118

119 **6,6'-azanediylbis-3-methoxybenzoic acid (1a').** 2-amino-5-methoxybenzoic acid (2.00 g,

120 8.66 mmol), 2-bromo-5-methoxybenzoic acid (0.11 g, 1.73 mmol), copper (0.11 g, 1.73 mmol),

121 cuprous oxide (0.12 g, 0.87 mmol) and potassium carbonate (7.74 g, 56 mmol) were added to

122 15 mL DMF, the mixture was stirred at 80 °C overnight. The resulting slurry was cooled to

123 room temperature, and 2 M HCl was added into the mixture until the system became acidic

124 and a large amount of solid was precipitated. After filtration, the precipitate was washed with

125 water and dried to give compound **1a'** (2.00 g, 73%) as a green solid. **1a'** was used in next

126 step without further purification.

127 **9-chloro-2,7-dimethoxyacridine-4-carboxylic acid (1b').** Compound **1a'** (1.00 g, 3.16 mmol)

128 was added in a sealed tube, and 10 mL of phosphorus oxychloride was added under argon

129 atmosphere. The reaction was heated at 130 °C for 8 h. The resulting slurry was poured onto

130 ice with vigorous stirring, and a large amount of a yellow solid was precipitated. After filtration,

131 the precipitate was washed with water and dried to give compound **1b** (1.00 g, 95%) as an

132 orange solid. **1b'** was used in next step without further purification.

133 **methyl 9-chloro-2,7-dimethoxyacridine-4-carboxylate (1c).** To a suspension of **1b'** (0.89 g,

134 2.67 mmol) in 10 mL of dry dichloromethane, 0.40 mL of oxalyl chloride was added followed by

135 one drop of DMF, and a large amount of bubbles was generated. The mixture was allowed to

136 react at room temperature for 0.5 h until the system became a brownish black solution. Then

137 the reaction was quenched by dry menthol at room temperature for 2 h, followed by the

138 addition of triethylamine until the mixture became neutral. The system was diluted with

139 dichloromethane, washed twice with brine, dried over anhydrous Na_2SO_4 and concentrated *in*

140 *vacuo*. The residue was purified by chromatography on a silica gel column (dichloromethane /

141 ethyl acetate = 95/5) to gave compound **1c** as a yellow solid (0.74 g, 80%).^{1}H NMR (400 MHz,

142 $CDCl_3$) δ 8.12 (d, $J = 9.3$ Hz, 1H), 7.70 (s, 1H), 7.64 (s, 1H), 7.46 (s, 1H), 7.42 (d, $J = 9.4$ Hz,

143 1H), 4.09 (s, 3H), 4.03 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.9, 159.0, 157.1, 144.8, 141.3,

144 135.9, 134.1, 132.6, 125.8, 125.7, 125.0, 124.9, 103.0, 99.6, 56.0, 55.8, 52.3. HRMS(ESI) [M

145 + H][+] calculated for $C_{17}H_{15}ClNO_4$: 332.0684, found: 332.0680.

146

147 **Appendix 1-scheme 4** Synthesis route of C7.

148

149 **methyl-9-(((1-(tert-butoxycarbonyl)piperidin-4-yl)methyl)thio)-2,7-dimethoxyacridine-4-c**

150 **aboxylate (7a).** To a solution of **1c** (0.38 g, 1.16 mmol) in 10 mL of anhydrous DMF, sodium

151 hydrosulfide hydrate powder (70%, 0.10 g, 1.21 mmol) was added under argon atmosphere,

152 and the reaction was stirred at 50 °C for 2 h until full conversion of **1c**.

153 1-Boc-4-bromomethylpiperidine (0.64 g, 2.31 mmol) and potassium carbonate (0.40 g, 2.89

154 mmol) were added into the slurry and the reaction was allowed to react at room temperature

155 overnight. The solvent was then evaporated and the residue was dissolved with

156 dichloromethane and washed with water. The combined organic extracts were dried over

157 anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by chromatography

158 on a silica gel column (dichloromethane / ethyl acetate = 95/5) to give compound **7a** as a

159 yellow solid (0.38g, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, $J = 9.4$ Hz, 1H), 8.08 (d, $J =$

160 2.0 Hz, 1H), 7.89 (s, 1H), 7.68 (d, $J = 1.9$ Hz, 1H), 7.41 (dd, $J = 9.4, 1.9$ Hz, 1H), 4.09 (s, 3H),

161 4.02 (s, 6H), 2.78 (d, $J = 6.7$ Hz, 2H), 2.55 (t, $J = 12.2$ Hz, 2H), 1.82 (br s, 2H), 1.45-1.35(m,

162 3H), 1.43 (s, 9H), 1.20-1.09 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.2, 158.9, 157.0, 154.8,

163 144.7, 141.2, 137.0, 134.6, 133.0, 130.7, 130.5, 124.4, 124.3, 105.11, 101.74, 79.6, 56.0, 55.7,

164 52.8, 43.8, 43.1 (br s), 36.8, 31.8, 28.5. HRMS(ESI) [M + H][+] calculated for $C_{28}H_{35}N_2O_6S$:

165 527.2210, found: 527.2212.

166 **Compounds 16a-19a**. By employment of the above-described procedure, starting from **1c'**

167 and using suitable bromides, compounds **16a-19a** were prepared.

168 **methyl**

169 **9-((3-((tert-butoxycarbonyl)amino)propyl)thio)-2,7-dimethoxyacridine-4-carboxylate**

170 **(16a). Yield 76%.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (d, $J = 9.4$ Hz, 1H), 8.09 (d, $J = 2.8$ Hz,

171   1H), 7.89 (d, J = 2.8 Hz, 1H), 7.68 (dd, J = 2.9, 0.8 Hz, 1H), 7.41 (dd, J = 9.3, 2.8 Hz, 1H), 4.40

172   (br s, 1H), 4.09 (s, 3H), 4.04 (s, 3H), 4.03 (s, 3H), 3.17 (q, J = 6.6 Hz, 2H), 2.92 (t, J = 7.3 Hz,

173   2H), 1.61-1.59 (m, 2H), 1.39 (s, 9H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 168.2, 158.8, 157.0, 155.9,

174   144.57, 141.08, 136.2, 134.5, 132.9, 130.8, 130.6, 124.3, 124.3, 105.1, 101.8, 55.9, 55.7, 52.8,

175   39.5, 33.7, 30.7, 28.4. HRMS(ESI) [M + H]$^+$ calculated for C$_{25}$H$_{31}$N$_2$O$_6$S: 487.1889, found:

176   487.1897.

177   **methyl-(S)-9-(((1-(tert-butoxycarbonyl)pyrrolidin-3-yl)methyl)thio)-2,7-dimethoxyacridin**

178   **e-4-carboxylate (17a). Yield 76%.** ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.13 (d, J = 9.4 Hz, 1H), 8.08

179   (d, J = 2.4 Hz, 1H), 7.88 (d, J = 2.6 Hz, 1H), 7.68 (d, J = 2.7 Hz, 1H), 7.42 (dd, J = 9.4, 2.6 Hz,

180   1H), 4.10 (s, 3H), 4.05 (s, 3H), 4.04 (s, 3H), 3.61-3.14 (m, 2H), 3.27-2.1 (m, 4H), 2.10-2.01 (m,

181   1H), 1.90-1.85 (m, 1H), 1.68-1.59 (m, 1H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 168.2,

182   159.0, 157.1, 154.5, 144.6, 141.1, 135.9, 134.7, 133.1, 130.7, 130.5, 124.4, 124.3, 104.9,

183   101.6, 79.4, 55.9 (d, J = 96 Hz), 52.8, 51.0 (d, J = 104 Hz), 45.2 (d, J = 104 Hz), 39.6-39.3 (m),

184   38.4, 31.3, 30.7, 28.6. HRMS(ESI) [M + H]$^+$ calculated for C$_{27}$H$_{33}$N$_2$O$_6$S: 513.2052, found:

185   513.2054.

186   **methyl-(R)-9-(((1-(tert-butoxycarbonyl)pyrrolidin-3-yl)methyl)thio)-2,7-dimethoxyacridin**

187   **e-4-carboxylate (18a). Yield 84%.** ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.13 (d, J = 9.4 Hz, 1H), 8.07

188   (s, 1H), 7.88 (s, 1H), 7.68 (s, 1H), 7.41 (d, J = 8.8 Hz, 1H), 4.09 (s, 3H), 4.04 (s, 6H), 3.62-3.31

189   (m, 2H), 3.26-3.18 (m, 1H), 3.10-2.79 (m, 3H), 2.11-1.99 (m, 1H), 1.97-1.86 (m, 1H), 1.65-1.56

190   (m, 1H), 1.43 (s, 9H). ^{13}C NMR (126 MHz, CDCl$_3$) δ 168.2, 159.1, 157.2, 154.5, 144.7, 141.3,

191   135.9, 134.8, 133.2, 130.8, 130.6, 124.4, 124.3, 105.0, 101.7, 79.4, 55.9(d, J = 105 Hz), 52.8,

192   51.1 (d, J = 115 Hz), 45.2 (d, J = 135 Hz), 39.7-39.4 (m), 38.5, 31.4, 30.7, 28.6. HRMS(ESI) [M

193   + H]$^+$ calculated for C$_{27}$H$_{33}$N$_2$O$_6$S: 513.2052, found: 513.2054.

194   **methyl-9-(((1-(tert-butoxycarbonyl)azetidin-3-yl)methyl)thio)-2,7-dimethoxyacridine-4-ca**

195   **rboxylate (19a). Yield 75%.** ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.14 (d, J = 9.4 Hz, 1H), 8.03 (s, 1H),

196   7.84 (s, 1H), 7.68 (d, J = 1.6 Hz, 1H), 7.42 (d, J = 9.4 Hz, 1H), 4.10 (s, 3H), 4.04 (s, 6H), 3.85 (t,

197   J = 8.3 Hz, 2H), 3.60 (s, 2H), 3.10 (d, J = 7.8 Hz, 2H), 2.26-2.16 (m, 1H), 1.40 (s, 9H). ^{13}C NMR

198   (126 MHz, CDCl$_3$) δ 168.2, 159.2, 157.3, 156.3, 144.7, 141.2, 134.9, 134.8, 133.2, 130.9,

199   130.7, 124.5, 124.3, 104.9, 101.6, 79.7, 56.0, 55.8, 54.0(br s), 52.8, 39.9, 29.0, 28.5.

