1 **GFI1 cooperates with IKAROS/IKZF1 to activate gene expression in T-cell acute lymphoblastic**

2 **leukemia**

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19 **KEYWORDS:** GFI1; IKAROS; NOTCH3; T-cell Acute Lymphoblastic Leukemia (T-ALL)

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25   **Abstract (168 words)**

26   Growth factor independence-1 (GFI1) is a transcriptional repressor and master regulator of normal and

27   malignant hematopoiesis. Repression by GFI1 is attributable to recruitment of LSD1-containing protein

28   complexes via its SNAG domain. However, the full complement of GFI1 partners in transcriptional control

29   is not known. We show that in T-ALL cells, GFI1 and IKAROS are transcriptional partners that co-occupy

30   regulatory regions of hallmark T cell development genes. Transcriptional profiling reveals a subset of genes

31   directly transactivated through the GFI1—IKAROS partnership. Among these is *NOTCH3*, a key factor in

32   T-ALL pathogenesis. Surprisingly, *NOTCH3* transactivation by GFI1 and IKAROS requires the GFI1

33   SNAG domain but occurs independent of SNAG—LSD1 binding. GFI1 variants deficient in LSD1 binding

34   fail to transactivate *NOTCH3*, but conversely, small molecules that disrupt the SNAG—LSD1 interaction

35   while leaving the SNAG primary structure intact stimulate *NOTCH3* expression. These results identify a

36   non-canonical transcriptional control mechanism in T-ALL which supports GFI1-mediated transactivation

37   in partnership with IKAROS and suggest competition between LSD1-containing repressive complexes and

38   others favoring transactivation.

Introduction

Growth factor independence-1 (GFI1) is a zinc finger transcription factor which plays essential roles in normal and malignant myeloid and lymphoid hematopoiesis (Hock, Hamblen et al., 2004, Zeng, Yucel et al., 2004). Germline *GFI1* mutations cause severe congenital neutropenia (Person, Li et al., 2003), while *Gfi1* null mice show impaired T cell and neutrophil differentiation (Hock, Hamblen et al., 2003, Karsunky, Zeng et al., 2002, Yucel, Karsunky et al., 2003). In acute myelogenous leukemia (AML), *GFI1* mRNA expression can be used to stratify patient survival, while *GFI1* displays a dose-dependent impact on the pace of leukemic progression brought on by onco-fusion proteins, MLL-AF9 and NUP98-HOXD13 (Hones, Botezatu et al., 2016, Volpe, Walton et al., 2017). Notably, a GFI1 variant, GFI136N, generated from a single nucleotide polymorphism expressed in 3-7% of the Caucasian population, is disproportionately observed in AML patients and increases risk for AML development by 60% relative to the more common GFI136S variant (Khandanpour, Krongold et al., 2012). G*FI1* mRNA is also elevated in samples from patients with early T cell precursor acute lymphoblastic leukemia (ETP-ALL) who display a positive NOTCH signature (Khandanpour, Phelan et al., 2013). ETP-ALL is a high-risk subgroup of T-ALL (Coustan-Smith, Mullighan et al., 2009, Zhang, Ding et al., 2012), which itself is an aggressive form of acute leukemia characterized by the expansion of immature lymphoid precursor cells (Terwilliger & Abdul-Hay, 2017). The precise role of GFI1 in T-ALL is not clear.

T-ALL has a high incidence of relapse, and survival following disease recurrence is dismal. Abberant activation of NOTCH signaling is a unifying theme in T-ALL, and arises either from mutations in NOTCH receptors or NOTCH regulators. Normally, in response to ligand binding, NOTCH receptors are cleaved by γ-secretase to liberate their intracellular domains (NICD). NICD then partners with nuclear factors to direct the expression of NOTCH target genes. *NOTCH1*-activating mutations are found in approximately 60% of T-ALL cases (Ferrando, 2010). While γ-secretase inhibitors (GSIs) have shown anti-leukemic activity *in vitro* and in murine models, they have not been integrated into T-ALL treatment protocols because of dose limiting gastrointestinal toxicity and poor anti-leukemic efficacy (Golde, Koo et al., 2013).

65   Like NOTCH1, NOTCH3 promotes T cell lineage specification and leukemogenesis. *NOTCH3*-activating

66   mutations have been identified in approximately 5% of T-ALL cases and NOTCH3 blocking antibodies

67   exhibit potent anti-leukemic effect in T-ALL (Bellavia, Campese et al., 2000, Bellavia, Campese et al.,

68   2002, Bernasconi-Elias, Hu et al., 2016, Waegemans, Van de Walle et al., 2014, Xu, Choi et al., 2015).

69   Moreover, abnormal expression and/or activation of *NOTCH3* is seen in T-ALL patient samples lacking

70   *NOTCH1*-activating mutations, reinforcing NOTCH signaling as critical for T-ALL pathogenesis and

71   suggesting *NOTCH3* and factors controlling its expression could represent alternative therapeutic targets in

72   this disease (Choi, Severson et al., 2017, Tottone, Zhdanovskaya et al., 2019). The development of new

73   therapeutic strategies for T-ALL depends upon deeper understanding of its molecular underpinnings.

74

75   Here, we identify IKAROS as a frequent DNA binding partner for GFI1. GFI1 and IKAROS do not interact

76   in classical co-immunoprecipitation (co-IP) assays, but their proximity relationship is impaired by the

77   N383S mutation that impairs GFI1 DNA binding and the N159A mutation that impairs IKAROS DNA

78   binding. In contrast, their interaction is not affected by LSD1 binding-deficient GFI1 variants including

79   GFI1-P2A, -K8L or -ΔSNAG. We identify a strong, genome-wide correlation between GFI1- and

80   IKAROS-regulated genes through ChIP-Seq. Genes co-occupied by GFI1 and IKAROS encode hallmark

81   T cell development proteins such as NOTCH3, CD3, GFI1 itself, c-MYC, C-MYB and HES1. Gene

82   expression profiling by RNA-Seq identifies a cluster of genes activated by ectopic GFI1 expression and

83   repressed with IKAROS knockout. Interestingly, these genes include the direct GFI1/IKAROS target

84   *NOTCH3,* a critical oncogenic factor in T-ALL. Using both CCRF-CEM and SUP-T1 T-ALL cells, we

85   show that inducible expression of either GFI1 or IKAROS elevates NOTCH3 cell surface expression, while

86   acute degradation of IKAROS through an IKAROS inhibitor or CRISPR/Cas9-mediated IKAROS

87   knockout significantly attenuates GFI1-mediated NOTCH3 induction. Increased NOTCH3 cell surface

88   expression depends upon SNAG domain amino acids that enable interaction with LSD1. Yet, LSD1

89   inhibition and disruption of the SNAG—LSD1 interaction augments *NOTCH3* expression, suggesting that

90   LSD1 competes with activators of NOTCH3 expression mediated by the GFI SNAG domain. Together,

91   these results identify a noncanonical transativation mechanism for GFI1, working in partnership with

92   IKAROS to promote expression of *NOTCH3* and related T cell development genes, and providing new

93   insights for therapeutic targeting in T-ALL.

