Title:

CRISPR/Cas9 screens Reveal Dasatinib Targets of Inhibiting T cell Activation and Proliferation

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Abstract (< 250 words)

Immune response by T cells is essential for a healthy body against cancer, infection, and pathophysiological alteration. The activation and expansion of T cells can be inhibited by dasatinib, a tyrosine inhibitor, thus improving the outcome of diseases, such as autoimmune disease, graft-versus-host disease, and transplant rejection. The underlying mechanism of inhibition by dasatinib is elusive. Here, we designed and synthesized a CRISPR/Cas9 screening library that includes 6,149 genes. Using the library, we performed dasatinib CRISPR/cas9 screening in Jurkat cell, a T lymphocyte cell. We firstly identified survival essential genes for Jurkat cells. Comparing with other CRISPR/Cas9 screenings, we obtained Jurkat cell specific essential genes. By comparing dasatinib treatment to control, we identified a set of dasatinib targets, which includes known targets: CSK, LCK, ZAP70, and previously unknown targets: ZFP36L2, LRPPRC, CFLAR, PD-1, CD45 et al. Visualizing these target genes on T cell receptor signaling pathway, we found several genes could be inhibited by dasatinib. Furthermore, we introduced a framework, 9-square, to classify genes and found a group of genes that are associated with dasatinib resistance, possibly linking the side effects of dasatinib. These data reveal a set of dasatinib targets and demonstrate the molecular potential functions of dasatinib. Identification of dasatinib targets will broaden our understanding to its molecular mechanism, and thus benefits to clinical outcome.

Introduction

T cells are critical for human against cancer, infection, and autoimmune diseases. Immune responses by T cells are tightly regulated by pathogen-, tumor-antigens, and self-peptides [1]. The activations of T lymphocytes are intimately implicated in several pathophysiological processes of diseases, such as cancer, infection, autoimmune, graft-versus-host disease (GVHD), and transplant rejection. The activations are determined by the major histocompatibility complex (MHC) that binds to antigens derived from pathogens and displays them on the cell surface for recognition by T cells that express appropriate T cell receptor (TCR). The interaction between TCR and MHC along with additional potential signal transduction networks determines the fate of immune response. It is established that 4 families of protein tyrosine kinases: Src family kinases, ZAP-70/Syk, Tec, and Csk, are involved in TCR signaling. Two Src kinases, Lck and Fyn, involving in the initial process of TCR activation, phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of CD3- and ζ-chains within the TCR:CD3 complex. This phosphorylated ITAMs recruit Zap70 that is further activated by Lck-mediated phosphorylation, fully activated Zap70 to form an effective signaling complex, which recruits a number of signaling proteins, including GADS, Grb2, SOS, Vav1, ITK, and PLC-γ1 [2]. The activations of T cells by the TCR signaling initiate a series of complicated biochemical cascades. However, if the activation of the T cell is aberrant, the immune system could be over-response to refrain from protecting and even attacking the body in diseases, such as autoimmune disease, GVHD, and transplant rejection. In such cases, the immune responses are required to be mitigated by inhibiting the activations of T cells with agents.

Dasatinib, a second-generation tyrosine kinase inhibitor (TKI), inhibits T cell activations by targeting multiple kinases, such as Src family. Dasatinib as salvage therapy for sclerotic chronic graft-vs-host disease was reported. Dasatinib is a first-line therapy for chronic myelogenous leukemia (CML) patients [3]. CML is a hematopoietic stem cell disorder frequently caused by chromosomal abnormality, called Philadelphia (Ph) chromosome, a translocation between the abelson (ABL) tyrosine kinase gene at chromosome 9 and the breakpoint cluster region (BCR) at chromosome 22, which forms a fusion oncogene BCR-ABL. Dasatinib is a potent multi-kinase inhibitor that can target and inhibit the BCR-ABL, SRC family (SRC, LCK, HCK, YES, FYN, FGR, BLK, LYN, FRK), receptor tyrosine kinases (c-KIT, PDGFR, DDR1/2, c-FMS, ephrin receptors), and TEC family kinases (TEC and BTK). The Src family kinases are involved in T cell activation, signal transductions, blocking cell duplications, adhesion et al [6]. Dasatinib potently inhibits Src family kinases, diminishing metastatic spread

62 of tumor cells, trigging apoptosis of tumor cells, and inhibiting T cell over-activation [7]. These discoveries suggest that
63 dasatinib can inhibit multiple targets and consequently determine cancer cell fate.
64
65 The CRISPR/Cas9 is a gene-editing technology that can cause double-strand breaks (DSBs) by single-guide RNAs (sgRNAs)
66 binding. A sgRNA usually contains 18-20 nucleotides complementary to its target and guides the Cas9 enzyme to a specific
67 DNA location where Cas9 induces a DSB. Repairs of such DSBs by the cell induce mutations in sgRNA-targeted genes,
68 leading to knockouts of these targeted genes[8]. Genome-wide or pooled CRISPR/Cas9 screening is a high-throughput
69 technology to investigate new biological mechanisms, such as drug resistance and cell survival-essential genes. In the
70 screening experiment, sgRNAs are designed, synthesized, and cloned into a lentivirus library, which is subsequently
71 transfected into cells at a low multiplicity of infection to ensure each cell takes up only one sgRNA. Cells are cultured under
72 different experimental settings, such as with or without drug treatment. The sgRNAs that incorporated into the host genome
73 are replicated with the host cell division during passages. By sequencing sgRNAs between initial cells and survived cells
74 using high-throughput sequencing and data analysis, survival essential candidate genes can be identified in comparison
75 between sgRNA in survived cells and initial cells. If one cell carrying a sgRNA is dead or slow-growing, the gene targeted
76 by the sgRNA is possibly essential for the cell survival. Drug-targeting genes can be identified by comparison of CRISPR
77 screenings between with and without drug treatment. Positively selected genes induce drug-treated cells growing faster than
78 control, might be associated with drug resistance, while negatively selected genes induce cell death or growing slowly, might
79 be promising candidates of the drug target. The CRISPR/Cas9 screening has been successfully used for identifying cell-
80 essential, non-essential genes, and drug-targeted genes in genome-scale [9-12].
81
82 Despite these progress in finding the regulators for T cell activation and proliferation during the past 20 years, the detailed
83 molecular mechanism underlying TCR signaling for T cell function remains elusive. To address this, we designed and
84 synthesized a gene-pooled CRISPR/Cas9 screening library, which includes 6,149 genes. Using the library, we performed
85 CRISPR/Cas9 screening in human Jurkat cells with dasatinib treatment. By bioinformatics analysis, we identified survival
86 essential genes for Jurkat cells and found the positively selected genes were significantly enriched in the TCR signaling
87 pathway. By comparing with other more than 33 CRISPR/Cas9 screenings, we obtained Jurkat cell specific essential genes.
88 Comparing dasatinib treatment to control, we identified a set of dasatinib targets. Importantly, we introduce a framework, 9-
89 square, to classify screening genes and define a group of genes that are associated with dasatinib resistance and possibly links