200   HRMS(ESI) [M + H]$^+$ calculated for C$_{26}$H$_{31}$N$_2$O$_6$S: 499.1897, found: 499.1895.

201   ***tert*-butyl-4-(((4-(hydroxymethyl)-2,7-dimethoxyacridin-9-yl)thio)methyl)piperidine-1-car**

202   **boxylate (7b).** 3.1 mL of 1.5 M DIBAL-H solution in toluene was added to a solution of **7a** (348

203   mg, 0.661 mmol) in 10 mL of dry dichloromethane at 0 °C, and the reaction was stirred at room

204   temperature for 4 h. The reaction was quenched by adding saturated potassium hydrogen

205   tartrate solution, diluted with dichloromethane, washed twice with brine, dried over anhydrous

206   Na_2SO_4 and concentrated *in vacuo*. The residue was purified by chromatography on a silica

207   gel column (dichloromethane / ethyl acetate = 90/10) to gave compound **7b** (180 mg, 55%) as

208   yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, *J* = 9.3 Hz, 1H), 7.88 (s, 1H), 7.81 (s, 1H),

209   7.39 (d, *J* = 9.3 Hz, 1H), 7.27 (s, 1H), 5.41 (s, 1H), 5.21 (s, 2H), 4.02 (s, 3H), 4.00 (s, 3H) 2.79

210   (d, *J* = 6.7 Hz, 2H), 2.57 (t, *J* = 11.7 Hz, 2H), 1.84 (br s, 2H), 1.47-1.25 (m, 3H), 1.43 (s, 9H),

211   1.20-1.09 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.5, 157.9, 154.8, 143.3, 142.7, 140.4,

212   137.6, 131.9, 131.1, 130.5, 124.1, 122.1, 102.0, 101.4, 79.6, 65.0, 55.7, 55.7, 43.6, 43.1, 36.8,

213   31.8, 28.5. HRMS(ESI) [M + H][+] calculated for $C_{27}H_{35}N_2O_5S$: 499.2258, found: 499.2261.

214   **Compounds 16b-19b**. By employment of the above-described procedure, starting from

215   **16a-19a**, compounds **16b-19b** were prepared.

216   ***tert*-butyl (3-((4-(hydroxymethyl)-2,7-dimethoxyacridin-9-yl)thio)propyl)carbamate (16b).**

217   **Yield 68%.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, *J* = 9.3 Hz, 1H), 7.92 (d, *J* = 2.8 Hz, 1H), 7.85

218   (d, *J* = 2.8 Hz, 1H), 7.41 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.27 (d, *J* = 2.7 Hz, 1H), 5.42 (br s, 1H), 5.21

219   (s, 2H), 4.43 (br s, 1H), 4.04 (s, 5H), 4.01 (s, 5H), 3.19 (d, *J* = 6.7 Hz, 2H), 2.93 (t, *J* = 7.3 Hz,

220   2H), 1.62 (t, *J* = 7.0 Hz, 3H), 1.39 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.6, 158.0, 156.0,

221   143.4, 142.7, 132.0, 131.3, 130.8, 124.2, 122.2, 102.1, 101.6, 65.1, 55.8, 55.8, 39.6, 33.7,

222   30.8, 28.5. HRMS(ESI) [M + H][+] calculated for $C_{24}H_{31}N_2O_5S$: 459.1948, found: 459.1950.

223   ***tert*-butyl-(S)-3-(((4-(hydroxymethyl)-2,7-dimethoxyacridin-9-yl)thio)methyl)pyrrolidine-1**

224   **-carboxylate (17b). 68%.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, *J* = 9.3 Hz, 1H), 7.92 (d, *J* =

225   2.6 Hz, 1H), 7.85 (d, *J* = 2.3 Hz, 1H), 7.42 (dd, *J* = 9.3, 2.5 Hz, 1H), 7.29 (s, 1H), 5.36 (br s, 1H),

226   5.21 (s, 2H), 4.05 (s, 3H), 4.02 (s, 3H), 3.62-3.32 (m, 2H), 3.27 - 2.84 (m, 4H), 2.15-2.05 (d, *J* =

227   6.4 Hz, 1H), 2.01 - 1.92 (m, 1H), 1.70-1.60 (m, 1H), 1.43 (d, *J* = 4.8 Hz, 9H). ^{13}C NMR (126

228   MHz, $CDCl_3$) δ 158.6, 158.0, 154.4, 143.4, 142.7, 140.5, 136.5, 131.9, 131.1, 130.5, 124.1,

229   122.1, 101.9, 101.3, 79.3, 64.9, 55.7, 51.1 (d, *J* = 115 Hz), 45.1 (d, *J* = 135 Hz), 39.4, 38.4,

230   31.2, 30.6, 28.5. HRMS(ESI) [M + H][+] calculated for $C_{26}H_{33}N_2O_5S$: 485.2105, found: 485.2110.

231   ***tert*-butyl-(R)-3-(((4-(hydroxymethyl)-2,7-dimethoxyacridin-9-yl)thio)methyl)pyrrolidine-1**

232   **-carboxylate (18b). 64%.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (d, *J* = 9.4 Hz, 1H), 7.90 (d, *J* =

233   2.7 Hz, 1H), 7.83 (d, *J* = 2.6 Hz, 1H), 7.41 (dd, *J* = 9.3, 2.7 Hz, 1H), 7.28 (s, 1H), 5.38 (br s, 1H),

234   5.21 (s, 2H), 4.04 (s, 3H), 4.02 (s, 3H), 3.64 - 3.20 (m, 4H), 3.08 - 2.87 (m, 2H), 2.14 - 2.03 (m,

235   1H), 1.99-1.90 (m, 1H), 1.68 - 1.57 (m, 1H), 1.42 (d, *J* = 5.2 Hz, 9H). ^{13}C NMR (126 MHz,

236      $CDCl_3$) δ 158.6, 158.0, 154.4, 143.3, 142.7, 140.5, 136.4, 131.9, 131.1, 130.5, 124.1, 122.0,

237   101.9, 101.3, 79.3, 64.9, 55.7, 50.9 (d, *J* = 115 Hz), 45.1 (d, *J* = 140 Hz), 39.4, 38.4, 31.2, 30.6,

238   28.5. HRMS(ESI) [M + H][+] calculated for $C_{26}H_{33}N_2O_5S$: 485.2105, found: 485.2102.

239 **_tert_-butyl-3-(((4-(hydroxymethyl)-2,7-dimethoxyacridin-9-yl)thio)methyl)azetidine-1-carb**

240 **oxylate (19b). 50%.** ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.08 (d, J = 9.3 Hz, 1H), 7.87 (d, J = 2.7 Hz,

241 1H), 7.80 (d, J = 2.7 Hz, 1H), 7.43 (dd, J = 9.3, 2.7 Hz, 1H), 7.30 (d, J = 2.6 Hz, 1H), 5.22 (s,

242 2H), 4.04 (s, 3H), 4.02 (s, 3H), 3.85 (t, J = 8.4 Hz, 2H), 3.59 (dd, J = 8.8, 5.2 Hz, 2H), 3.12 (d, J

243 = 7.9 Hz, 2H), 2.24 (td, J = 7.9, 4.1 Hz, 1H), 1.39 (s, 9H). ^{13}C NMR (126 MHz, CDCl$_3$) δ 158.7,

244 158.1, 156.2, 143.0, 142.5, 140.5, 135.6, 131.8, 131.2, 130.7, 124.3, 122.3, 101.8, 101.2, 79.6,

245 64.8, 55.7, 55.7, 39.7, 29.0, 28.3. HRMS(ESI) [M + H]$^+$ calculated for C$_{25}$H$_{31}$N$_2$O$_5$S: 471.1948,

246 found: 471.1939.

247 **(2,7-dimethoxy-9-((piperidin-4-ylmethyl)thio)acridin-4-yl)methanol (7).** Compound **7b**

248 (24.2 mg, 0.048 mmol) was dissolved in 2 mL of 5% trifluoroacetic acid dichloromethane

249 solution and the mixture was allowed to react at room temperature for 2 h. The solvent was

250 then evaporated and the residue was dissolved with methanol, then purified by HPLC/MS on a

251 Waters Auto Purification LC/MS system (ACQUITY UPLC ® BEH C18 17 μm 2.1X50 mm

252 column) to afford **7** (11.2 mg, 45%) as a dark red solid. ^{1}H NMR (400 MHz, MeOD) δ 8.09 (d, J

253 = 9.4 Hz, 1H), 7.76 (d, J = 2.2 Hz, 1H), 7.71 (d, J = 2.2 Hz, 1H), 7.54 (d, J = 1.1 Hz, 1H), 7.50

254 (dd, J = 9.4, 2.5 Hz, 1H), 5.23 (s, 2H), 4.04 (s, 3H), 4.03 (s, 3H), 3.33 - 3.30 (m, 2H), 2.95 (d, J

255 = 6.7 Hz, 2H), 2.83 (td, J = 12.8, 1.6 Hz, 2H), 2.07-1.99 (m, 2H), 1.66 - 1.55 (m, 1H), 1.51 -

256 1.40 (m, 2H). ^{13}C NMR (126 MHz, MeOD) δ 160.0, 159.8, 144.0, 143.1, 143.0, 137.7, 132.9,

257 131.7, 131.4, 124.9, 121.8, 102.8, 101.7, 62.2, 56.2, 56.1, 44.8, 42.9, 35.7, 29.4. HRMS(ESI)

258 [M + H]$^+$ calculated for C$_{22}$H$_{27}$N$_2$O$_3$S: 399.1737, found: 399.1732.

259 **Compounds 16-19**. By employment of the above-described procedure, starting from **16b-19b**,

260 compounds **16-19** were prepared.

261 **(9-((3-aminopropyl)thio)-2,7-dimethoxyacridin-4-yl)methanol (16). Yield 88%.** ^{1}H NMR

262 (400 MHz, MeOD) δ 8.13 (d, J = 9.4 Hz, 1H), 7.94 (d, J = 26.6 Hz, 2H), 7.58 - 7.54 (m, 1H),

263 7.50 (dd, J = 9.4, 2.7 Hz, 1H), 5.31 (s, 2H), 4.05 (s, 3H), 4.05 (s, 3H), 3.09 (t, J = 7.4 Hz, 2H),

264 2.95 - 2.88 (t, J = 7.4 Hz, 2H), 1.79 - 1.70 (m, 2H). ^{13}C NMR (151 MHz, MeOD) δ 160.3, 160.0,

265 141.4, 140.9, 140.6, 139.8, 132.1, 131.7, 129.9, 127.0, 124.3, 103.2, 102.3, 62.1, 56.4, 56.3,

266 39.5, 34.1, 29.4.HRMS(ESI) [M + H]$^+$ calculated for C$_{19}$H$_{23}$N$_2$O$_3$S: 359.1424, found: 359.1428.