Results

GFI1 proximitome proteomics in T-ALL cells

94 To identify potential GFI1 cooperating proteins in T-ALL, we applied the BioID (Biotin IDentification)

97 proximity-dependent labeling method to screen for vicinal proteins (Figure 1A). We generated doxycycline-

98 inducible, GFI1-BirA*-expressing CCRF-CEM cells. In parallel, cells transduced with empty vector or

99 BirA* only were generated as controls (Figure 1B). Cells were incubated in biotin-containing medium,

100 treated with doxycycline and lysates prepared. Biotinylated proteins were collected with streptavidin

101 Sepharose beads and surveyed for known GFI1 interacting partners. As expected, we detected comparable

102 biotinylation of GFI1-BirA* and BirA*, suggesting the presence of GFI1 protein structure does not interfere

103 with the formation of reactive biotin-AMP by BirA* in the fusion protein. Moreover, we find an altered

104 pattern of biotinylated proteins when BirA* activity is tethered to GFI1, and enrichment for known GFI1

105 interacting partners, LSD1 and CoREST among biotinylated proteins in cells expressing GFI1-BirA* vs.

106 BirA* control (Figure 1C). These data validate the technique for detecting GFI1 proximity partners

107 proteome-wide.

108 To identify the complete cohort of GFI1 proximity partners, biotinylated proteins were purified on

109 streptavidin (SAv)-Sepharose beads and subjected to unbiased, proteome-wide LC-MS/MS (Figure 1A).

110 Three replicates were performed for each condition (Vector, BirA* and GFI1-BirA*). A total of 502

111 interacting proteins were identified that demonstrated both increased mass spectrometry intensity in the

112 presence of doxycycline compared to empty vector control and a BirA* vs GFI1- BirA* P-value of <0.05

113 (Supplemental Table S1). Proximity partners were analyzed as previously described (McClellan, Casey et

114 al., 2019). A volcano plot showing fold change in average sum read intensities ($\log_2$ (GFI1-WT/BirA*))

115 relative to P-value (-$\log_{10}$ P-value) is shown in Figure 2A. Being covalently tethered to BirA*, the

116 placement of GFI1 in the top right corner of the plot is an important quality control, signifying the most

117 statistically significant P-value and most abundantly enriched protein in the data set. Among biotinylated

118 proteins, known GFI1 partners LSD1 (KDM1A), CoREST (RCOR1), STAG1, BCL11A and HMG20B

119 were enriched when comparing GFI1-BirA* to BirA* only (Figure 2A, shown in red). Among newly-

120   identified GFI1 proximity partners, the strongest overall by fold-enrichment was for IKZF1/IKAROS

121   (Figure 2A). To identify possible functional protein associations, we clustered the top 40 GFI1-proximate

122   proteins using the STRING functional protein—protein association network (Szklarczyk, Gable et al.,

123   2019). The majority of these proteins were annotated as being nuclear and involved in regulating gene

124   expression (Figure 2B, red and blue circles respectively). Notably, STRING output linked GFI1 directly to

125   LSD1 and CoREST, as expected (Figure 2B), but placed IKAROS more remotely, arguing against a direct

126   interaction with GFI1 (Figure 2B).

127

128   **The GFI1—IKAROS interaction requires intact GFI1 and IKAROS DNA binding**

129   We validated the proximity relationship between IKAROS and GFI1 through Western blotting. First, we

130   tested if N-terminal GFI1 mutations known to block binding to LSD1 also block the ability to transfer biotin

131   moieties to IKAROS. GFI1 mutations that block LSD1 binding did not affect IKAROS proximity labeling

132   (Figure 3A). Additionally, GFI1 and IKAROS failed to interact using a variety of traditional co-

133   immunoprecipitation conditions (Figure 3B and data not shown). One possible explanation for the

134   discrepancy could be that GFI1 and IKAROS co-occupy nearby DNA binding sites at common target genes

135   such that their proximity enables biotin moieties to be frequently transferred from GFI1-BirA* to IKAROS

136   even though the two proteins do not interact directly in solution. Similarly, GFI1 and IKAROS could

137   occupy sites distant from one another but brought together through interactions involving a shared protein

138   complex. Both models predict that the proximity relationship between GFI1 and IKAROS would require

139   both proteins to bind DNA and that a defect in DNA binding by either protein would attenuate the proximity

140   relationship between them. Previous work on GFI1 and IKAROS has identified specific domains and

141   mutants that control DNA binding activity (Kuehn, Boisson et al., 2016, Zarebski, Velu et al., 2008). We

142   established these DNA-binding deficient mutations in GFI1 (N383S in rat GFI1, which corresponds to

143   N382S in human GFI1) and IKAROS (N159A) and used them to interrogate the GFI1—IKAROS proximity

144   relationship. GFI1-N383S-BirA* reduced proximity labeling of wild type IKAROS (Figure 3C). Likewise,

145   we observed a comparable reduction in proximity labeling of IKAROS-N159A when when tested with wild

146   type GFI1-BirA*. When both DNA-binding deficient mutants were combined, the proximity relationship

147   between GFI1 and IKAROS is abolished (Figure 3C). These results indicate that the proximity relationship

148   between GFI1 and IKAROS relies upon their shared ability to bind DNA and suggests a mechanism for

149   GFI1 and IKAROS to cooperate to control a common set of genes through near or distant regulatory regions.

150

151   **GFI1 and IKAROS associate with common genes, including genes associated with T cell development**

152   To further study the interplay between GFI1 and IKAROS, we conducted ChIP-Seq using CCRF-CEM

153   cells expressing 3×FLAG-tagged GFI1 or IKAROS (GFI1-3×FLAG or IKAROS-3×FLAG) under