90 the side effects of dasatinib. Collectively, our results reveal a group of dasatinib-targets, demonstrating the potential dasatinib
91 mechanism on inhibition of T cell activation and proliferation. Deciphering dasatinib molecular basis is significantly benefit
92 to patients with diseases, such as autoimmune, GVHD, transplant rejection, and CML. These data extend our knowledge
93 regarding the dasatinib molecular mechanism.
94

95 Results

96 CRISPR/Cas9 Screening with a custom library

97 To identify the dasatinib targets with CRISPR/Cas9 screen, we designed and synthesized a custom CRISPR/Cas9 library.
98 Using the library, we performed CRISPR/Cas9 screens with dasatinib treatment in Jurkat cells. Jurkat cells are
99 an immortalized line of human T lymphocyte cells. The library contained 6,149 genes with 63,949 sgRNAs, ~10 sgRNAs
100 per gene. The number of sgRNAs per gene is greater than that in GeCKO (v2) libraries that contains ~6 sgRNAs per gene
101 [11]. Screening results from more sgRNAs per gene are usually more robust than less sgRNAs per gene. Of the 6,149 genes,
102 there are three genomic safe harbors: AAVS1, ROSA26, and CCR5 that are non-essential targeting regions, which are treated
103 as a positive controls for data normalization, because depletion of these genes could not obviously influence cell growth [13].
104 These 6,149 genes were involved in several important KEGG pathways, including ribosome, cell cycle, pathways in cancer,
105 CML, AML, and et al (Fig 1b). All sgRNAs were synthesized and cloned into a lentivirus library that was then infected into
106 cells at a low multiplicity of infection to ensure each cell takes up only one sgRNA as possible (Fig 1a). The concentration
107 of dasatinib treatment was identified with the half maximal inhibitory concentration (IC50) experiments (Fig 1c). The IC50
108 of dasatinib was 300 nano-molar in jurkat cells. SgRNAs that were incorporated into the host genome can be replicated with
109 the host cell division. These virally infected cells were selected and split into 3 populations at a starting time point. One
110 population representing the initial sgRNA status (day0) was harvested for sequencing. The remaining 2 populations were
111 respectively treated with dasatinib (treatment) and vehicle (control) and allowed to grow under same experimental conditions
112 for 10 double time (~4 weeks). The screening operations were conducted in parallel to minimize the technical variations. At
113 the end of the screen, genomic DNA from the infected cells was extracted and the sgRNA-encoded regions that viruses had
114 integrated to host genome were sequenced using single-end Illumina platform. 43.67 million reads were generated in the 3

115 samples in total. The read count of each sgRNA was a proxy for the cell growth characteristics under a knockout of the gene
116 targeted by the sgRNA.
117
118 The sequencing data were analyzed using the MAGeCK (v0.5.7) package[14] that uses negative binomial distribution to
119 calculate beta score, which represents CRISPR screening performance on each genes. The beta score is similar to the log2-
120 transferred fold-change in gene differential expression analysis. By comparing dasatinib-treated and vehicle-treated sample
121 to day0 sample, we obtained treatment and control beta score (CBS), respectively. Dasatinib-targeting genes were identified
122 on the basis of comparison of treatment beta scores (TBS) to CBS. Mapping these sequencing reads onto the CRISPR library,
123 we found that at least 85% sgRNAs were sequenced with more than or equal 53 reads in the 3 samples (Fig 1d). 79.4% reads
124 in day0 sample, 81.9% in control, and 81.6% in treatment were mappable (SFig 1a). The mapping rate indicates the successes
125 in construction and sequencing of the CRISPR libraries. Principle component analysis (PCA) and cumulative distribution
126 function (CDF) shown that the 3 samples can be separated from each other and with similar frequency tendency, respectively
127 (SFig 1b, c). Collectively, these data suggest that the 3 samples qualify for downstream analysis with the success in the
128 CRISPR library design, synthesis, screening, sequencing, and bioinformatic analysis.
129

130 **Identification of Jurkat-specific essential genes**

131 We firstly identified essential genes for Jurkat cell survival. Identification of essential genes can enable us to understand T
132 cell proliferation. By comparing the control sample to day0, we calculated CBS. Using 1-quantile standard deviation (QSD)
133 as cutoff, we identified 238 positively selected genes (PSGs) with beta scores greater than the cutoff, indicating these gene
134 could suppress T-cell proliferation (Fig 2a and Supplementary table 1). We also identified 1,714 negatively selected genes
135 (NSGs) with beta scores less than the cutoff (Fig 2a and Supplementary table 1), indicating these genes are required for T-
136 cell proliferation. As expected, these NSGs were enriched in ribosome, spliceosome, RNA transport, and cell cycle pathways
137 (SFig2 d, e), indicating these pathways are essential for T-cell proliferation, which suggests the Jurkat cells share common
138 essential genes with other cells. Interestingly, these PSGs were enriched in TCR signaling pathway, insulin resistance, and
139 viral carcinogenesis (SFig2 c). Visualizing of control beta scores in TCR signaling pathway, we found that genes, including
140 CTLA4, CD45, LCK, ZAP70, and et al, were negatively selected, suggesting these genes are essential for T-cell proliferation,
141 while genes, such as NCK, PAK, NFAT, AP1, and et al, were positively selected, suggesting these genes are required to be
142 suppressed for TCR signaling pathway activation. These data suggest these selected genes fine-tune T-cell activation and