267 **(S)-(2,7-dimethoxy-9-((pyrrolidin-3-ylmethyl)thio)acridin-4-yl)methanol (17). Yield 93%.**

268 ^{1}H NMR (400 MHz, MeOD) δ 8.11 (d, J = 9.4 Hz, 1H), 7.81 (s, 1H), 7.75 (s, 1H), 7.57 (s, 1H),

269 7.51 (d, J = 9.4 Hz, 1H), 5.27 (s, 2H), 4.07 (s, 3H), 4.07 (s, 3H), 3.36 - 3.27 (m, 2H), 3.19 - 3.05

270 (m, 3H), 2.96 (dd, J = 11.4, 8.3 Hz, 1H), 2.25 (hept, J = 7.6 Hz, 1H), 2.17 - 2.05 (m, 1H), 1.80 -

271 1.70 (m, 1H). ^{13}C NMR (126 MHz, MeOD) δ 160.3, 160.1, 141.8, 141.4, 140.7, 139.9, 131.8,

272   131.4, 130.8, 126.4, 123.6, 103.0, 102.0, 62.1, 56.3, 56.2, 50.7, 46.2, 40.0, 39.5, 30.9.

273   HRMS(ESI) [M + H]$^+$ calculated for $C_{21}H_{25}N_2O_3S$: 385.1580, found: 385.1580.

274   **(R)-(2,7-dimethoxy-9-((pyrrolidin-3-ylmethyl)thio)acridin-4-yl)methanol (18). Yield 64%.**

275   ^{1}H NMR (400 MHz, MeOD) δ 8.14 (d, $J = 9.3$ Hz, 1H), 7.96 (s, 1H), 7.89 (s, 1H), 7.58 (s, 1H),

276   7.53 - 7.48 (m, 1H), 5.32 (s, 2H), 4.06 (s, 6H), 3.35 - 3.26 (m, 2H), 3.20 - 3.08 (m, 3H), 2.97 -

277   2.92 (m, 1H), 2.26 (hept, $J = 7.6$ Hz, 1H), 2.17 - 2.07 (m, 1H), 1.80 - 1.71 (m, 1H). ^{13}C NMR

278   (126 MHz, MeOD) δ 160.2, 160.0, 141.4, 141.1, 140.3, 140.2, 131.7, 131.3, 130.5, 126.5,

279   123.7, 103.0, 102.0, 62.1, 56.3, 56.2, 50.6, 46.1, 39.9, 39.6, 30.9. HRMS(ESI) [M + H]$^+$

280   calculated for $C_{21}H_{25}N_2O_3S$: 385.1580, found: 385.1579.

281   **(9-((azetidin-3-ylmethyl)thio)-2,7-dimethoxyacridin-4-yl)methanol (19). Yield 56%.** ^{1}H

282   NMR (400 MHz, MeOD) δ 8.07 (d, $J = 9.4$ Hz, 1H), 7.80 (d, $J = 2.2$ Hz, 1H), 7.73 (d, $J = 2.1$ Hz,

283   1H), 7.53 (s, 1H), 7.46 (dd, $J = 9.3, 2.2$ Hz, 1H), 5.28 (s, 2H), 4.04 (s, 3H), 4.03 (s, 3H), 3.82 (t,

284    $J = 9.7$ Hz, 2H), 3.69 - 3.62 (m, 2H), 3.26 (d, $J = 7.9$ Hz, 2H), 2.71 - 2.58 (m, 1H). ^{13}C NMR

285   (101 MHz, MeOD) δ 160.4, 160.2, 142.4, 142.0, 141.3, 138.0, 132.0, 131.7, 131.5, 126.2,

286   123.2, 102.7, 101.6, 62.0, 56.3, 56.2, 51.9, 38.9, 33.9. HRMS(ESI) [M + H]$^+$ calculated for

287    $C_{20}H_{23}N_2O_3S$: 371.1424, found: 371.1431.

288

289   **Appendix 1-scheme 5.** Synthesis route of C8.

290   **2,7-dimethoxy-9-((piperidin-4-ylmethyl)thio)acridine-4-carboxylic acid (8).** 0.4 mL of

291   aqueous1.0 M lithium hydroxide solution of was added into a solution of compound **7a** (42 mg,

292   0.080 mmol) 2 mL of tetrahydrofuran, and the mixture was reacted at 40 ° C for 20 h. After

293   cooling to room temperature, 5 mL of a 10% solution of trifluoroacetic acid in dichloromethane

294   was added to the system, and the mixture was reacted at room temperature for 6 h. The

295   solvent was then evaporated and the residue was dissolved with methanol, then purified by

296   HPLC/MS on a Waters Auto Purification LC/MS system (ACQUITY UPLC ® BEH C18 17 µm

297   2.1X50 mm column) to afford **8** (21.6 mg, 51%) as a dark red foam. ^{1}H NMR (600 MHz, MeOD)

298   δ 8.39 (d, $J = 2.8$ Hz, 1H), 8.23 (d, $J = 2.7$ Hz, 1H), 8.12 (d, $J = 9.3$ Hz, 1H), 7.97 (d, $J = 2.5$ Hz,

299   1H), 7.62 (dd, $J = 9.3, 2.6$ Hz, 1H), 4.09 (s, 3H), 4.07 (s, 3H), 3.36-3.33 (m, 2H), 3.02 (d, $J =$

300   6.8 Hz, 2H), 2.89 - 2.82 (m, 2H), 2.11 (d, $J = 14.0$ Hz, 2H), 1.70 - 1.64 (m, 1H), 1.50 - 1.41 (m,

301   2H). ^{13}C NMR (126 MHz, MeOD) δ 168.7, 160.7, 158.9, 141.9, 141.4, 140.9, 131.6, 131.5,

302   130.7, 130.5, 128.0, 126.7, 108.6, 103.1, 56.6, 56.5, 44.8, 43.4, 35.7, 29.4. HRMS(ESI) [M +

303   H]$^+$ calculated for $C_{22}H_{25}N_2O_4S$: 413.1530, found: 413.1526.

304

305   **Appendix 1-scheme 6.** Synthesis route of C9.

306

tert-butyl 4-(((4-formyl-2,7-dimethoxyacridin-9-yl)thio)methyl)piperidine-1-carboxylate

(9a). 20.4 µl of oxalyl chloride was dissolved in 1 mL of dry tetrahydrofuran and the mixture

was cooled to -78 ° C. 25.6 µl of dimethyl sulfoxide was slowly added to the system, the

reaction was stirred at -78 ° C for 30 min, followed by addition of a solution of compound **7b**

(60 mg, 0.12 mmol) in 1 mL of tetrahydrofuran solution, and the reaction was continued at -78 °

C for 1 h. 0.10 mL of triethylamine was then added, and the reaction was allowed to return to

room temperature for 2 h. The solvent was then evaporated and the residue was purified by

chromatography on a silica gel column (dichloromethane / ethyl acetate = 95/5) to gave

compound **9a** (56 mg, 94%) as a light yellow solid. ^{1}H NMR (400 MHz, CDCl$_3$) δ 11.54 (s, 1H),

8.19 (d, J = 2.9 Hz, 1H), 8.09 (d, J = 9.3 Hz, 1H), 7.98 (d, J = 2.9 Hz, 1H), 7.86 (d, J = 2.6 Hz,

1H), 7.44 (dd, J = 9.4, 2.7 Hz, 1H), 4.10-3.98 (m, 2H), 4.04 (s, 3H), 4.03 (s, 3H), 2.80 (d, J =

6.8 Hz, 2H), 2.58 (t, J = 12.2 Hz, 2H), 1.83 (m, 2H), 1.52 - 1.44 (m, 1H), 1.43 (s, 9H), 1.23-1.08

(m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 192.8, 158.9, 157.6, 154.8, 144.5, 142.9, 137.3, 133.5,

132.5, 130.7, 130.7, 125.0, 123.0, 109.0, 101.9, 79.7, 56.0, 55.8, 43.8 (br s), 43.2, 36.9, 31.8,

28.5. HRMS(ESI) [M + H]$^+$ calculated for C$_{27}$H$_{33}$N$_2$O$_5$S: 497.2105, found: 497.2101.

tert-butyl-4-(((4-(aminomethyl)-2,7-dimethoxyacridin-9-yl)thio)methyl)piperidine-1-carbo

xylate (9b). Under argon atmosphere, compound **9a** (55 mg, 0.11 mmol), ammonium acetate

(85 mg, 1.11 mmol), sodium cyanoborohydride (6.9 mg, 0.11 mmol), 4 mg of 4A molecular

sieves was dissolved in 3 mL of dry methanol at -5 ^{o}C, then the reaction was stilled at the

same temperature overnight. After the removal of solid by filtration, the solvent was diluted

with methylene chloride, washed with brine, and then evaporated under vacuum. The residue

was purified by chromatography on a silica gel column (dichloromethane /methanol = 95/5) to

gave compound **9b** (25 mg, 46%) as a light yellow solid. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.54 (d,

J = 2.6 Hz, 1H), 7.52 (d, J = 2.5 Hz, 1H), 7.30 (d, J = 9.3 Hz, 1H), 7.08 (d, J = 2.3 Hz, 1H), 6.90

(dd, J = 9.3, 2.6 Hz, 1H), 4.86 (s, 2H), 4.06 - 3.95 (m, 2H),4.04 (s, 3H), 3.96 (s, 3H), 2.62 (d, J

= 6.7 Hz, 2H), 2.52 (t, J = 12.0 Hz, 2H), 1.85-1.70 (m, 2H), 1.41 (s, 9H), 1.35-1.30 (m, 1H),

1.18-1.04 (m, 2H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 158.7, 157.2, 154.8, 142.5, 140.9, 137.2,

131.2 (overlapped), 130.0, 129.6, 126.0, 124.0, 103.2, 101.5, 79.7, 55.7 (overlapped), 50.4,

43.6 (br s), 43.1, 36.8, 31.8, 28.5. HRMS(ESI) [M + H]$^+$ calculated for C$_{27}$H$_{36}$N$_3$O$_4$S: 498.2421,

found: 498.2420.