154   doxycycline-inducible control. FLAG immunoblotting confirmed inducible and comparable expression of

155   the two proteins (Figure 4A). Two replicates each for GFI1 and IKAROS ChIP were performed. Sequencing

156   of the ChIP material resulted in 25,674 total GFI1 and 52,759 IKAROS peaks. The signals from input, GFI1

157   ChIP-Seq and IKAROS ChIP-Seq replicates were highly correlated, with Spearman correlation R-

158   values >0.8. Further, the GFI1 and IKAROS ChIP-Seq signals were also highly correlated, with R

159   values >0.75. (Supplementary Figure S1A). Principal component analysis of the bound peaks also indicated

160   that the inputs and ChIP replicates from the two cells lines were more similar to each other compared to the

161   other samples (Supplementary Figure S1B). Approximately 80% of GFI1-bound peaks overlap (at least one

162   bp) with peaks bound by IKAROS (Figure 4B), suggesting that they regulate common targets. To

163   investigate this more closely, we centered binding peaks from either or both ChIP experiments (57,841

164   peaks) by peak summit, and arranged them as a heatmap from strongest to weakest GFI1 binding (Figure

165   4C, left side) and IKAROS binding (Figure 4C, right side), indicating an overall correlation between the

166   strength of GFI1 and IKAROS binding. Motif analysis using all identified peaks showed that GFI1 and

167   IKAROS shared four of the top five most significant binding motifs (Supplementary Figure S1C), again

168   suggesting common gene binding. Among the top shared motifs is FLI1, an Ets transcription factor

169   (consensus TTCC or GGAA reverse complement). Out of the top 20 enriched motifs for the two datasets,

170   14 and 15 Ets transcription motifs were present (Supplementary Figure S1C and data not shown). IKAROS

171   is known to interact with Ets motifs (Zhang, Jackson et al., 2011).

172   GFI1 and IKAROS play crucial roles in hematopoiesis, including in T cells (Georgopoulos, Bigby

173   et al., 1994, Shi, Kalupahana et al., 2013, Tinsley, Hong et al., 2013). Consistently, gene ontology (GO)

174   analysis of genes within 6 kb of the GFI1- and IKAROS-bound peaks (using GREAT v4.0.4) identifies

175   hematopoiesis and TCR recombination as common terms (Supplemental Table S2). Example bound targets

176   are shown in Figure 4D, including genes encoding key transcription factors required for T cell development

177   such as *GFI1* itself, *MYC*, *MYB*, *HES1, RUNX3* and *TCF3*. Other examples include *NOTCH3* and genes in

178   the *CD3* cluster. Figure 4D also shows previously published RNA-Seq data (Quentmeier, Pommerenke et

179   al., 2019) indicating that these genes are all expressed in CCRF-CEM cells.

180

181   **GFI1 and IKAROS positively regulate a subset of target genes**

182   We used bulk RNA-Seq with GFI1-3×FLAG CCRF-CEM cells to identify changes in gene expression

183   associated with either ectopic GFI1 expression or CRISPR-mediated IKAROS knockout. For knockout, we

184   transfected GFI1-3×FLAG cells with CRISPR RNPs containing Cas9 protein, fluorescently-conjugated

185   tracrRNA and either a nonspecific or *IKAROS*-specific sgRNA. Successfully transfected cells were sorted

186   on the following day and cultured for an additional 9 days before treatment with doxycycline or vehicle for

187   24 hrs (see methods). This resulted in robust knockout using specific but not control RNPs (Figure 5A). A

188   similar knockout strategy targeting GFI1 resulted in lethality in CCRF-CEM cells (not shown),

189   underscoring its critical pro-survival role, and for this reason was not pursued further. Three independent

190   replicates were performed for each of the four conditions, with the exception of vehicle-treated IKAROS

191   knockout with two replicates. Between 21.7 and 28.2 million RNA-Seq reads were generated for each

192   condition, wherein 88.6 to 90.3% of these aligned uniquely to the human *Hg38* reference genome.

193   Approximately 99% of the reads within coding regions aligned to the correct strand (Supplemental Table

194   S3). Hierarchical clustering of the top 500 most significantly differentially regulated genes across all the

195   conditions (ranked based on *P*-value) revealed groups of genes repressed and activated by the different

196   conditions. For example, a large number of genes are de-repressed with IKAROS knockout and/or repressed

197   with GFI1 overexpression (Figure 5B, clusters 1, 2 ,3). Another group, not strongly affected by GFI1, was

198   repressed with IKAROS knockout (cluster 5). However, a small group of 38 genes (cluster 4) were induced

199   by GFI1 overexpression (Figure 5B). This same group of genes was also repressed with IKAROS knockout.

200   Interestingly, when ectopic GFI1 expression and IKAROS knockout were combined, the induction of these

201   genes was significantly blunted, suggesting IKAROS is required for GFI1-mediated transactivation (Figure

202   5B). These genes include *RAG1* and *NOTCH3* (Supplemental Table S4). Intersecting this set of positively

203   regulated genes with the ChIP-Seq data reveals that 68.4% (26 of 38 total genes in cluster 4) have peaks

204   for GFI1 and IKAROS located <10 kb from their transcription start sites, strongly suggesting they are direct

205   targets of both GFI1 and IKAROS (Supplemental Table S4). 34.2% of these genes (13/38) show promoter

206   binding (<500 bp from TSS) of both proteins. RNA-Seq genome tracks for *NOTCH3* are shown in Figure

207   5C alongside the GFI1 and IKAROS ChIP-Seq reads. Strong and overlapping GFI1 and IKAROS peaks

208   are present in the 5' region of the gene. In the RNA-seq, *NOTCH3* is induced by ectopic GFI1 expression

209   and inhibited by IKAROS knockout, while the combination significantly blunts induction by GFI1. No such

210   changes were observed for *NOTCH1* or *NOTCH2*, which show some GFI1 and IKAROS association but

211   constitutive expression, or for *NOTCH4*, which shows no binding and is not expressed in CCRF-CEM cells

212   (Supplementary Figure 2).