143 proliferation through the TCR signaling pathway (SFig2 j). Collectively, we identified positively and negatively selected
144 genes for T-cells and demonstrated these genes are involved in T-cell proliferation.
145
146 We next identified genes that are essential for Jurkat cells specifically, which can extend our understanding to the Jurkat cells
147 characteristics. We analyzed CRISPR/Cas9 screening data from a prior study that carried out on 33 cell lines [15] by
148 MAGeCK package. These cell lines represent a wide diversity of cells to a certain extent. We selected 6,089 common
149 screening genes across our Jurkat cells and the 33 cell lines. Among the 33 cell lines, we identified 1,085 NSGs and 181
150 PSGs across at least more than 20 cell lines at a cutoff of 1 QSD (Fig 2b), suggesting that these genes were selected from the
151 majority of cell lines. Thus, we defined these genes as common positively and common negatively selected genes. Excluding
152 these common positive and negative selection genes from the PSGs and NSGs of Jurkat cells, we obtained 845 Jurkat-specific
153 NSGs (group A) and 223 Jurkat-specific PSGs (group D), as well as 842 common negatively (group B) and 11 positively
154 (group C) selected genes across these cell lines (Fig 2c, SFig 2a, and Supplementary table 2). These 845 NSGs were
155 specifically essential for Jurkat cell survival, reflecting the characteristics of Jurkat cells. We also analyzed the gene
156 expression profile of Jurkat cells using public data in GEO database (GSM2068619 and GSM2068620). Gene expression
157 profile analysis shown that the gene expression abundance in group A and B from Jurkat cells was greater than that in group
158 C, D, and non-selected genes (SFig 2b), and group B greater than group A, which suggests the Jurkat-specific and common
159 negatively selected genes are required high expression relative to other genes for maintaining the cell growth. Performing
160 GO term and KEGG pathway enrichment analysis, we found that these negative Jurkat-specific selected genes were
161 pertaining to such pathways as fanconi anemia pathway, alcoholism, and et al (SFig 2f, g). Visualizing control beta scores
162 onto fanconi anemia pathway, we found almost all screened genes, excepting PMS2, were negative selected, indicating these
163 Jurkat-specific negatively selected genes in fanconi pathway are essential for the cell survival. Taken together, these data
164 suggest that Jurkat cell survival is associated with a group of essential genes, in which the Jurkat-specific selected genes are
165 involved in TCR signaling that possibly links the Jurkat identity.
166

167 **Identification of dasatinib-targeting genes.**

168 To identify dasatinib-targeting genes, we identified treatment beta scores (TBS) by comparing dasatinib-treated library to
169 day0, and then comparing TBS to CBS. We identified a list of beta score differences between TBS and CBS, which reflects

170 dasatinib influence in the screenings. Similarly, using 1-QSD as a cutoff, we identified 1,053 positively and 902 negatively
171 selected genes (Fig 3a, SFig 3a, and supplementary table 2). By ranking the delta beta scores (treat-control) from high to low,
172 we found that the top 5 genes: CSK (C-Terminal Src Kinase), LCK (LCK proto-oncogene, Src family tyrosine kinase),
173 ZFP36L2 (zinc finger protein 36, C3H type-like 2), LRPPRC (leucine rich pentatricopeptide repeat containing), and CFLAR
174 (CASP8 and FADD like apoptosis regulator), were positively selected. This suggests that the genes more essential in control
175 cells than in dasatinib-treated cells (Fig 3b), indicating that these genes as cell fitness genes whose functions were inhibited
176 by dasatinib treatment. Thus, these genes are potential targets of dasatinib. CSK and LCK as tyrosine kinases, were previously
177 known to be dasatinib targets[16]. By contrast, the bottom 5 genes: SIK3 (SIK Family Kinase 3), CDK6 (cyclin dependent
178 kinase 6), BCL2L1 (BCL2 like 1), KMT2C (lysine methyltransferase 2C), and TONSL (tonsoku like, DNA repair protein),
179 were negatively selected. This suggests that these genes were more essential in dasatinib-treated cells than in control cells
180 (Fig 3b), indicating that the loss of these genes sensitized the cells to dasatinib. Therefore, these genes when inhibited would
181 synergistically enhance dasatinib treatment for T cells. Examining the sgRNA changes between the 3 samples, we found that
182 sgRNAs of LCK, CSK, and CFLAR in dasatinib-treated sample were higher than in control sample, supporting positive
183 selection by dasatinib, while SIK3 and CDK6 in treatment were lower than in control, supporting negative selection by
184 dasatinib (Fig 3c). We performed KEGG pathway enrichment analysis for the bottom 400 negatively selected genes and
185 found these genes were significantly enriched in chronic myeloid leukemia (CML), acute myeloid leukemia (AML), and
186 TGF-beta signaling pathways, suggesting that dasatinib treatment is implicated in the alterations on these pathways (SFig
187 3b), thus potentially improving the pathology of CML and AML. Performing GSEA analysis, we found many genes in CML
188 and AML pathways were negatively selected (SFig 3d, e, g, h), indicating that CML and AML pathways were suppressed
189 when cells were treated with dasatinib, which supports the therapy of dasatinib in CML and AML patients. More importantly,
190 we found 13 positively and 13 negatively selected genes in TCR signaling pathway at cutoff of 1-QSD (Fig 3d). The positive
191 selected genes include LCK, ZAP70, CD45, GSK3B, CBLB, PD-1, ITK et al, suggesting that the functions of these genes
192 can be inhibited by dasatinib, while IFNG, HRAS, RELA, RAF1, AKT1 et al were negatively selected, indicating these genes
193 could synergistically enhance dasatinib treatment function (SFig 3c). LCK and ZAP70 have previously found to be targets
194 of dasatinib. PD-1 is a checkpoint playing a critical role in regulating TCR signaling pathway. ITK encodes an intracellular
195 tyrosine kinase expressed in T-cells, regulates the development, function and differentiation of T-cells, thus ITK targeted by
196 dasatinib can inhibit T cell activation and proliferation.

197 Next, we ask if there were interactions between these identified dasatinib-targets. Based on the annotation of protein-
198 protein interactions (PPI) from GeneMANIA[17], we performed PPI analysis for these dasatinib targets. we highlighted a
199 sub-network that includes ranked top 5 (CSK, LCK, ZFP36L2, LRPPRC, and CFLAR), bottom 5 genes (SIK3, CDK6,
200 BCL2L1, KMT2C, and TONSL), and genes that were interacted with the 10 genes (SFig 3f), suggesting that these dasatinib
201 targets are possibly interacted to mediate dasatinib functions. We, based on these data, concluded that the dasatinib targets
202 the tyrosine kinases and TCR related genes to mediate gene functions, and consequently suppress T cell activation and
203 proliferation.
204