(2,7-dimethoxy-9-((piperidin-4-ylmethyl)thio)acridin-4-yl)methanamine (9). Compound **9b**

(13.5 mg，0.027 mmol) was dissolved in 2 mL of 5% trifluoroacetic acid dichloromethane

solution and the mixture was allowed to react at room temperature for 2 h. The solvent was

then evaporated and the residue was dissolved with methanol, then purified by HPLC/MS on a

Waters Auto Purification LC/MS system (ACQUITY UPLC ® BEH C18 17 μm 2.1X50 mm

column) to afford **9** (6.3 mg, 45%) as a dark red foam. ^{1}H NMR (400 MHz, MeOD) δ 7.55 (dd, J

$= 18.2, 2.6$ Hz, 2H), 7.47 (d, $J = 9.3$ Hz, 1H), 7.17 (d, $J = 2.5$ Hz, 1H), 6.95 (dd, $J = 9.3, 2.7$ Hz,

1H), 4.14 - 4.07 (m, 3H), 4.02 (s, 3H), 2.86 - 2.72 (m, 4H), 2.07 - 1.98 (m, 3H), 1.57 - 1.31 (m,

4H). ^{13}C NMR (151 MHz, MeOD) δ 160.2, 158.7, 143.5, 141.6, 137.9, 132.0, 131.7, 130.9,

130.4, 127.6, 125.2, 104.1, 102.5, 56.3, 56.2, 50.7, 44.8, 42.9, 35.6, 29.4. HRMS(ESI) [M + H][+]

calculated for $C_{22}H_{28}N_3O_2S$: 398.1897, found: 398.1896.

Appendix 1-scheme 7. Synthesis route of C10.

(9-chloro-2,7-dimethoxyacridin-4-yl)methanol (10a). 0.28 mL of 1.0 M DIBAL-H solution in

toluene was added to a solution of **1c** (40 mg, 0.12 mmol) in 5 mL of dry dichloromethane at

-70 °C, then the reaction was stilled at the same temperature overnight. The reaction was

quenched by adding saturated potassium hydrogen tartrate solution, diluted with

dichloromethane, washed twice with brine, dried over anhydrous Na_2SO_4 and concentrated *in*

vacuo. The residue was purified by chromatography on a silica gel column (dichloromethane /

ethyl acetate = 90/10) to gave compound **10a** (180 mg, 55%) as a light yellow solid. ^{1}H NMR

(400 MHz, CDCl₃) δ 8.00 (d, $J = 9.3$ Hz, 1H), 7.44 - 7.34 (m, 3H), 7.27 (s, 1H), 5.28 (br s, 1H),

5.19 (s, 2H), 4.02 (s, 3H), 4.00 (s, 3H). ^{13}C NMR (101 MHz, CDCl₃) δ 158.6, 158.0, 143.4,

142.9, 140.1, 136.3, 131.5, 126.0, 125.6, 124.7, 122.5, 99.8, 99.2, 64.8, 55.8, 55.8. HRMS(ESI)

[M + H][+] calculated for $C_{16}H_{15}ClNO_3$: 304.0735, found: 304.0732.

9-chloro-4-(difluoromethyl)-2,7-dimethoxyacridine (10b). To a solution of compound **10a**

(30 mg，0.10 mmol) in 3 mL of dichloromethane, Dess-Martin oxidant (60 mg, 0.14 mmol) was

added and the reaction was allowed to stir at room temperature for 2 h. The solution was

diluted with dichloromethane, washed twice with brine, dried over anhydrous Na_2SO_4 and

concentrated *in vacuo*. Under argon atmosphere, the crude product was dissolved in 2 mL of

dry dichloromethane, 0.1 mL of diethylaminosulfur trifluoride was added, and the mixture was

reacted at room temperature overnight. The reaction was quenched by sodium bicarbonate

aqueous solution, then diluted with dichloromethane, washed twice with brine, dried over

anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by chromatography

on a silica gel column (petroleum ether / ethyl acetate = 5/1) to afford **10b** (7.4mg, 23%) as

white solid. ^{1}H NMR (400 MHz, CDCl₃) δ 8.08 (d, $J = 9.3$ Hz, 1H), 7.91 (t, $J = 55.4$ Hz, 1H),

7.76 - 7.73 (m, 1H), 7.59 (d, $J = 2.6$ Hz, 1H), 7.48 - 7.41 (m, 2H), 4.05 (s, 3H), 4.03 (s, 3H). [13]C

374 NMR (151 MHz, CDCl$_3$) δ 158.9, 157.6, 144.3, 141.2 (t, J = 19Hz), 135.9, 133.8 (t, J = 85 Hz),

375 132.2, 125.9, 125.6, 125.0, 121.8 (t, J = 27 Hz), 112.1 (t, J = 942 Hz), 102.4, 99.7, 56.0, 55.9.

376 HRMS(ESI) [M + H]$^+$ calculated for C$_{16}$H$_{13}$ClF$_2$NO$_2$: 324.0597, found: 324.0597.

377 **tert-butyl**

378 **4-(((4-(difluoromethyl)-2,7-dimethoxyacridin-9-yl)thio)methyl)piperidine-1-carboxylate**

379 **(10c).** To a solution of **10b** (7.4 mg，0.023 mmol) in 2 mL of anhydrous DMF, sodium

380 hydrosulfide hydrate powder (70%, 1.9 mg，0.034 mmol) was added under argon atmosphere,

381 and the reaction was stirred at 60 °C overnight. 1-Boc-4-bromomethylpiperidine (12.7 mg，

382 0.0461 mmoll) and potassium carbonate (15.8 mg，0.114 mmol) were added into the slurry and

383 the reaction was allowed to react at room temperature overnight. The solvent was then

384 evaporated and the residue was dissolved with dichloromethane and washed with water. The

385 combined organic extracts were dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The

386 residue was purified by flash chromatography on a silica gel column (Petroleum ether / ethyl

387 acetate = 3/1) to give compound **10c** as a yellow solid (5.3 mg, 45%) and used for next step.

388 **4-(difluoromethyl)-2,7-dimethoxy-9-((piperidin-4-ylmethyl)thio)acridine (10).** Compound

389 **10c** (5.3 mg，0.010 mmol) was dissolved in 2 mL of 5% trifluoroacetic acid dichloromethane

390 solution and the mixture was allowed to react at room temperature for 2 h. The solvent was

391 then evaporated and the residue was dissolved with methanol, then purified by HPLC/MS on a

392 Waters Auto Purification LC/MS system (ACQUITY UPLC ® BEH C18 17 μm 2.1X50 mm

393 column) to afford **10** (3.8 mg, 70%) as a yellow solid. ^{1}H NMR (400 MHz, MeOD) δ 8.08 (d, J =

394 6.3 Hz, 1H), 7.93 (d, J = 2.8 Hz, 1H), 7.89 (t, J = 59.6Hz, 1H) 7.89 (d, J = 4.1 Hz, 1H), 7.68 (dd,

395 J = 2.8, 1.4 Hz, 1H), 7.47 (dd, J = 9.4, 2.8 Hz, 1H), 4.09-4.05(overlapped, m, 2H), 4.06 (s, 3H),

396 4.04 (s, 3H), 2.96 (d, J = 6.7 Hz, 2H), 2.90 - 2.78 (m, 2H), 2.11 (d, J = 14.4 Hz, 2H), 1.68 - 58

397 (m, 1H)，1.52 - 1.43 (m, 2H). ^{13}C NMR (151 MHz, MeOD) δ 160.4, 158.9, 145.1, 142.0, 137.7,

398 135.5 (t, J = 85 Hz), 133.4, 131.7, 131.4, 125.6, 122.0 (t, J = 27 Hz), 113.3 (t, J = 937 Hz),

399 105.3, 102.7, 56.4, 56.2, 44.8, 43.0, 35.7, 29.5. HRMS(ESI) [M + H]$^+$ calculated for

400 C$_{22}$H$_{25}$F$_2$N$_2$O$_2$S: 419.1599, found: 419.1587.

401

402 **Appendix 1-scheme 8.** General procedure of C11-C14 synthesis.

403

404 General procedures of synthesizing **1c'/1d;** To a suspension of compound **1b** (1.65 g, 6.05

405 mmol) in 200 mL of dry dichloromethane was slowly added 30 mL of boron tribromide, and the

406 mixture was reacted at 0 ° C for 2 h. Methanol was added to the reaction system to quench the

407 reaction, and the solvent was removed under reduced pressure. The crude product was

408 separated and purified with a silica gel column (dichloromethane / methanol = 92/8) to obtain

409   **1c'** (0.22 g, 14%) and the reported compound **1d** (0.80 g, 56%), with 0.70 g of the starting

410   material recovered. **1c':** ^{1}H NMR (400 MHz, MeOD) δ 8.08 (dd, $J = 9.4$, 4.8 Hz, 2H), 7.64 (dd,

411    $J = 7.6$, 4.4 Hz, 4H), 4.07 (s, 3H). ^{13}C NMR (151 MHz, MeOD) δ 160.8, 159.2, 129.2, 127.8,

412   127.2, 104.6, 101.3, 56.6. HRMS(ESI) [M + H][+] calculated for $C_{14}H_{11}ClNO_2$: 260.0473, found:

413   260.0471.

414

415   General procedures of synthesizing **11a**,**12a**: To a solution of **1c'** (94.5 mg, 0.364 mmol, 100

416   mol%) in 10 mL of anhydrous DMF, potassium carbonate (75.4 mg, 0.546 mmol, 150 mol%)

417   silver oxide (126.5 mg, 0.546 mmol, 150 mol%) and suitable bromides (0.546 mmol, 150 mol%)

418   was added. The reaction was allowed to react at 40 ^{o}C until full conversion. The reaction

419   solution was spin-dried under reduced pressure, and water / dichloromethane was separated.

420   The solvent was then evaporated and the residue was dissolved with dichloromethane and

421   washed with water. The combined organic extracts were dried over anhydrous Na_2SO_4 and

422   concentrated *in vacuo*. The residue was purified by chromatography on a silica gel column

423   (Petroleum ether / ethyl acetate = 9/1) to give the desired compound.