213

214   **GFI1 and IKAROS cooperatively regulate NOTCH3**

215   A model in which GFI1 and IKAROS interact on DNA to induce the expression of specific genes predicts

216   that overexpression of either protein should induce target gene expression. Both GFI1 and IKAROS interact

217   with the *NOTCH3* gene. In addition, NOTCH3 activating mutations can trigger T-ALL development

218   (Bernasconi-Elias et al., 2016, Choi et al., 2017, Tottone et al., 2019). For these reasons, and because its

219   surface expression can easily be measured using flow cytometry, we used *NOTCH3* as an example target

220   gene. We treated CCRF-CEM cells that inducibly express GFI1-3×FLAG with doxycycline and followed

221   surface NOTCH3 expression as a time course using flow cytometry. NOTCH3 was low but detectable in

222   untreated cells (Figure 6A, 0h), with expression increasing during the 48 hour treatment course. Expression

223   increased both as measured by the percentage of positive cells (not shown), and as measured by mean

224   fluorescence intensity (MFI). CCRF-CEM cells inducibly expressing IKAROS-3×FLAG were similarly

225   able to increase cell surface expression of NOTCH3 (Figure 6B). We then replaced the GFI1 lentiviral

226   construct with a truncation mutant in which the SNAG domain was deleted (ΔSNAG) or with a SNAG

227   domain point mutant that no longer interacts with LSD1 to mediate transcriptional repression (P2A)

228   (Grimes, Chan et al., 1996, Velinder, Singer et al., 2017). Unexpectedly, these mutant forms of GFI1 no

229   longer augment surface NOTCH3 expression (Figure 6C). These results suggest that GFI1 and IKAROS

230   collaborate to transactivate the *NOTCH3* gene, and that GFI1 does so, at least in part, though residues that

231   are also important to recruit LSD1. To more directly determine the role of LSD1 in GFI1-mediated

232   NOTCH3 induction in CCRF-CEM cells, we utilized the non-competitive LSD1 inhibitor, SP-2509 (Fiskus,

233   Sharma et al., 2017, Inui, Zhao et al., 2017). Surprisingly, NOTCH3 expression significantly increased in

234   cells treated with SP-2509 compared to DMSO vehicle control even without doxycycline treatment (Figure

235   6D). Doxycycline-induced GFI1 expression cooperated with SP-2509 to further increase NOTCH3

236   expression at 24 but not 48 hr (Figure 6D). We also obtained similar results using SUP-T1, a different T-

237   ALL cell line. Doxycycline-induced GFI1 expression or LSD1 inhibition with SP-2509 elevated surface

238   levels of NOTCH3 (Figure 7A).

239   Notably, results obtained using SNAG domain mutants (ΔSNAG and P2A) are the reverse of those

240   obtained with SP-2509, where the SNAG domain remains intact. We hypothesized this positive impact of

241   SP-2509 could represent targeted disruption of the interaction between LSD1 and the SNAG domain to

242   enable alternative interactions involving candidate co-activators. Small molecule inhibitors of LSD1 can

243   disrupt binding between GFI1 and LSD1(Maiques-Diaz, Spencer et al., 2018). Further, we have previously

244   shown the SNAG domain is sufficient for LSD1 binding and that dimethyl modification at lysine 8 (K8me2)

245   of the SNAG domain strongly favors LSD1binding in an *in vitro* binding assay. We deployed this assay

246   (Figure 7B, left panel) to test the impact of SP-2509 on SNAG—LSD1 binding. We find that SP-2509

247   completely abolishes binding between LSD1 and a biotinylated K8me2-SNAG peptide (Figure 7B, right

248   panel). These results suggest the SNAG—LSD1 interaction can be modulated to enable competition

249   between LSD1 and one or more unknown proteins for SNAG domain binding and regulation of GFI1-

250   mediated transcriptional output.

251   We then tested the effect of IKAROS loss on GFI1-induced NOTCH3 expression using GFI1-

252   3×FLAG CCRF-CEM cells. Lenalidomide is well-documented to rapidly and efficiently trigger

253   degradation of IKAROS (Lu, Middleton et al., 2014). Induction of cell surface NOTCH3 expression was

254   significantly impaired by Lenalidomide treatment compared with DMSO control (Figure 7C).

255   Immunoblotting confirmed the rapid loss of IKAROS protein and unaltered expression of ectopic GFI1 in

256   the presence of Lenalidomide (Supplementary Figure S3A-B). We obtained similar results using the same

257   cells instead electroporated with IKAROS CRISPR RNPs (Figure 7D, Supp. Figure 3C-D). These results

258   strongly suggest that IKAROS and GFI1 act in a cooperative fashion to transactivate a specific subset of

259   genes which includes *NOTCH3*, and given the tumor promoting role of constitutively active NOTCH3

260   could provide a new direction for therapeutic development in T-ALL (Figure 8).

Discussion

Identifying the molecular determinants of T cell development furthers understanding of T-ALL pathogenesis (Rothenberg & Taghon, 2005). The zinc finger transcription factor GFI1 plays critical roles in myeloid and lymphoid development and is an important pro-survival factor in T-ALL (Khandanpour et al., 2013, van der Meer, Jansen et al., 2010). The protein partnerships on which GFI1 depends for this role may offer potential therapeutic targets in T-ALL, but are as yet ill-defined. Here, using an unbiased quantitative proximity labeling approach, we identify GFI1 interacting proteins in T-ALL cells. Among these, IKAROS serves as a cooperating partner for GFI1-mediated gene regulation. Functional studies using T-ALL cells reveal a previously unrecognized role for GFI1 in transcriptional activation of genes that are among its targets, and several of which are involved in T cell development.