205 **Dasatinib resistance and response gene analysis**

206 To further explore dasatinib molecular function, we assigned all genes into 9 squares using 1-QSD as a cutoff for CBS
207 and TBS (Fig 4a). The delta score (TBS-CBS) reflects dasatinib treatment. Genes selected by CBS were associated with cell
208 growth, while genes selected in TBS were associated with both dasatinib treatment and cell growth. We highlighted 4 groups:
209 A, B, C and D (Supplementary Table 3), which were differentially selected between control and treatment screenings. Group
210 A and C were positively and negatively selected in TBS, respectively, but not selected in CBS, suggesting these genes were
211 associated with the drug treatment, but not associated with cell growth. Conversely, group B and D were positively and
212 negatively selection in CBS, respectively, but not selection in TBS, suggesting these genes were associated with cell survival,
213 but not associated with dasatinib treatment. In group A, dasatinib treatment induced cell growing faster than control,
214 indicating genes in group A may be associated with dasatinib resistance, while in group C, cell grew lower than control,
215 indicating may be associated with dasatinib response. Ranking the beta score difference, the top 5 genes in group A are
216 NUDCD1, CST4, DLX5, SIGMAR1, and NLRP7, and bottom 5 genes in group C are SIK3, KMT2C, RPL41, SLC25A22,
217 and PRKDC. We performed GO biological process and KEGG pathway enrichment analysis for group A and C (Fig 4b, c).
218 The top 10 enriched KEGG pathways includes Hedgehog signaling pathway, long-term potentiation, and cholesterol
219 metabolism pathway, and top 10 GO biological processes includes regulation of cell fate commitment, which are consistent
220 with previous studies [18, 19]. Similarly, group B were less positive selection after dasatinib treatment than control,
221 suggesting these genes may be synergy effect with dasatinib, while group D were less essential after dasatinib treatment than
222 control, indicating these genes may be dasatinib targets. We performed enrichment analysis for group B and D as well,
223 indicating that these genes were pertaining into pathways, including systemic lupus erythematosus, viral carcinogenesis,
224 Alcoholism, Th1 and Th2 cell differentiation, et al. Clinical treatment found that CML with long-term taking dasatinib could

225 suffer from side-effects, such as systemic lupus erythematosus, viral infection, increase disease risk when drink alcohol, and
226 immune inhibition of Th1 and Th2. These results provide links to the potential mechanism of the drug side effects. Taken
227 together, Group A and C maybe associated with the dasatinib resistance and response, and group B and D associated with
228 synergy and inhibition effects of dasatinib.
229

230 # Discussion

231 Our goal is to identify dasatinib targets of inhibiting T-cell activation and proliferation. To achieve these goals, we designed
232 and synthesized a CRISPR/Cas9 screen library, which contains 6,149 genes, covering key cancer-related pathways. SgRNAs
233 in the library were lentivirally transduced into Jurkat cells. 238 and 1,714 genes were respectively positively and negatively
234 selected by comparison to the starting plasmid library after 4-week unperturbed growth. Comparing with 33 published
235 screening datasets, we identified a set of genes that are essential for Jurkat specifically. By comparing dasatinib-treatment to
236 control beta score, we identified a group of potential targets of dasatinib, which are closely associated with TCR, CML and
237 AML pathways. These dasatinib targets included previously known targets: LCK, CSK, and unknown targets: ZFP36L2,
238 LRPPRC, CFLAR et al. Assigning genes into 9-square, we highlighted 4 group genes that are possibly associated with
239 dasatinib resistance, response, and side effects. These findings extend our knowledge regarding T-cell activation, proliferation,
240 and dasatinib molecular function.
241
242 T cell activation and proliferation are critical for adaptive immune. Dasatinib can inhibit tyrosine kinase and T cell activation.
243 Although the underlie mechanisms of T cell activation, proliferation, and dasatinib remain to be investigated, several
244 inferences can be drawn from the existing data.
245
246 First, we used CRISPR screens to identify 238 positive and 1,714 negative selection genes that are involved in Jurkat cell
247 survival. Jurkat cells are an immortalized line system of human T lymphocyte cells that are used to study acute T cell
248 leukemia, T cell signaling, and the expression of various chemokine receptors susceptible to viral entry, particularly HIV[20].
249 Jurkat cells can be used to determine the mechanism of differential susceptibility of cancers to drugs. These negatively
250 selected essential genes were enriched in ribosome, spliceosome, and RNA transport pathways, suggesting these genes are
251 strongly associated with T cell survival. These positively selected genes are associated with TCR signaling pathways,

252 indicating that these genes may be associated with the immunomodulation. The positively and negatively selected genes are
253 involved in T cell vulnerabilities. These data facilitate the understanding of T cell activation and proliferation.
254
255 Second, to identify Jurkat-specific essential genes, we compared the identified gene set with that from other 33 cell lines. We
256 obtained 845 negatively and 223 positively selected Jurkat-specific genes. The 845 genes are pertaining to Fanconi anemia
257 pathway, Alcoholism, Cysteine and methionine metabolism, and systemic lupus erythematosus pathways, while the 223
258 associated with B cell and TCR signaling pathway, indicating these pathways may be associated with the identity of Jurkat.
259 Token gather, the Jurkat-specific selected genes will benefit to the community for understanding the T cell characteristics.
260
261 Third, as a second-generation TKI inhibitor, the dasatinib is an effective therapy to CML patients carrying a fusion of BCL-
262 ABL. However, the potential mechanism of dasatinib is elusive, requiring further investigations. By comparison of dasatinib-
263 treated screen to control screen, we identified a group of targets that are potentially involved in dasatinib therapy, including
264 CSK, LCK, BCL, and ABL. These genes are targeted and inhibited by dasatinib[7, 21]. These dasatinib-targeted genes were
265 associated with CML, AML, and TCR signaling pathways. Visualizing these targeting genes on KEGG pathways enables
266 intuitive displaying genes in the context of regulation networks.
267
268 Fourth, assigning all genes into 9-square with a cutoff of 1-QSD, we identified 4 group genes. Genes positively and negatively
269 selected in dasatinib treatment but not in control may be associated dasatinib resistance and response. These genes are
270 associated with systemic lupus erythematosus, viral carcinogenesis, alcoholism, Th17 cell differentiation, Th1 and Th2 cell
271 differentiation pathways. Clinical evidences shown that patients with long term treatment of dasatinib may suffer severe side
272 effects, including system lupus erythematosus, alcoholism, and immune suppression[22]. These data suggest that these genes
273 may be associated with dasatinib side effects.
274
275 Finally, while we believe that these identified genes are important for T cell survival and dasatinib treatment, some issues
276 remain to be addressed. We used Jurkat cells as model, which is different from Ph+ CML cells. The screen library covers
277 6149 genes only. it is possibly missing some important genes. Although these limitations are existing in the data, these data
278 are largely extending our understanding to dasatinib mechanism, which will facilitate an improvement on dasatinib treatment
279 in clinic. Overall, although our study has certain limitations, because of the cell line in the experiments, requiring additional