424   **2-(benzyloxy)-9-chloro-7-methoxyacridine (11a). Yield 100%.** ^{1}H NMR (400 MHz, CDCl₃) δ

425   8.08 (dd, $J = 9.3$, 7.6 Hz, 2H), 7.60 (d, $J = 2.6$ Hz, 1H), 7.56 - 7.35 (m, 8H), 5.27 (s, 2H), 4.02 (s,

426   3H). ^{13}C NMR (101 MHz, CDCl₃) δ 158.4, 157.5, 144.4, 144.3, 136.2, 135.7, 131.6, 131.5,

427   128.3, 127.9, 128.0, 125.6, 125.6, 124.7, 124.6, 101.2, 99.9, 70.6, 55.8. HRMS(ESI) [M + H][+]

428   calculated for $C_{21}H_{17}ClNO_2$: 350.0942, found: 350.0941.

429   **9-chloro-2-isopropoxy-7-methoxyacridine (12a). Yield 78%.** ^{1}H NMR (400 MHz, CDCl₃) δ

430   8.08 (dd, $J = 9.4$, 2.2 Hz, 2H), 7.51 (dd, $J = 13.1$, 2.7 Hz, 2H), 7.41 (ddd, $J = 9.3$, 7.5, 2.7 Hz,

431   2H), 4.85 (p, $J = 6.0$ Hz, 1H), 4.03 (s, 3H), 1.48 (d, $J = 6.0$ Hz, 6H). ^{13}C NMR (151 MHz, CDCl₃)

432   δ 158.5, 156.7, 144.4, 144.3, 135.5, 131.7, 131.7, 125.8, 125.6, 125.5, 124.5, 101.8, 99.9,

433   70.5, 55.8, 22.0. HRMS(ESI) [M + H][+] calculated for $C_{17}H_{17}ClNO_2$: 302.0942, found: 302.0942.

434

435   General procedures of synthesizing **11-14**: To a solution of suitable acridine chloride (100

436   mol%) in 2 mL of anhydrous DMF, sodium hydrosulfide hydrate powder (150 mol%) was added

437   under argon atmosphere, and the reaction was stirred at 50 °C for 3 h. Suitable bromides (200

438   mol%) and potassium carbonate (500 mol%) were added into the slurry and the reaction was

439   allowed to react at room temperature overnight. The solvent was then evaporated and the

440   residue was dissolved with dichloromethane and washed with water. The combined organic

441   extracts were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was

442   dissolved in 2 mL of 5% trifluoroacetic acid dichloromethane solution and the mixture was

443   allowed to react at room temperature for 2h. The solvent was then evaporated and the residue

444   was dissolved with methanol, then purified by HPLC/MS on a Waters Auto Purification LC/MS

445   system (ACQUITY UPLC ® BEH C18 17 μm 2.1X50 mm column) to afford desired product.

446   **3-((2-(benzyloxy)-7-methoxyacridin-9-yl)thio)propan-1-amine (11). Yield 9.6%.** ^{1}H NMR

447   (400 MHz, MeOD) δ 8.13 (dd, J = 12.1, 9.4 Hz, 2H), 8.01 (dd, J = 8.5, 2.6 Hz, 2H), 7.81 (dd, J

448   = 9.4, 2.7 Hz, 1H), 7.72 (dd, J = 9.4, 2.7 Hz, 1H), 7.60-7.34 (m, 5H), 5.42 (s, 2H), 4.08 (s, 3H),

449   3.00 (t, J = 7.6Hz, 2H), 2.85(t, J = 7.6Hz, 2H), 1.70 (quint, J = 7.6Hz, 2H). ^{13}C NMR (151 MHz,

450   MeOD) δ 160.9, 159.6, 146.7, 138.5, 138.4, 137.6, 132.4, 132.0, 130.0, 129.9, 129.8, 129.4,

451   128.7, 126.4, 126.2, 105.7, 103.8, 71.8, 56.7, 39.4, 34.6, 29.4. HRMS(ESI) [M + H][+] calculated

452   for $C_{24}H_{25}N_2O_2S$: 405.1631, found: 405.1630.

453   **2-(benzyloxy)-7-methoxy-9-(piperidin-4-ylthio)acridine (12). 24%.** ^{1}H NMR (400 MHz,

454   MeOD) δ 7.99 (dd, J = 14.3, 9.4 Hz, 2H), 7.89 (d, J = 2.4 Hz, 2H), 7.58-7.50 (m, 3H), 7.47-7.40

455   (m, 3H), 7.39-7.33 (m, 1H), 5.36 (s, 2H), 4.01 (s, 3H), 3.26 (d, J = 13.1 Hz, 2H), 3.19 - 3.10 (m,

456   1H), 2.81 (t, J = 10.8 Hz, 2H), 1.86 (d, J = 11.5 Hz, 2H), 1.70 (td, J = 14.4, 3.7 Hz, 2H). [13]C

457   NMR (101 MHz, MeOD) δ 159.9, 158.7, 144.7, 144.6, 138.3, 135.9, 132.6, 132.2, 131.8, 131.7,

458   129.8, 129.1, 128.3, 125.8, 125.6, 105.0, 103.3, 71.2, 56.2, 45.5, 45.1, 32.0. HRMS(ESI) [M +

459   H][+] calculated for $C_{26}H_{27}N_2O_2S$: 431.1788, found: 431.1781.

460   **2-(benzyloxy)-7-methoxy-9-((piperidin-4-ylmethyl)thio)acridine (13). 19%.** ^{1}H NMR (400

461   MHz, MeOD) δ 8.01 - 7.89 (m, 2H), 7.86-7.75 (m, 2H), 7.58 - 7.31 (m, 7H), 5.32 (s, 2H), 3.99 (s,

462   3H), 3.27 (d, J = 13.1Hz, 2H), 2.75 (t, J = 12.5 Hz, 2H), 2.68 (d, J = 6.4 Hz, 2H), 1.94 (d, J =

463   13.2 Hz, 2H), 1.44 - 1.28 (m, 3H). ^{13}C NMR (151 MHz, MeOD) δ 160.0, 158.7, 144.7, 144.7,

464   138.6, 138.3, 132.0, 131.9, 131.7, 131.3, 129.8, 129.2, 125.8, 125.6, 104.9, 103.0, 71.4, 56.2,

465   45.1, 43.0, 36.0, 30.0. HRMS(ESI) [M + H][+] calculated for $C_{27}H_{29}N_2O_2S$: 445.1944, found:

466   445.1945.

467   **2-isopropoxy-7-methoxy-9-((piperidin-4-ylmethyl)thio)acridine (14). 26%.** ^{1}H NMR (400

468   MHz, MeOD) δ 8.15 (dd, J = 9.4, 3.6 Hz, 2H), 8.06 (t, J = 3.0 Hz, 2H), 7.71 (td, J = 9.3, 2.6 Hz,

469   2H), 4.98 - 4.92 (m, 1H), 4.09 (s, 3H), 3.36 (d, J = 12.9 Hz, 2H), 3.11 (d, J = 6.8 Hz, 2H), 2.88 (t,

470   J = 11.9 Hz, 2H), 2.12 (d, J = 14.0 Hz, 2H), 1.79 - 1.69 (m, 1H), 1.54-1.41 (overlapped, m, 2H),

471   1.51 (s, 3H), 1.49 (s, 3H). ^{13}C NMR (151 MHz, MeOD) δ 160.8, 158.9, 139.5, 139.3, 132.1,

472   129.9, 129.0, 127.4, 127.3, 105.6, 103.7, 72.2, 56.6, 44.8, 43.8, 36.0, 29.4, 22.0. HRMS(ESI)

473   [M + H][+] calculated for $C_{23}H_{29}N_2O_2S$: 397.1944, found: 397.1943.

474

475   **Appendix 1-scheme 9.** Synthesis route of C15.

476

2,7-bis(benzyloxy)-9-chloroacridine (15a). To a solution of 1d (94.5 mg, 0.364 mmol) in 10 mL of anhydrous DMF, potassium carbonate (150.8 mg, 1.092 mmol) silver oxide (253.0 mg, 1.092 mmol) and benzyl bromide (100 μl) was added. The reaction was allowed to react at 40 $^{\circ}$C until full conversion. The reaction solution was spin-dried under reduced pressure, and water / dichloromethane was separated. The solvent was then evaporated and the residue was dissolved with dichloromethane and washed with water. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by chromatography on a silica gel column (Petroleum ether / ethyl acetate = 9/1) to give 15a (163.9 mg, quant.) as a yellow solid. ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.10 (d, J = 9.4 Hz, 2H), 7.62 (d, J = 2.7 Hz, 2H), 7.57 - 7.36 (m, 12H), 5.28 (s, 4H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 157.6, 144.5, 136.3, 135.9, 131.8, 128.9, 128.5, 128.0, 125.6, 124.8, 101.2, 70.6. HRMS(ESI) [M + H]$^{+}$ calculated for $C_{27}H_{21}ClNO_2$: 426.1255, found: 426.1255.

2,7-bis(benzyloxy)-9-((piperidin-4-ylmethyl)thio)acridine (15). To a solution of **15a** (20 mg, 0.047 mmol) in 2 mL of anhydrous DMF, sodium hydrosulfide hydrate powder (70%, 5.6 mg, 0.070 mmol) was added under argon atmosphere, and the reaction was stirred at 50 °C for 3 h. *tert*-butyl 4-(bromomethyl)piperidine-1-carboxylate (26.1 mg, 0.094 mmol) and potassium carbonate (32.4 mg, 0.235 mol) were added into the slurry and the reaction was allowed to react at room temperature overnight. The solvent was then evaporated and the residue was dissolved with dichloromethane and washed with water. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was dissolved in 2 mL of 5% trifluoroacetic acid dichloromethane solution and the mixture was allowed to react at room temperature for 2h. The solvent was then evaporated and the residue was dissolved with methanol, then purified by HPLC/MS on a Waters Auto Purification LC/MS system (ACQUITY UPLC ® BEH C18 17 μm 2.1X50 mm column) to afford **15** (10.8 mg, 36%). ^{1}H NMR (400 MHz, MeOD) δ 8.09 (d, J = 9.4 Hz, 2H), 7.85 (d, J = 2.5 Hz, 2H), 7.73 (dd, J = 9.4, 2.6 Hz, 2H), 7.43 (ddd, J = 28.6, 28.0, 7.3 Hz, 10H), 5.37 (s, 4H), 3.22 (d, J = 12.8 Hz, 2H), 2.73 - 2.62 (m, 4H), 1.75 (d, J = 13.6 Hz, 2H), 1.41 - 1.31 (m, 1H), 1.30 - 1.16 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 159.4, 137.6, 137.6, 131.5, 130.3, 129.9, 129.4, 128.5, 125.8, 105.5, 71.8, 44.6, 43.6, 35.8, 29.1. HRMS(ESI) [M + H]$^{+}$ calculated for $C_{33}H_{33}N_2O_2S$: 521.2257, found: 521.2258.