GFI1 and GFI1B have been largely described as a transcriptional repressors, and indeed can substitute for one another *in vivo*. Repression requires a highly conserved 20-amino acid N-terminal SNAG domain capable of recruiting LSD1 complexed with CoREST (Saleque, Kim et al., 2007). In previous studies, point mutations or truncations in the SNAG domain that disrupt GFI1/1B—LSD1 binding impair GFI1/1B functions in multiple assays, pinpointing LSD1 as a central cofactor in GFI1/1B-mediated transcriptional repression and the establishment of downstream phenotypes (Grimes et al., 1996, McClellan et al., 2019, Saleque et al., 2007, Velinder et al., 2017). However, it is not clear whether GFI1/1B can regulate gene expression through other mechanisms, especially in cooperation with other factors. We applied BioID proximity labeling to identify GFI1-interacting proteins where the promiscuous biotin ligase (BirA*) (Kim, Jensen et al., 2016, Roux, Kim et al., 2012) is fused to GFI1. Relative to co-immunoprecipitation, this method has the advantage that transient and indirect interactions can be efficiently captured. We utilized this method together with human CCRF-CEM T-ALL cells to systematically label proteins spatially close to GFI1, identifying GFI1 itself and some 500 direct or indirect interacting partners. Among these are previously identified GFI1-interacting proteins HMG20B, STAG1 and components of the BHC complex including LSD1, CoREST, and GSE1. We segregated interacting proteins into functional groups and analyzed their network relationships. Through this analysis we

287   identified IKAROS, encoded by the *IKZF1* gene, as a top hit. IKAROS is also a zinc finger transcription

288   factor that plays multiple, key roles in hematopoiesis, including in the development of B and T cells

289   (Georgopoulos et al., 1994, Georgopoulos, Moore et al., 1992, Hahm, Ernst et al., 1994). As with GFI1,

290   IKAROS has also been linked to mouse and human T-ALL. In T-ALL, IKAROS functions as a tumor

291   suppressor, and its deletion is a poor prognostic indicator for affected patients (Marcais, Jeannet et al., 2010,

292   Sun, Crotty et al., 1999, Winandy, Wu et al., 1995). This impact on prognosis takes on a new dimension

293   when considered in the context of the GFI1—IKAROS partnership.

294   GFI1 and IKAROS do not co-precipitate in conventional co-IP assays, suggesting their interaction

295   is indirect and transient in nature. Biotin transfer between the two proteins depends on DNA binding but

296   does not depend on the GFI1 SNAG domain. Because GFI1 and IKAROS are both sequence-specific DNA

297   binding proteins, we performed ChIP-Seq to identify potential common target genes. This effort

298   identified >25,000 GFI1-bound and >50,000 IKAROS-bound peaks, and with considerable overlap. The

299   large number of IKAROS peaks is consistent with previous reports for IKAROS binding in human K562

300   and myeloma cell lines (Barwick, Neri et al., 2019, Consortium, 2012) and may be attributable to its ability

301   to bind DNA in multimeric configurations(McCarty, Kleiger et al., 2003, Molnar, Wu et al., 1996).

302   Approximately 80% (19966/25674) of GFI1 bound peaks overlap with IKAROS peaks, supporting the idea

303   that the two proteins frequently coregulate target gene expression. Co-occupied targets include *GFI1* itself,

304   as well as *NOTCH3*, the *CD3* gene cluster (*CD3E*, *CD3D* and *CD3G*), *TCF3*, *MYC*, *RUNX3*, *MYB* and

305   *HES1*, each with fundamental roles in T-cell development. Interplay between GFI1 and other transcription

306   factors has been described. For example, GFI1 and HOXA9 compete for overlapping binding sites at a

307   distinct class of targets in AML (Velu, Chaubey et al., 2014). Unlike HOXA9, our results suggest that

308   IKAROS cooperates with GFI1.

309   RNA-Seq gene expression profiling using CCRF-CEM cells with ectopic GFI1 expression and/or

310   with IKAROS knockout revealed a group of genes positively regulated by both proteins. Among these

311   genes are *NOTCH3* and *RAG1*, while other NOTCH family members *NOTCH1*, *NOTCH2*, and *NOTCH4*

312   were not affected by enforced expression of GFI1. This indicates *NOTCH3* is the specific NOTCH gene

313   targeted for induction by the GFI1—IKAROS partnership. Moreover, genes making up this group are

314   enriched for GFI1 and IKAROS occupancy, strongly suggesting they are direct targets and that the positive

315   effects of the GFI1—IKAROS partnership on transcription are also direct in nature.

316   In CCRF-CEM cells *NOTCH1*-activating mutation and *FBXW7* loss-of-function drive constitutive

317   NOTCH signaling (O'Neil, Grim et al., 2007). Similarly, SUP-T1 cells carry a t(7;9) translocation driving

318   expression of a truncated NOTCH variant to produce constitutive, ligand-independent NOTCH activity

319   (Reynolds, Smith et al., 1987). Using both cell lines, we find that GFI1 and IKAROS promote NOTCH3

320   cell surface expression. Moreover, mutations in the GFI1 SNAG domain that block the interaction with

321   LSD1 also render GFI1 incapable of inducing NOTCH3. It is not clear whether LSD1 itself is important

322   for this activation (e.g., through demethylating repressive protein modifications such as H3K9 methylation),

323   or whether interactions between the SNAG domain and some other positively-acting cofactor become

324   dominant in the case of GFI1—IKAROS coregulated genes. Interestingly, SP-2509 and SNAG mutants,

325   both of which disrupt GFI1—LSD1 binding, yield qualitatively opposite results in NOTCH3 cell surface

326   expression. Because SP-2509 leaves SNAG domain primary structure intact, our findings could indicate

327   that LSD1 and an as yet unidentified co-activator compete for SNAG domain binding in an IKAROS-

328   dependent manner. To address this possibility, we used lenalidomide to acutely abolish IKAROS expression.

329   Lenalidomide is a thalidomide derivative that is active in multiple myeloma by enabling targeted

330   degradation of IKAROS (Lu et al., 2014). We find that acute IKAROS depletion blunts GFI1-mediated

331   transactivation and cell surface expression of NOTCH3, consistent with GFI1 and IKAROS providing a

332   platform for SNAG-dependent recruitment of a transcriptional co-activating principle, leading to

333   transactivation of a gene expression program represented by the *NOTCH3* response.

334   Cumulatively, the results support a model in which the presence of IKAROS allows for activation

335   of target genes by GFI1. This model is consistent with the identification of SWI/SNF, a chromatin

336   remodeling complex associated with positive regulation of gene expression (Hirschhorn, Brown et al., 1992,

337   Imbalzano, Kwon et al., 1994, Kwon, Imbalzano et al., 1994, Peterson & Herskowitz, 1992), as a GFI1

338   proximity partner. An activating potential for both IKAROS and GFI1 has been described previously.