280 validations, we nonetheless identified a group of T cell essential genes and found that dasatinib-treatment is closely associated
281 with CML and AML pathways. These findings refine our understanding to T cell proliferation, and dasatinib mechanism,
282 which will facilitate the advantage in the clinic outcome.
283

284 Conclusions

285 Immune response by T cell activation and expansion is of importance to the body against cancer-antigens, pathogen-, antigens,
286 and self-peptides. if over activation or proliferation of T cell, the immune system could fail to protect and even attack the
287 body with diseases, such as autoimmune disease and GVHD. We have presented CRISPR screens with dasatinib-treatment
288 in T cells. We identified a set of essential genes for T cell survival, which will contribute to the field with extending the
289 understanding of T cell survival mechanism. With dasatinib treatment, we identify dasatinib targets and demonstrating the
290 potential reason of dasatinib inhibition on T cell activation. Collectively, these results and the demonstrations of CRISPR
291 screen have set the stage for further understanding the T cell survival and the mechanism of inhibition by dasatinib on T cell
292 activation and proliferation.
293

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298

299 Materials and Methods

300 1. Design and synthesis of CRISPR/Cas9 screening library

301 To design a smaller scale CRISPR/Cas9 knockout screen library focusing on cancer-related genes, we selected 6000
302 genes based on reported relevancies with cancers using multiple literature, including Cosmic[23] and Oncopanel [24]. For
303 each gene, we designed ten 19-nucleotide sgRNA against its coding region with optimized cutting efficiency and minimized

304 off-target potentials. We used sequence features of the spacers to calculate the efficiency score for each sgRNA using our
305 predictive model [25] to improve cutting efficiency and utilized Bowtie to map all candidate sgRNAs to hg38 reference
306 genome and chose those with least alignment to reduce potential off-targets. We selected the top 10 best sgRNAs for each
307 gene based on the considerations above. The library also contains both positive controls and two types of negative controls:
308 non-targeting controls and non-essential regions-targeting sgRNAs.

309 （1） Positive controls: we included 1466 sgRNAs targeting 147 positive control genes, which are significantly negatively
310 selected in multiple screen conditions.

311 （2） Non-targeting negative controls: 795 sgRNAs with sequences not found in human genome (hg38).

312 （3） non-essential regions-targeting negative controls: 1891 sgRNAs targeting AAVS1, ROSA26, and CCR5, which
313 have been reported as safe-harbor regions where knock-in leads to few detectable phenotypic and genotypic changes.

Targets	Cancer – related	Positive controls	Non-targeting	Non-essential targeting regions	Total
Number of genes	6000	146	-	3	6149
Number of sgRNAs	59797	147	795	1891	63949

314

315 **2. Cell culture and CRISPR screening.**

316 （1） **Cell culture.** Jurkat cells were cultured in RPMI1640 (Invitrogen) supplemented with 10% fetal bovine serum
317 (Hyclone) and penicillin/streptomycin and maintained at 37℃ in a humidified atmosphere at 5% CO2. The cells were
318 suspension lymphoblasts. When cells were 6-8×105cells/ml, split them 1:4 with fresh media (T75=50ml).

319 （2） **Dasatinib treatment.** Dasatinib was purchased from Selleck, USA. Dasatinib was prepared as 10mM stock
320 solutions in dimethyl sulfoxide, stored in aliquots at -20 °C until further use. Jurkat cells (5×10^5 cells/ml) were treated with
321 or without different concentrations of dasatinib about 3 days (0.10, 50, 100, 500, 1000nM). Proliferation was measured after
322 3 days of culture by CCK8 assay (Dojindo Molecular Technologies, USA).

323 （3） **CRISPR screening.** $1.2\text{x}10^8$ Jurkat cells (5×10^5 cells/ml) were transfected with the customized pooled lentiviral
324 sgRNA library at multiplicity of infection of 0.3. To select for sgRNA-expressing cells, transfected cells were treated with
325 0.5 μg/mL puromycin, commencing 48h after transduction. Puromycin was withdrawn at day 3, and cells were divided into
326 three groups (Day 0 group, vehicle group, and dasatinib treatment group). The cells in vehicle group were cultured without
327 dasatinib and dasatinib treatment group were cultured with 100nM dasatinib for about 1 month.

328 (4) **Genomic DNA sequence.** Genomic DNA (gDNA) of three groups was extracted using a Blood & Cell Culture Midi
329 kit (Qiagen). SgRNA-coding regions integrated into the chromosomes were PCR-amplified with Q5 High-Fidelity DNA
330 Polymerase (Biolabs). To keep the fold representation at 300 for each of the sgRNA, 200μg of gDNA per each sample was
331 used for the PCR. SgRNA sequences were amplified by two rounds of PCR. For the first PCR, perform 25-30 separate 100
332 μl reaction with 6-8 μg gDNA in each reaction and then combined the resulting amplicons. The forward primer of the first
333 PCR is AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG and the reverse primer of the first PCR is
334 TCTACTATTCTTTCCCCTGCACTGTACCTGTGGGCGATGTGCGCTCTG. To attach Illumina adaptors and to barcode
335 samples, the second PCR was performed in a 100 μl reaction volume using 5 μl of the product from the first PCR. Forward
336 primer of the second PCR is AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC
337 TCTTCCGATCTTCTTGTGGAAAGGACGAAACACCG and reverse primer of the second PCR is CAAGCAGAAGA
338 CGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT (NNNNNNNN, 8 bp-barcode) TACTATT
339 CTTTCCCCTGCACTGTACC. PCR bands were gel purified using Qiagen gel purification kit. The resulting libraries were
340 sequenced with pair-end 150bp reads on an Illumina Hiseq 2500.
341

342 **3. CRISPR screening data analysis**

343 The CRISPR/Cas9 screening data were analyzed using the MAGeCK and MAGeCK-VISPR algorithms [14] [26]. We
344 use the MAGeCK-VISPR algorithm to compare the gene selections across different conditions. MAGeCK-VISPR uses a
345 metric, "β score", to measure gene selections. The definition of β score is similar to the term of 'log fold change' in differential
346 expression analysis, and β>0 (or <0) means the corresponding gene is positively (or negatively) selected, respectively.
347 MAGeCK-VISPR models the gRNA read counts as a negative binormal variable, which mean value is determined by the
348 sequencing depth of the sample, the efficiency of the gRNA, and a linear combination of β scores of the genes. MAGeCK-
349 VISPR builds a maximum likelihood (MLE) model to model all gRNA read counts of all samples, and iteratively estimate
350 the gRNA efficiency and gene β scores using the Expectation-Maximization (EM) algorithm. A detailed description of the
351 MAGeCK-MLE algorithm can be found in the original study[14].