Appendix 1-scheme 10. Synthesis route of C20.

*t*ert-butyl-(S)-3-(((4-(bromomethyl)-2,7-dimethoxyacridin-9-yl)thio)methyl)pyrrolidine-1-carboxylate (20a). 17b** (13 mg, 0.027mmol), Triphenylphosphine (10.6 mg, 0.040 mmol),

511   N-bromosuccinimide (7.2 mg, 0.040 mmol) was dissolved in 1 mL of anhydrous

512   tetrahydrofuran and the reaction was stirred at room temperature for 30 min. The reaction was

513   concentrated *in vacuo* and the residue was purified by chromatography on a silica gel column

514   (pure dichloromethane) to give compound **20a** (9.6 mg, 65%) as a yellow solid. ^{1}H NMR (400

515   MHz, CDCl$_3$) δ 8.16 (d, $J = 9.3$ Hz, 1H), 7.94 (s, 1H), 7.91 (s, 1H), 7.59 (s, 1H), 7.43 (d, $J = 9.2$

516   Hz, 1H), 5.31 (d, $J = 8.9$ Hz, 2H), 4.05 (s, 3H), 4.03 (s, 3H), 3.64 - 3.33 (m, 2H), 3.30 - 2.84 (m,

517   4H), 2.12 (br s, 1H), 2.01 - 1.92 (m, 1H), 1.67-1.60 (m, 1H), 1.43 (s, 9H). ^{13}C NMR (101 MHz,

518   CDCl$_3$) δ 158.8, 157.8, 154.6, 143.8, 141.7, 138.8, 135.8, 132.9, 130.8, 130.7, 124.7, 124.1,

519   102.9, 101.8, 79.4, 55.8, 51.0 (d, $J = 108$Hz), 50.9, 45.2 (d, $J = 112$Hz), 39.5, 31.4, 30.7, 29.7,

520   28.6. HRMS(ESI) [M + H]$^+$ calculated for C$_{26}$H$_{32}$BrN$_2$O$_4$S: 547.1261, found: 547.1255.

521   **(S)-2-((2,7-dimethoxy-9-((pyrrolidin-3-ylmethyl)thio)acridin-4-yl)methyl)guanidine (20).**

522   Under argon atmosphere, N, N'-di-Boc-guanidine (3.1 mg, 12.1 μmol) and potassium

523   carbonate (1.7 mg, 12.1 μmol) was added into the solution of **20a** (5.5 mg, 10.0 μmol) in 1 mL

524   of anhydrous DMF. The reaction was performed at 50 ^{o}C for 2.5 h. The reaction was

525   concentrated *in vacuo* and the residue was dissolved in 4 mL of dichloromethane containing 5%

526   trifluoroacetic acid and reacted at room temperature for 2 h. The solvent was then evaporated

527   and the residue was dissolved with methanol, then purified by HPLC/MS on a Waters Auto

528   Purification LC/MS system (ACQUITY UPLC ® BEH C18 17 μm 2.1X50 mm column) to give

529   compound **20** (2.0 mg, 27%). ^{1}H NMR (400 MHz, MeOD) δ 8.11 (d, $J = 9.4$ Hz, 1H), 7.96 (d, J

530     $= 2.7$ Hz, 1H), 7.94 (d, $J = 2.7$ Hz, 1H), 7.52 - 7.48 (m, 2H), 5.04 (s, 2H), 4.07 (s, 3H), 4.06 (s,

531   3H), 3.39 - 3.32 (m, 2H), 3.19 - 2.98 (m, 4H), 2.28 (hept, $J = 7.6$ Hz, 1H), 2.20 - 2.09 (m, 1H),

532   1.83 - 1.73 (m, 1H). ^{13}C NMR (151 MHz, MeOD) δ 160.5, 159.6, 159.0, 144.3, 142.9, 138.5,

533   137.4, 132.8, 132.1, 131.8, 125.7, 125.1, 103.2, 102.8, 56.3, 50.8, 46.2, 43.1, 40.0, 39.4, 31.0.

534   HRMS(ESI) [M + H]$^+$ calculated for C$_{22}$H$_{28}$N$_5$O$_2$S: 426.1958, found: 426.1946.

535

536   **Appendix 1-scheme 11.** Synthesis route of C21.

537   **ethyl 5-((4-methoxyphenyl)amino)-2-(methylthio)thiazole-4-carboxylate (21a).** Under

538   argon atmosphere, a solution of a known compound 23 (1.04 g, 5.02 mmol) in 4 mL of

539   anhydrous tetrahydrofuran was added dropwise to a solution of potassium tert-butoxide (0.79

540   g, 7.02 mmol) in THF at -78 ^{o}C, followed by a solution of compound 24 (0.83 g, 5.02 mmol) in 4

541   mL of anhydrous tetrahydrofuran. The reaction was performed at -78 ^{o}C for 0.5 h and then

542   slowly warmed up to room temperature and reacted overnight. The reaction was quenched by

543   adding saturated ammonium chloride, diluted with dichloromethane, washed twice with brine,

544   dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The residue was purified by

545   chromatography on a silica gel column (pure dichloromethane) to give compound **21a** (0.29 g,

546   18%) as a light yellow solid. ^{1}H NMR (400 MHz, CDCl$_3$) δ 9.39 (s, 1H), 7.17 - 7.11 (m, 2H),

547   6.91 - 6.83 (m, 2H), 4.38 (q, $J = 7.1$ Hz, 2H), 3.77 (s, 3H), 2.57 (s, 3H), 1.39(t, $J = 7.1$ Hz, 3H).

548   ^{13}C NMR (101 MHz, CDCl$_3$) δ 164.7, 158.5, 156.8, 145.4, 134.2, 121.9, 121.5, 114.9, 60.8,

549   55.5, 17.7, 14.6. HRMS(ESI) [M + H]$^+$ calculated for C$_{14}$H$_{17}$N$_2$O$_3$S$_2$: 325.0675, found:

550   325.0673.

551   **9-chloro-7-methoxy-2-(methylthio)thiazolo[5,4-b]quinolone (21b).** Compound **21a** (198

552   mg, 0.61 mmol) was added in a sealed tube, and 5 mL of phosphorus oxychloride was added

553   under argon atmosphere. The reaction was heated at 130 °C overnight. The resulting slurry

554   was poured onto ice with vigorous stirring to make full quenching. The mixture was diluted with

555   dichloromethane, washed twice with brine, dried over anhydrous Na$_2$SO$_4$ and concentrated *in*

556   *vacuo*. The residue was purified by chromatography on a silica gel column (pure

557   dichloromethane) to give compound **21b** (74 mg, 41%) as a light yellow solid. ^{1}H NMR (400

558   MHz, CDCl$_3$) δ 7.97 (d, $J = 9.2$ Hz, 1H), 7.55 (d, $J = 2.8$ Hz, 1H), 7.40 (dd, $J = 9.2$, 2.8 Hz, 1H),

559   4.01 (s, 3H), 2.88 (s, 3H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 172.1, 158.5, 157.6, 142.6, 142.4,

560   130.2, 129.5, 126.2, 123.1, 101.8, 55.8, 15.5. HRMS(ESI) [M + H]$^+$ calculated for

561   C$_{12}$H$_{10}$ClN$_2$OS$_2$: 296.9918, found: 296.9917.

562   **tert-butyl-4-(((7-methoxy-2-(methylthio)thiazolo[5,4-b]quinolin-9-yl)thio)methyl)piperidin**

563   **e-1-carboxylate (21c).** To a solution of **21b** (0.020 g, 0.067 mmol) in 5 mL of anhydrous DMF,

564   sodium hydrosulfide hydrate powder (70%, 10.8 mg, 0.135 mmol) was added under argon

565   atmosphere and the reaction was stirred at 50 °C for 2 h until full conversion.

566   1-Boc-4-bromomethylpiperidine (37.5 mg, 0.135 mmol) and potassium carbonate (27.9 mg,

567   0.202 mmol) were added into the slurry and the reaction was allowed to react at room

568   temperature overnight. The solvent was then evaporated and the residue was dissolved with

569   dichloromethane and washed with water. The combined organic extracts were dried over

570   anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The residue was purified by chromatography

571   on a silica gel column (dichloromethane / ethyl acetate = 95/5) to give compound **21c** (14.9 mg,

572   45%) as a light yellow solid. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.94 (d, $J = 9.2$ Hz, 1H), 7.85 (d, $J =$

573   2.8 Hz, 1H), 7.38 (dd, $J = 9.2$, 2.8 Hz, 1H), 4.12 - 4.01 (m, 2H), 4.00 (s, 3H), 3.47 (d, $J = 6.9$ Hz,

574   2H), 2.84 (s, 3H), 2.61 (t, $J = 12.7$ Hz, 2H), 1.86 (d, $J = 13.1$ Hz, 2H), 1.65 - 1.57 (m, 1H), 1.44

575   (s, 9H), 1.23 - 1.14 (m, 2H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 169.9, 158.1, 158.0, 154.9, 145.1,

576   141.7, 132.9, 130.3, 128.7, 122.4, 103.5, 79.6, 55.8, 43.9 (br s), 41.1, 36.9, 31.7, 28.6, 15.5.

577   HRMS(ESI) [M + H]$^+$ calculated for C$_{23}$H$_{30}$N$_3$O$_3$S$_3$: 492.1444, found: 492.1435.

578   **7-methoxy-2-(methylthio)-9-((piperidin-4-ylmethyl)thio)thiazolo[5,4-b]quinolone (21).**

579   **21c** (14.9 mg, 0.030 mmol) was dissolved in 2 mL of 5% trifluoroacetic acid dichloromethane

solution and the mixture was allowed to react at room temperature for 2 h. The solvent was then evaporated and the residue was dissolved with methanol, then purified by HPLC/MS on a Waters Auto Purification LC/MS system (ACQUITY UPLC ® BEH C18 17 μm 2.1X50 mm column) to afford **21** (14.7 mg, 96%). ^{1}H NMR (400 MHz, MeOD) δ 7.85 (d, $J = 9.2$ Hz, 1H), 7.81 (d, $J = 2.7$ Hz, 1H), 7.40 (dd, $J = 9.2, 2.8$ Hz, 1H), 3.98 (s, 3H), 3.62 (d, $J = 6.9$ Hz, 2H), 3.37 - 3.32 (m, 2H), 2.88 (s, 3H), 2.91 - 2.83 (overlapped, m, 2H), 2.12 (d, $J = 13.8$ Hz, 2H), 1.82-1.70 (m, 1H), 1.55-1.42 (m, 2H). ^{13}C NMR (151 MHz, MeOD) δ 172.0, 159.6, 158.8, 146.1, 142.4, 133.9, 130.7, 129.6, 123.5, 104.3, 97.5, 56.2, 44.9, 40.7, 35.7, 29.3, 15.7. HRMS(ESI) [M + H]$^+$ calculated for $C_{18}H_{22}N_3OS_3$: 392.0920, found: 392.0920.