339   IKAROS binds and activates *Cd3d* through an upstream enhancer region (Georgopoulos et al., 1994,

340   Georgopoulos et al., 1992). CD3 proteins form a central component of the T cell receptor signaling complex

341   (Ngoenkam, Schamel et al., 2018). Positive regulation of gene expression by GFI1 was found in mouse

342   granulocyte-monocyte precursor cells (GMPs) undergoing a binary monocyte/granulocyte fate decision. In

343   GMPs it was shown that GFI1 associates with granulocyte-specific targets such as *Per3* and *Ets1*, activating

344   their expression as part of a broader granulocyte fate-specifying program. Concurrently, GFI1 binding and

345   repression of monocyte-specific genes suppresses the monocyte program (Olsson, Venkatasubramanian et

346   al., 2016). It seems reasonable to consider that concurrent activation and repression of opposing gene

347   expression programs, such as those exemplified by the GFI1—IKAROS—LSD1 relationship could direct

348   alternative outcomes in developmental hematopoiesis. More work is necessary to define the molecular

349   mechanisms by which GFI1 switches between repressive and activating transcriptional potential to control

350   these binary fate decisions.

Materials and Methods

Cells and Culture conditions

CCRF-CEM and SUP-T1 cells were purchased from ATCC. Both cell lines were cultured in RPMI 1640 medium, 10% fetal bovine serum, 2 mM GlutaMAX-I, 100 U/mL penicillin and 100 μg/mL streptomycin. All cell culture materials were purchased from ThermoFisher Scientific, Waltham, MA.

Antibodies

Antibodies used for immunoblotting and immunoprecipitation were as follows: anti-LSD1 (CST, C69G12, 2184S); anti-CoREST (CST, D6I2U,14567S); anti-IKAROS (ThermoFisher Scientific, PA5-23728); anti-β-actin (Santa Cruz, C4, sc-47778); anti-α-tubulin (Santa Cruz, B-7, sc-5286); anti-FLAG (Sigma, M2, F1804); anti-HA antibody (Roche, 12CA5, 11583816001). Streptavidin-HRP was purchased from GE Healthcare (RPN1231).

Constructs and Cloning

In-frame GFI1 fusion proteins with BirA* were created by subcloning GFI1 from constructs in our previous study (Velinder et al., 2017) into the *Eco*RI and *Bam*HI sites of the MCS-BioID2-HA vector (a gift from Kyle Roux, Addgene #74224). Subsequently, GFI1-BirA*-HA PCR products were cloned into the *Not*I and *Eco*RI sites of the pLVX-Tight-Puro vector (Clontech Laboratories, Inc). We used the rat GFI1 ortholog for our experiments, which is >99% conserved at the amino acid level with human GFI1. IKZF1/IKAROS constructs were based on the human form. The N383S derivative of rat GFI1 is analogous to the N382S mutation in human GFI1.

Lentivirus Packaging

To generate lentiviral particles, HEK293T cells were transfected with lentiviral packing vectors, viral packaging (psPAX2) and viral envelope (pMD2G) constructs at a 4:2:1 ratio with 1 mg/mL polyethylenimine (PEI, linear MD 25,000 Da, Sigma, Cat# 408727). The ratio of total transfected DNA to

377   PEI was 1:3 (1 µg DNA:3 µg PEI). After 24 hr, 20 mL of fresh cell culture medium was added and the cells

378   were incubated for an additional 24 hr. Then the culture medium was replaced with virus collection medium

379   (culture medium with 20mM HEPES). Viral supernatants were collected after a further 8 and 24hr. The two

380   supernatants were combined and passed through a 0.45µm filter. Virus was directly used for infection or

381   stored at -80°C.

382

383   **BioID proximity purification and proteomic analysis (MassIVE File Identifier MSV000086405)**

384   CCRF-CEM cells were transduced with Tet-On lentiviral vectors (Lenti-XTM Tet-On Advanced, Clontech

385   Laboratories, Inc) and selected with 500 µg/mL G418 to generate CCRF-CEM-Tet-On cells. CCRF-CEM-

386   Tet-On cells were transduced with empty vector, BirA*-HA or GFI1-BirA*-HA viruses (pLVX-Tight-Puro,

387   pLVX-Tight-puro-BirA*-HA or pLVX-Tight-Puro-GFI1-BirA*-HA, respectively) and selected with 0.5

388   µg/mL puromycin to generate CCRF-CEM cells expressing doxycycline-inducible GFI1-BirA* fusion

389   proteins or BirA*. Cells were treated with 1 µg/mL doxycycline for 48 hr and 20 µM biotin for 16 hr. Cells

390   were lysed in buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 1% Triton X-100) and

391   incubated with streptavidin-Sepharose High Performance beads (Sigma, GE17-5113-01) on ice for 16hr.

392   After washing the beads five times with lysis buffer on ice, proteins were eluted with 2× Laemmli Sample

393   Buffer (LSB, 65.8 mM Tris-HCl pH 6.8, 26.3% (w/v) glycerol, 2.1% SDS, 0.01% bromophenol blue) and

394   boiled for 10 min. Proteins were resolved by SDS-PAGE. Whole lanes of SDS-PAGE gel were cut out and

395   subjected to LTQ Orbitrap Velos Pro ion-trap mass spectrometry (ThermoFisher Scientific, Waltham, MA).

396   Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment

397   ions for each peptide. Protein identity was determined by matching protein databases with the acquired

398   fragmentation pattern by the software program, Sequest (ThermoFisher Scientific, Waltham, MA). All

399   databases include a reversed version of all the sequences and the data was filtered to a 1-2% peptide false

400   discovery rate.

401

ChIP-Seq (Gene Expression Omnibus Series record GSE160183)

The CCRF-CEM-Tet-On cells described above were infected with GFI1-3×FLAG or IKAROS-3×FLAG viruses (packaged with pLVX-Tight-puro-GFI1-3×FLAG or pLVX-Tight-puro-IKAROS-3×FLAG plasmids). Cells were selected with 1µg/mL puromycin to produce CCRF-CEM stable cell lines inducibly expressing GFI1-3×FLAG and IKAROS-3×FLAG. These cells were treated with 1µg/mL doxycycline for 24 hr. 20 million CCRF-CEM cells were crosslinked using a final concentration of 1% formaldehyde in the medium for 10 min. Crosslinking was quenched with 0.125 M glycine for 2 min. Cells were washed with ice-cold PBS and Farnham lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, 1 mM PMSF and 10µg/mL Aprotinin pH 8.0). Cells were resuspended in 1 mL RIPA lysis buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF and 10 µg/mL Aprotinin pH 8.0). Cells were sonicated to shear the DNA to between 200 and 500 bp. Fifty µL of sonicated chromatin was saved. The leftover samples were precipitated with anti-FLAG antibody which was prebound to Protein G Dynabeads (Thermo Fisher). The 50 µL of saved chromatin and immunoprecipitated DNA were de-crosslinked in a 65°C water bath overnight. De-crosslinked samples were purified using a Zymo ChIP DNA clean & concentrator (Zymo Research). Five to 10 ng of precipitated DNA was used for library construction using NEBNext ChIP-Seq Library Prep Reagent Set. Sequencing libraries (25 pM) were chemically denatured and applied to an Illumina HiSeq v4 single read flow cell using an Illumina cBot. Hybridized molecules were clonally amplified and annealed to sequencing primers with reagents from an Illumina HiSeq SR Cluster Kit v4-cBot (GD-401-4001). Following transfer of the flow cell to an Illumina HiSeq 2500 instrument (HCSv2.2.38 and RTA v1.18.61), a 50-cycle single-read sequence run was performed using HiSeq SBS Kit v4 sequencing reagents (FC-401-4002).