352 We identified Jurkat essential genes by control sample compared with starting time sample, using MAGeCK, and
353 dasatinib target genes by treatment β scores compared with control β scores. The MAGeCK algorithm works as follows. It
354 first collects read counts of all gRNAs in all conditions from fastq files, and then normalizes the read counts of control and

355 treatment conditions using median normalization. After that, MAGeCK builds a linear model to estimate the variance of
356 gRNA read counts, evaluate the gRNA abundance changes between control and treatment conditions, and assigns a p-value
357 using the Negative Binomial (NB) model. Finally, the selection of genes is evaluated from the rankings of gRNAs (by their
358 p-values) using the α-RRA (α-Robust Rank Aggregation) algorithm. For each gene, α-RRA evaluates the rankings of all its
359 gRNAs, and assigns a lower score (RRA score) if the distribution is more skewed compared with uniform distribution. The
360 statistical significance of the RRA score is evaluated by permutation, and the Benjamini-Hochberg method is used for
361 multiple comparison adjustments. To increase the statistical power, genes that have fewer than 4 gRNAs, or genes that have
362 fewer than 2 significant gRNAs are excluded from the comparison. A detailed description of the MAGeCK algorithm can be
363 found in the original study [14, 26].

364 Gene enrichment was performed with clusterProfiler package, which allowed to analyze enrichment of GO biological
365 process terms and KEGG pathways. Pathview package was used to visualizing β scores or β score difference on the KEGG
366 pathways. R-scripts were made to visualize enrichment analysis.

367

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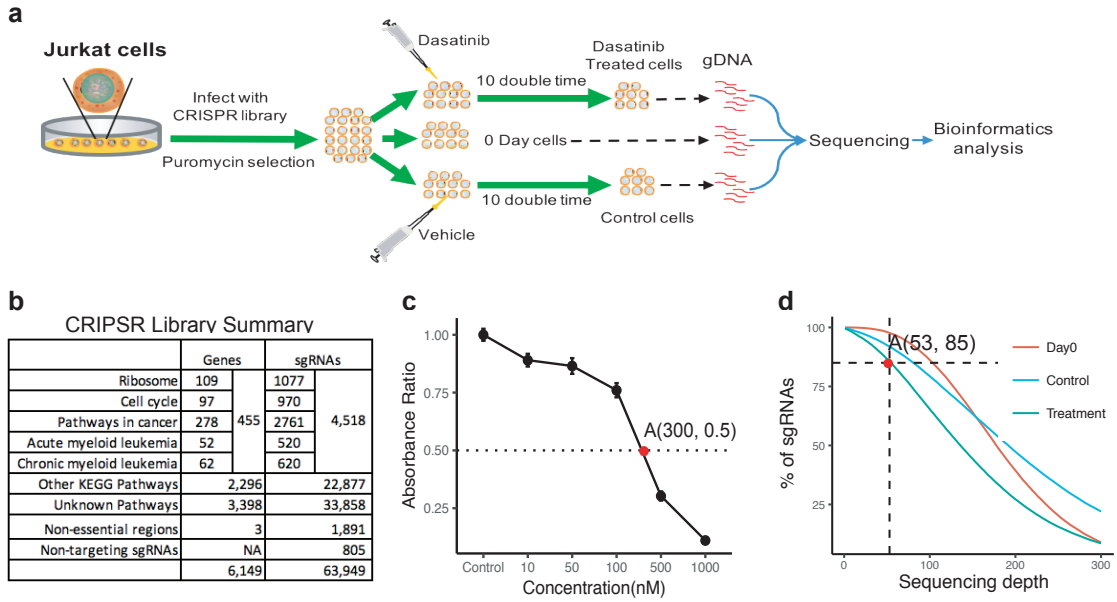
426
427

Figure 1



CRIPSR Library Summary

	Genes		sgRNAs	
Ribosome	109	455	1077	4,518
Cell cycle	97		970	
Pathways in cancer	278		2761	
Acute myeloid leukemia	52		520	
Chronic myeloid leukemia	62		620	
Other KEGG Pathways	2,296		22,877	
Unknown Pathways	3,398		33,858	
Non-essential regions	3		1,891	
Non-targeting sgRNAs	NA		805	
	6,149		63,949	

Figure 1. Schema of Dasatinib CRISPR/Cas9 screenings in Jurkat cells. (a) Schema of CRISPR/Cas9 library screening. Jurkat cells were transduced with lentivirus gRNA library, selected with puromysin for 2 weeks, and equally divided into 3 populations: one was treated as base line in a starting time point (Day 0), other 2 populations treated with dasatinib and vehicle as drug treatment and control, respectively. (b) CRISPR/Cas9 library composition. Genes in the library are associated with many essential and cancer related pathways. (c) Half maximal inhibitory concentration (IC50) of dasatinib in jurkat cells. X- and y-axis are dasatinib concentration (nM, nano-molar) and the dasatinib absorbance ratio, respectively. Point A represents the absorbance ratio was 50% when dasatinib concentration was 300nM. (d) sgRNA sequencing depth. In point A, 85% of sgRNAs was sequenced at least 53 converge in day0, control, and treatment samples.