Appendix 1-scheme 12. Synthesis route of C22.

Ethyl-5-((4-methoxy-2-(methoxycarbonyl)phenyl)amino)-2-(methylthio)thiazole-4-carbox ylate (22a). Under argon atmosphere, a solution of a known compound 23 (150 mg, 0.73 mmol) in 1 mL of anhydrous tetrahydrofuran was added dropwise to a solution of potassium tert-butoxide (112 mg, 0.92 mmol) in 8 mL of anhydrous tetrahydrofuran at -60 ^{o}C, followed by a solution of known compound 24' (150 mg, 0.66 mmol) in 1 mL of anhydrous tetrahydrofuran. The reaction was performed at -60 ^{o}C for 1.5 h and then slowly warmed up to room temperature and reacted overnight. The reaction was quenched by adding saturated ammonium chloride, diluted with dichloromethane, washed twice with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by chromatography on a silica gel column (pure dichloromethane) to give compound **22a** (69.2 mg, 28%) as a yellow solid. ^{1}H NMR (400 MHz, CDCl$_3$) δ 11.61 (s, 1H), 7.58 - 7.50 (m, 2H), 7.11 (dd, $J = 9.0, 3.2$ Hz, 1H), 4.49 (q, $J = 7.1$ Hz, 2H), 3.97 (s, 3H), 3.83 (s, 3H), 2.64 (s, 3H), 1.42 (t, $J = 7.1$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 167.1, 163.9, 154.3, 153.6, 147.0, 137.0, 125.6, 121.0, 117.9, 117.4, 115.8, 61.1, 55.9, 52.7, 17.7, 14.7. HRMS(ESI) [M + H]$^+$ calculated for $C_{16}H_{19}N_2O_5S_2$: 383.0730, found: 383.0729.

Methyl-9-chloro-7-methoxy-2-(methylthio)thiazolo[5,4-b]quinoline-5-carboxylate (22b). Compound **22a** (50 mg, 0.14 mmol) was added in a sealed tube, and 1 mL of phosphorus oxychloride was added under argon atmosphere. The reaction was heated at 130 °C overnight. The resulting slurry was poured onto ice with vigorous stirring to make full quenching. The mixture was diluted with dichloromethane, washed twice with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by chromatography on a silica gel column (pure dichloromethane) to give compound **22b** (15 mg, 32%) as a light yellow solid.

614 ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.69 (s, 2H), 4.05 (s, 3H), 4.02 (s, 3H), 2.88 (s, 3H). ^{13}C NMR

615 (101 MHz, CDCl$_3$) δ 173.4, 167.5, 157.2, 142.6, 139.3, 132.7, 129.4, 126.6, 123.5, 105.1, 56.1,

616 53.0, 15.5. HRMS(ESI) [M + H]$^+$ calculated for C$_{14}$H$_{12}$ClN$_2$O$_3$S$_2$: 354.9972, found: 354.9973.

617 **Methyl-9-(((1-(tert-butoxycarbonyl)piperidin-4-yl)methyl)thio)-7-methoxy-2-(methylthio)t**

618 **hiazolo[5,4-b]quinoline-5-carboxylate (22c).** To a solution of **22b** (15 mg, 0.042 mmol) in 2

619 mL of anhydrous DMF, sodium hydrosulfide hydrate powder (70%, 6.7 mg, 0.084 mmol) was

620 added under argon atmosphere, and the reaction was stirred at 50 °C for 2 h until full

621 conversion of **1c**. 1-Boc-4-bromomethylpiperidine (29.3 mg, 0.105 mmol) and potassium

622 carbonate (17.4 mg, 0.126 mmol) were added into the slurry and the reaction was allowed to

623 react at room temperature overnight. The solvent was then evaporated and the residue was

624 dissolved with dichloromethane and washed with water. The combined organic extracts were

625 dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The residue was purified by

626 chromatography on a silica gel column (dichloromethane / ethyl acetate = 95/5) to give

627 compound **22c** (16.2 mg, 70%) as a light yellow solid. ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.02 (d, *J* =

628 2.9 Hz, 1H), 7.65 (d, *J* = 2.8 Hz, 1H), 4.12-3.96 (overlapped, m, 2H), 4.04 (s, 3H), 4.00 (s, 3H),

629 3.44 (d, *J* = 6.9 Hz, 2H), 2.84 (s, 3H), 2.58 (t, *J* = 12.6 Hz, 2H), 1.82 (d, *J* = 12.9 Hz, 2H), 1.61 -

630 1.52 (m, 1H), 1.44 (s, 9H), 1.23 - 1.13 (m, 2H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 171.2, 167.8,

631 158.8, 156.6, 154.8, 145.2, 138.5, 132.7, 132.6, 129.1, 122.5, 106.7, 79.4, 55.9, 52.8,

632 44.3-43.2 (m), 41.1, 36.7, 31.5, 28.4, 15.4. HRMS(ESI) [M + H]$^+$ calculated for C$_{25}$H$_{32}$N$_3$O$_5$S$_3$:

633 550.1499, found: 550.1492.

634 **(7-methoxy-2-(methylthio)-9-((piperidin-4-ylmethyl)thio)thiazolo[5,4-b]quinolin-5-yl)met**

635 **haol (22).** 87 μl of 1.0 M DIBAL-H solution in toluene was added to a solution of **22c** (16.0 mg,

636 0.029 mmol) in 1 mL of dry dichloromethane at -60 °C, and the reaction was slowly warmed to

637 room temperature and reacted overnight. The reaction was quenched by adding saturated

638 potassium hydrogen tartrate solution, diluted with dichloromethane, washed twice with brine,

639 dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The residue was dissolved in 4 mL

640 of 5% trifluoroacetic acid dichloromethane solution and the mixture was allowed to react at

641 room temperature for 2 h. The solvent was then evaporated and the residue was dissolved

642 with methanol, then purified by HPLC/MS on a Waters Auto Purification LC/MS system

643 (ACQUITY UPLC ® BEH C18 17 μm 2.1X50 mm column) to afford **22** (5.3 mg, 34%). ^{1}H NMR

644 (400 MHz, MeOD) δ 7.78 (d, *J* = 2.8 Hz, 1H), 7.52 (d, *J* = 2.7 Hz, 1H), 5.20 (s, 2H), 3.98 (s, 3H),

645 3.58 (d, *J* = 6.9 Hz, 2H), 3.37-3.32 (m, 2H), 2.88 (s, 3H), 2.88 - 2.81 (m, 2H), 2.15 - 2.08 (m,

646 2H), 1.78 - 1.69 (m, 1H), 1.55 - 1.41 (m, 2H). ^{13}C NMR (151 MHz, MeOD) δ 172.2, 159.5,

647 157.9, 146.2, 142.2, 140.4, 133.5, 129.8, 120.6, 102.9, 61.7, 56.0, 44.9, 40.8, 35.7, 29.3, 15.6.

648   HRMS(ESI) $[M + H]^+$ calculated for $C_{19}H_{24}N_3O_2S_3$: 422.1025, found: 422.1013.

649

650

651

652

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
cell line (*Homo-sapiens*)	HEK293T	American Type Culture Collection	Cat#: CRL-3216, RRID: CVCL_0063	
cell line (*Homo-sapiens*)	HEK293A	Thermo Fisher	Cat#: R70507	
cell line (*Homo-sapiens*)	HEK293	American Type Culture Collection	Cat#: CRL-1573，RRID: CVCL_0045	
cell line (*Homo-sapiens*)	U266	American Type Culture Collection	Cat#: TIB-196，RRID: CVCL_0566	
cell line (*Homo-sapiens*)	HCT116	China Infrastructure of Cell Line Resources	Cat#: 1101HUM-PUMC000158	
antibody	anti-4EPB1(Rabbit polyclonal)	Cell Signaling Technology	Cat#: 9644, RRID: AB_2097841	WB (1:1000)
antibody	anti-phosphorylated 4E-BP1 (Thr37/46) (Rabbit monoclonal)	Cell Signaling Technology	Cat#: 2855, RRID: AB_560835	WB (1:1000)

antibody	anti-phosphorylated 4E-BP1 (Ser65) (Rabbit monoclonal)	Cell Signaling Technology	Cat#: 9451, RRID: AB_330947	WB (1:1000)
antibody	anti-phosphorylated 4E-BP1 (Thr70) (Rabbit monoclonal)	Cell Signaling Technology	Cat#: 13396, RRID: AB_2798206	WB (1:1000)
antibody	anti-HA (Rabbit monoclonal)	Cell Signaling Technology	Cat#: 3724, RRID: AB_1549585	WB (1:1000)
antibody	anti-FLAG (Mouse monoclonal)	Sigma-Aldrich	Cat#: F3165, RRID: AB_259529	WB (1:5000)
antibody	anti-FLAG (Rabbit monoclonal)	Abcam	Cat#: ab205606	WB (1:5000)
antibody	anti-GFP (Rabbit monoclonal)	Abcam	Cat#: ab183734	WB (1:5000)
antibody	anti-GFP (Mouse monoclonal)	Proteintech	Cat#: 66002-1-Ig, RRID: AB_11182611	WB (1:5000)
antibody	anti-RPT3 (Rabbit polyclonal)	Thermo Fisher Scientific	Cat#: A303-849A-M, RRID: AB_2781512	WB (1:1000)
antibody	anti-pThr25 (Rabbit polyclonal)	(Guo et al., 2016)	N/A	WB (1:500)
antibody	anti-GAPDH (Mouse monoclonal)	Transgene Biotechnology	Cat#: HC301-01	WB (1:5000)

antibody	anti-mouse-IgG-HRP (Goat monoclonal)	Transgene Biotechnology	Cat#: HS201-01	WB (1:5000)
antibody	anti-rabbit-IgG-HRP (Goat monoclonal)	Transgene Biotechnology	Cat#: HS101-01	WB (1:5000)
recombinant DNA reagent	GCaMP6f (plasmid)	Xiaowei Chen Lab (Peking University, China)	N/A	
recombinant DNA reagent	pEGFP-Orai1 (plasmid)	Xiaowei Chen Lab (Peking University, China)	N/A	
recombinant DNA reagent	mCherry-STIM1 (plasmid)	Xiaowei Chen Lab (Peking University, China)	N/A	
recombinant DNA reagent	pLL3.7-DYRK2-shRNA (plasmid)	Xing Guo Lab (Zhejiang University, China)	(Guo et al., 2016)	
recombinant DNA reagent	Flag-STIM1 (plasmid)	This paper	N/A	This plasmid was generated by modification of mCherry-STIM1 plasmid.
recombinant DNA reagent	pQlinkHx-DYRK2$^{208-552}$ (plasmid)	This paper	N/A	This plasmid was generated by modification of pEGFP-DYR