ChIP-Seq analysis

SAM alignments were generated from Illumina Fastq files aligned to human hg38 genome using Novocraft's novoalign aligner (http://www.novocraft.com) with the following parameters: -o SAM –r

427 Random. Peak calling was then performed using macs2 (https://github.com/taoliu/MACS, v2.1.1.20160309)

428 with the following settings: -g 2.7e9 –call -summit –f BAMPE –nomodel –B –SPMR –extsize 200.

429 Generated bedgraph files were then transformed to bw format using UCSC bedGraphToBigWig application

430 (v4). Heatmap clustering of ChIP-SEQ was carried out using deepTools (v3). Matrix was generated with

431 computeMatrix application using the following parameters: computeMatrix -S input_1.bw input_2.bw -R

432 peaks.bed–outFileName out.matrix–referencePoint center -a 10000 -b 10000 -bs 100 –sortRegions descend.

433 Correlation heatmap was generated using the deep Tools plot Heatmap application. PCA analysis was

434 performed using the deepTools plotPCA application. The peaks.bed was generated by combining peaks

435 from two ChIP-Seq experiments. plotHeatmap application with default settings was then used to plot

436 heatmap. Peak regions were further used for motif finding analysis, which was carried out using the

437 findMotifGenome.pl application (v4.8.3, HOMER, http://homer.ucsd.edu/homer/motif/) with default

438 settings. This resulted in between 92.6M and 86.4M reads, wherein between 81.8M and 73.6M (88.3 and

439 85.2%) uniquely mapped to the *Hg38* genome build.

440

441 ***IKAROS/IKZF1* knockout**

442 Nonspecific or *IKZF1*-specific CRISPR/Cas9 RNPs were generated from tracrRNA-ATTO550 (IDT),

443 crRNA (IDT) and Cas9 protein (QB3 MacroLab, UC Berkeley) using commercial guidelines (IDT) and

444 transfected into GFI1-3×FLAG CCRF-CEM cells by electroporation using the Neon transfection system

445 10 μL kit (ThermoFisher Scientific). Electroporation parameters were 1500V, 10 ms pulse width, 3 pulses.

446 Transfection efficiency was measured by flow cytometry to detect the tracrRNA-ATTO550 positive cells.

447 Approximately 95% transfection efficiency was obtained. Cells were cultured for >9 days to confirm stable

448 ablation of IKAROS protein by Western blotting. CRISPR guide RNAs were chosen targeting the 5' exons

449 of the *IKZF1* gene using the IDT predesigned CRISPR guide RNA database

450 (https://www.idtdna.com/site/order/designtool/index/CRISPR_PREDESIGN): *IKAROS/IKZF1*:

451 TCATCTGGAGTATCGCTTACagg; GACCTCTCCACCACCTCGGGagg;

452   CTCCAAGAGTGACAGAGTCGtgg. CRISPR/Cas9 negative control crRNA (IDT, 1072544) was used as

453   a control for transfection.

454

455   **RNA-Seq (Gene Expression Omnibus Series record GSE160183)**

456   Total RNA was extracted from GFI1-3×FLAG CCRF-CEM cells treated with or without doxycycline for

457   24 hr, and with IKAROS or control CRISPR RNP knockout using the RNeasy Mini kit (Qiagen) and RNase-

458   Free DNase Set (Qiagen). RNA integrity numbers (RIN) ranged from 9.4 to 9.9. Poly(A) RNA was purified

459   from total RNA samples (100-500 ng) with oligo(dT) magnetic beads followed by library construction

460   using the Illumina TruSeq Stranded mRNA Library Prep kit and TruSeq RNA UD Indexes. Purified

461   libraries were qualified on an Agilent Technologies 2200 TapeStation using a D1000 ScreenTape assay.

462   The molarity of adapter-modified molecules was defined by quantitative PCR using the Kapa Biosystems

463   Kapa Library Quant Kit. Individual libraries were normalized to 1.30 nM in preparation for Illumina

464   sequence analysis. NovaSeq 2×50 bp Sequencing_100 M Read-Pairs Sequencing libraries (1.3 nM) were

465   chemically denatured and applied to an Illumina NovaSeq flow cell using the NovaSeq XP chemistry

466   workflow. Following transfer of the flowcell to an Illumina NovaSeq instrument, a 2×51 cycle paired-end

467   sequence run was performed using a NovaSeq S1 reagent Kit.

468

469   **RNA-seq analysis**

470   Filtering and alignments were performed using the analysis pipeline developed by the Huntsman Cancer

471   Institute   (HCI)   Bioinformatic   Core   Facility

472   (https://huntsmancancerinstitute.github.io/hciRscripts/hciR_scripts.html). Briefly, fastq files were aligned

473   using STAR aligned (v2.7.3a) with the following settings: --twopassMode Basic --outSAMtype BAM

474   SortedByCoordinate --limitBAMsortRAM 64000000000 --outBAMsortingBinsN 100

475 --quantMode TranscriptomeSAM --outWigType bedGraph --outWigStrand Unstranded. The count matrix

476 was then calculated using the Subread FeatureCounts function (v1.6.3) DESeq2 (v1.28.1) was used for

477 differentially expressed genes analysis.