Figure 2

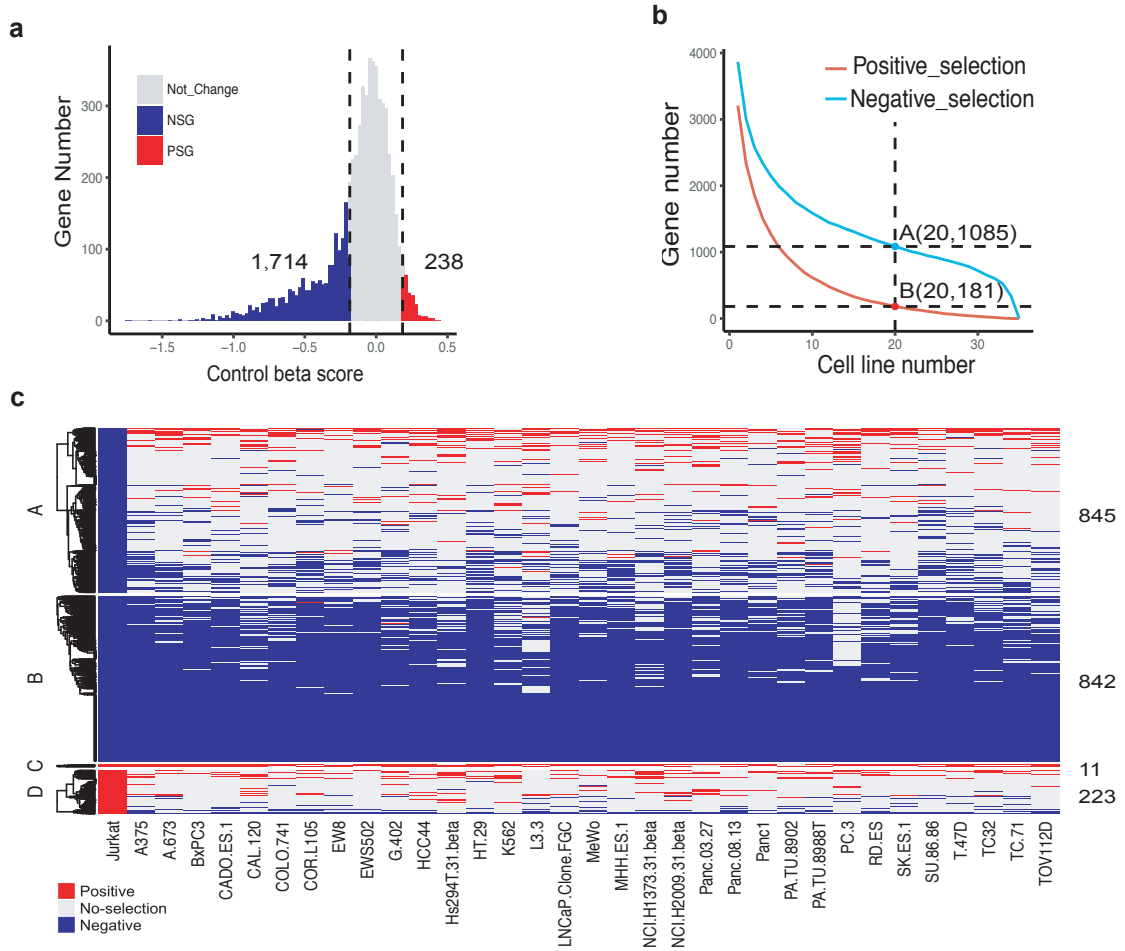


Figure 2. Identification of essential genes in Jurkat cells. (a) A distribution of essential genes of Jurkat cells. 1,714 and 238 are total NSG number and PSG number, respectively. Vertical dotlines are cutoff of +/- 1 QSD. (b) Common positive and negative selected gene numbers across 34 cell lines, including Jurkat. X- and y-axis are the cell line number and common selected-gene number, respectively. Point A represents 1,085 genes were negatively selected across at least 20 cell lines, and point B 181 positively selected across at least 20 cell lines. The 1,085 and 181 genes were defined as common negatively and positively selected genes across 34 cell lines, respectively. (c) A clustering heatmap showing Jurkat positively/negatively selected genes across 34 cell lines. Group A and D were 845 negatively and 223 positively Jurkat-specific selected genes, respectively. Group B and C were 842 negatively and 11 positively commonly selected genes across at least 20 cell lines, respectively. Red and blue are positive and negative selection in the heatmap, respectively. NSG: negatively selected gene; PSG: positively selected gene; QSD: quantile standard deviation.