				K2 plasmid.
recombinant DNA reagent	pQlinkGx-STIM1$^{235\text{-}END}$ (plasmid)	This paper	N/A	This plasmid was generated by modification of mCherry-STIM1 plasmid.
recombinant DNA reagent	HA-mcherry-DYRK2 (plasmid)	This paper	N/A	This plasmid was generated by modification of pEGFP-DYRK2 plasmid.
recombinant DNA reagent	HA-mcherry-DYRK2-D275N (plasmid)	This paper	N/A	This plasmid was generated by modification of pEGFP-DYRK2-D275N plasmid.
peptide, recombinant protein	Flag peptide: DYKDDDDK	Smart Lifesciences	Cat#: SLR01002	
peptide, recombinant protein	GST-MARK3 protein	Carna Biosciences	Cat#: 02-122	
peptide, recombinant protein	GST-Haspin protein	Carna Biosciences	Cat#: 05-111	
strain, strain background (*Escherichia coli*)	BL21(DE3)	Sigma-Aldrich	Cat#: CMC0016	Electrocompetent cells

chemical compound, drug	AKTi-1/2	Selleck	Cat#: S80837	
chemical compound, drug	PD0325901	Aladdin	Cat#: P125494	
chemical compound, drug	Thapsigargin	Aladdin	Cat#: T135258	
chemical compound, drug	X-tremeGENE 9 DNA Transfection Reagent	Roche	Cat#: 19129300	
chemical compound, drug	Lipofectamine 2000	Thermo Fisher Scientific	Cat#: 11668019	
chemical compound, drug	protease inhibitor mixture	Roche	Cat#: 11697498001	
chemical compound, drug	phosphatase inhibitor mixtures	Roche	Cat#: 04906837001	
chemical compound, drug	Ionomycin	Sigma-Aldrich	CAS: 56092-81-0	
chemical compound, drug	2-Bromo-5-methoxybenzoic acid	J&K Scientific	CAS: 22921-68-2	
chemical compound, drug	2-Amino-5-Methoxybenzoic acid	Energy Chemicals	CAS: 6705-03-9	
chemical compound, drug	p-Anisidine	J&K Scientific	CAS: 104-94-9	

chemical compound, drug	1-Boc-4-Bromomethylpiperidine	Bide Pharmatech	CAS: 158407-04-6	
chemical compound, drug	(S)-1-Boc-3-(Bromomethyl)pyrrolidine	Bide Pharmatech	CAS: 1067230-64-1	
chemical compound, drug	(R)-1-Boc-3-(Bromomethyl)pyrrolidine	Bide Pharmatech Ltd	CAS: 1067230-65-2	
chemical compound, drug	4-Methoxyphenyl isothiocyanate	Energy Chemicals	CAS: 2284-20-0	
chemical compound, drug	tert-butyl 4-bromopiperidine-1-carboxylate	J&K Scientific	CAS: 180695-79-8	
chemical compound, drug	DIBAL-H	Alfa Aesar Chemicals	CAS: 1191-15-7	
chemical compound, drug	Dess-Martin	Alfa Aesar Chemicals	CAS: 87413-09-0	
chemical compound, drug	Boron Tribromide	Sigma-Aldrich	CAS: 10294-33-4	
chemical compound, drug	Urea	Sigma-Aldrich	CAS: 57-13-6	
chemical compound, drug	2-Amino-2-(hydroxymethyl)-1,3-propanediol	Sigma-Aldrich	CAS: 77-86-1	
chemical compound, drug	Sodium orthovanadate	NEW ENGLAND BioLabs	Cat#: P0758S	

commercial assay or kit	Ni Sepharose 6 Fast Flow	GE healthcare	Cat#: 17531803	
commercial assay or kit	Glutathione Sepharose 4B beads	GE healthcare	Cat#: 17-0756-05	
commercial assay or kit	ANTI-FLAG® M2 Affinity Gel	Sigma-Aldrich	Cat#: A2220	
commercial assay or kit	Superdex 200 Increase 10/300 GL	GE healthcare	Cat#: 28990944	
commercial assay or kit	BCA Protein Assay Kit Pierce	Thermo-Pierce	Cat#: 23227	
commercial assay or kit	ADP-Glo™ kinase assay	Promega	Cat#: V9102	
software, algorithm	Chembiodraw	http://www.perkinelmer.co.uk/category/chemdraw	RRID:SCR_016768	v13
software, algorithm	GraphPad Prism	GraphPad Software	RRID:SCR_002798	v8.4.0
software, algorithm	ImageJ (Fiji)	(Schindelin et al., 2012)	RRID:SCR_003070	
software, algorithm	Matlab	https://ww2.mathworks.cn/products/matlab.html	N/A	v2014a
software, algorithm	HKL-2000	HKL Research	RRID:SCR_015547	

software, algorithm	Phenix	https://www.phenix-online.org/	RRID:SCR_014224	v1.19.2
software, algorithm	Coot	http://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/	RRID:SCR_014222	v0.9
software, algorithm	Maxquant	http://www.biochem.mpg.de/5111795/maxquant	RRID: SCR_014485	v1.5.5.1
software, algorithm	Perseus	http://coxdocs.org/doku.php?id=perseus:start	RRID: SCR_015753	v1.5.5.3
software, algorithm	Thermo Xcalibur	https://www.thermofisher.cn/order/catalog/product/OPTON-30965	RRID: SCR_014593	v4.1.50

654

655

656

A Compound 17

B

Kinase	IC$_{50}$ (nM)
DYRK2	9
DYRK1A	2145
DYRK1B	2272
DYRK3	68
Haspin	26
MARK3	87

A Control | Compound 17 10 μM, 1 h → Treatment +/- → Trypsin digestion → 500 μg peptides, Ti⁴⁺-IMAC, Phosphopeptide enrichment → Single-shot 1 h gradient → LC-MS/MS and label-free quantification analysis

B
- Y: 139, 1%
- T: 2798, 18%
- S: 12818, 81%
- Class III: 2401, 16%
- Class II: 2557, 16%
- Class I: 10647, 68%

C Total phosphosites: 7583
- Downregulated: 373
- Upregulated: 445
- STIM1_S521
- STIM1_S519
- EIF4EBP1_T37

x-axis: Log_2 Intensity Ratio (Treatment / Control)
y-axis: $-\text{Log}_{10}$ p-value

D
EIF4EBP1 T37
VVLGDGVQLPPGDYST**T**PGGTLFSTTPGGTR

STIM1 S519
DLTH**S**DSESSLHMSDR

STIM1 S521
DLTHSDS**E**SSLHMSDR

E
Upregulated:
- RNA transport
- Salmonella infection
- Yersinia infection
- Pathogenic Escherichia coli infection
- Epstein−Barr virus infection
- Mitophagy−animal
- Protein processing in endoplasmic reticulum
- Toll−like receptor signalling pathway
- Hepatitis B
- Spliceosome

Downregulated:
- AMPK signalling pathway
- Cell cycle
- Insulin signalling pathway
- RNA transport
- mTOR signalling pathway
- Chronic myeloid leukemia
- ErbB signalling pathway
- Longevity regulating pathway
- Proteoglycans in cancer
- Bladder cancer

x-axis: $-\text{Log}_{10}$ adjusted p-value

shRNA sequence

Name	Sequence
DYRK2 shRNA	gaacaagcaatgaagcaat

qRT-PCR primers

Name	Sequence
DYRK2-qPCR-Forward	TGCATTTTCCTCTCCAGCG
DYRK2-qPCR-Reverse	ACTGTTGAACCTGGATCTGTC
GAPDH-qPCR-Forward	CAAGCTCATTTCCTGGTATGACA
GAPDH-qPCR-Reverse	GGGAGATTCAGTGTGGTGGG

1c

1) 70% NaSH, DMF, 50 °C, 1h

2)
Br
NBoc
, K₂CO₃

DMF, r.t., overnight

7a

DIBAL, DCM

0 °C to r.t.
overnight

7b

5% TFA in DCM

r.t., 1h

7

7a

1) LiOH, THF/H₂O, 40 °C, 20h

2) 10% TFA in DCM, r.t., 6h

8

$(COCl)_2$, DMSO, TEA
THF, -78°C to r.t.

7b

NH$_4$Ac, NaBH$_3$CN
4A Molecular sieve
MeOH, -5°C, overnight

9a

9b

5% TFA in DCM, r.t., 1h

9

1b → BBr₃, DCM, -45 °C, overnight → **1c** + **1d**

1c → suitable bromides, Ag₂O, K₂CO₃, 40°C, DMF, 2h - overnight →

11a, R' = -Bn
12a, R' = -iPr

→ 1) 70% NaSH, DMF, 50 °C, 3h;
2) suitable bromides, K₂CO₃, DMF, r.t., overnight;
3) 5% TFA in DCM, r.t., 2h →

11-14

11	**12**	**13**	**14**
R = (propyl-NH₂)	(piperidine)	(CH₂-piperidine)	(CH₂-piperidine)
R' = -Bn	-Bn	-Bn	-iPr

1d → (BnBr, K$_2$CO$_3$, Ag$_2$O, DMF, 40 °C, 2h) → **15a** →

1) 70% NaSH, DMF, 50 °C, 2h

2) [4-(bromomethyl)piperidine-1-NBoc], K$_2$CO$_3$, DMF, r.t., overnight

3) 5% TFA in DCM, r.t., 2h

→ **15**