Figure 3

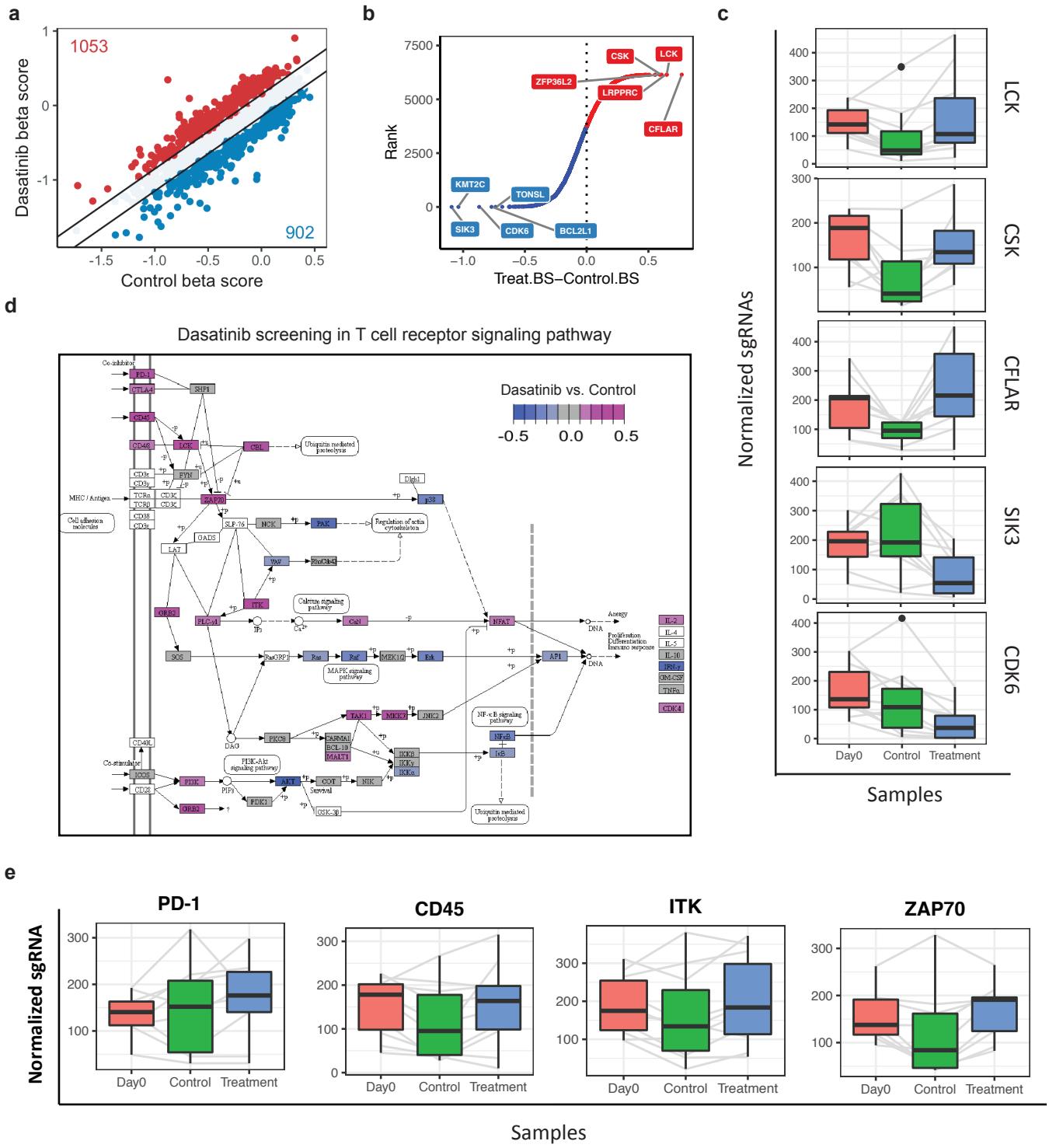


Figure 3. Dasatinib CRISPR screening. (a) A scatterplot shows the distribution of control and dasatinib beta score. (b) Rank of value of treatment minus control beta score. Treat.BS and Control.BS are dasatinib and control beta score. (c) Boxplots show normalized sgRNA count distributions in representive genes: LCK, CSK, CFLAR, SIK3, and CDK6. The normalization is based on the amount of sequencing data. (d) visualization of beta score change of dasatinib/control beta score in T cell receptor signaling pathway. The pathway was adapted from chronic myeloid leukemia KEGG pathway. (e) Boxplots show normalized sgRNA counts in TCR pathway genes: PD-1, CD45, ITK, and ZAP70.

Figure 4

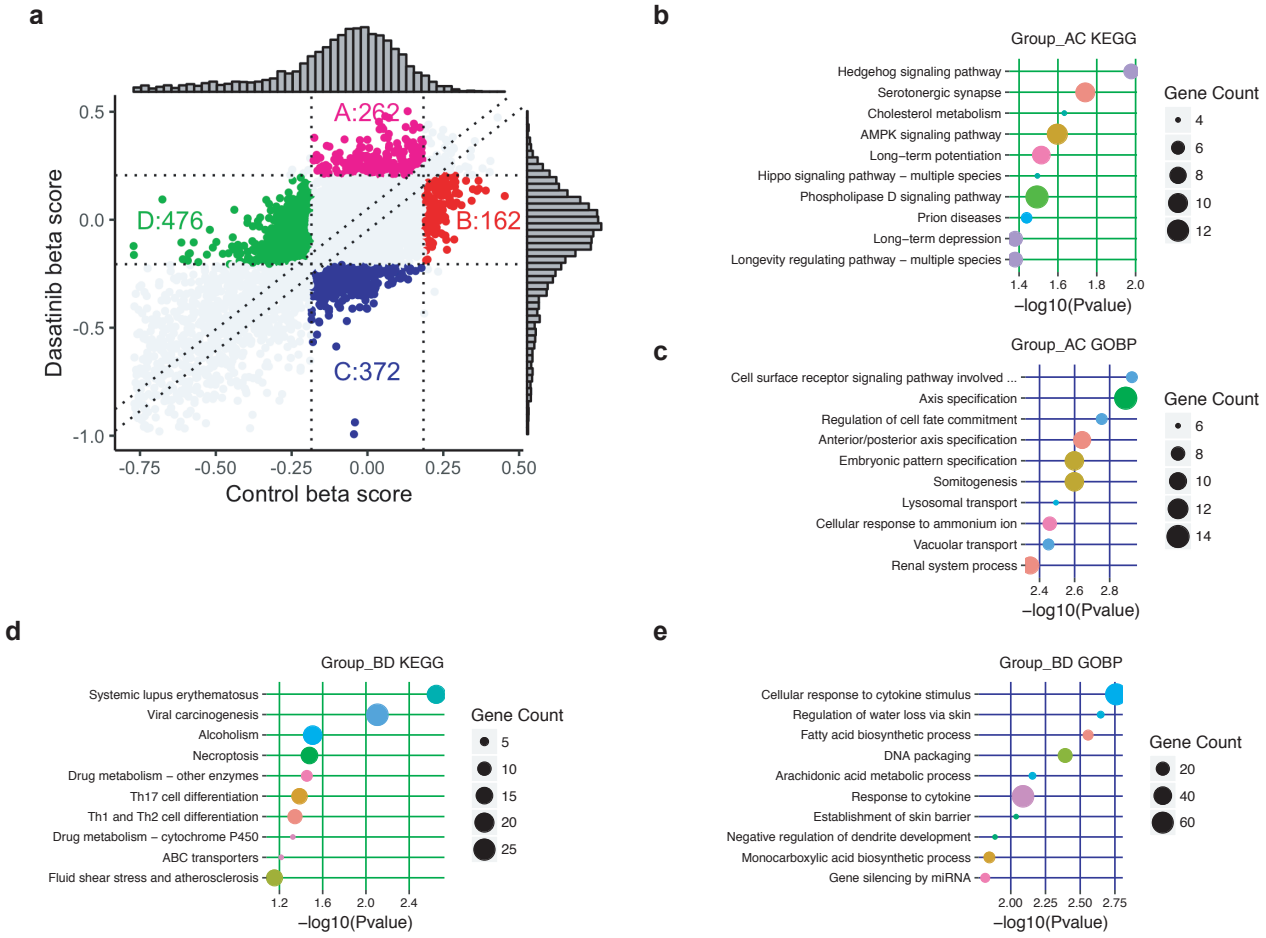


Figure 4. gene classification with control and dasatinib beta score and KEGG and GO enrichment analysis. (a) gene classifications with control and dasatinib beta score. Vertical dot lines are control cutoff on +/- 1 QSD. Horizontal dot lines are dasatinib cutoff on +/- 1 QSD. According to these vertical and horizontal dot lines, all genes are assigned into 9 groups. Diagonal dot lines are lines of dasatinib/control cutoff +/- 0.05 for separating group A and B as well as D and C. (b & c) KEGG pathway (b) and GO biological process enrichment analysis (c) for genes in group A and C. (d & f) KEGG pathway (d) and GO biological process enrichment analysis (f) for genes in group B and D. (e) Values of dasatinib minus control beta score on hedgehog signaling pathway. (g & h) Group A and C gene beta score difference visualization in AMPK signaling pathway (g) and Long-term potentiation (h). (i-k) Group B and D gene beta score difference visualization in system lupus erythematosus (i), viral carcinogenesis (j), and alcoholism (k) KEGG pathways