478

479 ***In vitro* SNAG—LSD1 binding assay**

480 Biotinylated, lysine(K)-8 dimethylated SNAG peptide (Bio-K8me2-SNAG) was commercially synthesized

481 and deployed as previously described in CCRF-CEM extracts in either the absence or presence of SP-2509

482 (Velinder et al., 2017). Biotinylated peptides were collected on streptavidin-Sepharose beads whose non-

483 specific binding sites were blocked in 1% bovine serum albumin (BSA) in 1X phosphate buffered saline

484 (PBS). LSD1 was detected by western blotting in pellets and supernatants.

485

486 **Data Availability**

487 ChIP-Seq and RNA-Seq datasets are available at the Gene Expression Omnibus (GEO) website

488 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160183. The following secure token has been

489 created to allow reviewer access of the data while it remains in private status: gjircyuifxgxfaf. Should the

490 manuscript by accepted for publication, the authors will direct GEO to release the data publicly. BioID

491 mass spectrometry data were submitted to MassIVE with MassIVE File Identifier MSV000086405 at

492 website: https://massive.ucsd.edu/ProteoSAFe/private-

493 dataset.jsp?task=ff3728eabdb84663981aafe4e44df11b. The data is still in private status. The following

494 reviewer login credentials will allow reviewers to access. Username :MSV000086405_reviewer; password:

495 a.

496

507

508   Author contributions

509   WS, DM, DB, AP and MC performed the experiments analyzed the data. JG analyzed ChIP-Seq and RNA-

510   Seq data. MEE and DT conceived and supervised the study and interpreted data. BRC provided technical

511   support. All authors were involved in the writing of the manuscript.

512

513   Funding

517

518   Conflict of interest

519   The authors declare no conflicts of interest, direct or indirect, associated with this manuscript.

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687

688   **Figure legends**

689   **Figure 1.** BioID proximity labeling identifies GFI1-interacting proteins. (**A**) Scheme for BioID proximity

690   labeling method to identify GFI1-proximate proteins. Gray circle reflects the radius for biotinylation of

691   nearby molecules such as GFI1 itself and its known binding partner LSD1, as well as unknown proteins A,

692   B and C but not D. (**B**) CCRF-CEM cells inducibly express BirA*-HA alone or as a fusion with GFI1.

693   Biotin-treated CCRF-CEM cells transduced with empty vector (lanes 1-2), BirA*-HA (lanes 3-4), or GFI1-

694   BirA*-HA (lanes 5-6) were treated with doxycycline (Doxy) (+) or vehicle control (-). Whole cell lysates

695   (WCL) were resolved by SDS-PAGE and immunoblotted using HA antibodies. α-tubulin is shown as a

696   loading control. (**C**) Biotinylation of GFI1-associated proteins LSD1 and CoREST is enriched using GFI1-

697   BirA*-HA expressing CCRF-CEM cells relative to empty vector or BirA*-HA controls. Whole cell lysates

698   prepared as in (B) were used for immunoblotting or were first precipitated using streptavidin Sepharose

699   beads (SAv-P), and subsequently analyzed by immunoblotting with anti-HA, anti-LSD1 or anti-CoREST

700   antibodies, or streptavidin-HRP (SAv:HRP) to detect biotinylation.

701

702   **Figure 2.** Top hits of GFI1 proximal partners identified by proteome-wide BioID labeling. (**A**) Volcano

703   plot of proteins identified by BioID proximity labeling. Thresholds (red dashed lines) were fold-

704   difference >2 and *P*-value <0.05. (**Inset**) Magnified portion of (A) depicting significant hits. Known GFI1

705   partners and GFI1 itself are labeled in red. (**B**) Genes labeled in (A) were subjected to STRING (version

706   10.5) functional protein association network analysis. Edges represent protein—protein associations. Blue

707   indicates annotated nuclear proteins. Red indicates annotated proteins associated with gene regulation. The

708   network was clustered using the MCL algorithm with inflation parameter 3. Dotted line indicates the

709   proteins belong to different clusters.

710

711   **Figure 3.** The GFI1—IKAROS interaction requires DNA binding. (**A**) Validation and non-LSD1 binding

712   dependence of the GFI—IKAROS proximity relationship. Biotinylated proteins in whole cell lysates from

713   CCRF-CEM cells transduced with the indicated constructs and treated as in Figure 1B were isolated with

714   streptavidin beads and immunoblotted with anti-IKAROS and anti-HA antibodies targeting the epitope tag

715   in GFI1-BirA* fusion protein variants. (**B**) GFI1—IKAROS binding is not observed in traditional

716   coprecipitation methods. Lysates from CCRF-CEM cells inducibly expressing wild type GFI1-3×FLAG or

717   variants (K8L,P2A and ΔSNAG) were immunoprecipitated with anti-FLAG antibody and Protein G-

718   Sepharose. The presence or absence of IKAROS, CoREST, or GFI1 in the precipitate was detected by

719   immunoblotting with anti-IKAROS, anti-CoREST or anti-FLAG antibodies. 2% input whole cell lysate

720   (WCL) is shown as a control. (**C**) The proximity relationship between GFI1 and IKAROS requires DNA

721   binding activity of both proteins. HEK293T cells were transiently transfected with empty vector or GFI1-

722   BirA*-HA (wild type or N383S) together with human IKAROS-3×FLAG (wild type or N159A) expression

723   constructs. IKAROS and control GFI1 biotinylation was monitored by precipitation with streptavidin beads

724   and IKAROS immunoblotting as described in panel A. A GFI1 immunoblot is shown to confirm equivalent

725   precipitation with streptavidin-Sepharose beads.

726

727   **Figure 4.** Identification of GFI1 and IKAROS targets in CCRF-CEM cells. (**A**) Validation of GFI1 and

728   IKAROS expression in CCRF-CEM cell lines by Western blotting. CCRF-CEM cells expressing GFI1-

729   3×FLAG and IKAROS-3×FLAG were used for ChIP-Seq. (**B**) Venn diagram showing enumeration of

730   unique and shared GFI1 and IKAROS ChIP-Seq peaks. (**C**) Left: GFI1 ChIP-Seq heatmap showing all peak

731   sites, centered by the ChIP peak summit and arranged from strongest to weakest. Right: the same set of

732   peak summits in the same order shown as an IKAROS binding heatmap. (**D**) Example Integrative Genomics

733   Viewer (IGV) tracks of GFI1 ChIP-Seq and input controls (purple), IKAROS ChIP-Seq and input controls

734   (teal), together with previously published RNA-Seq (peach) of CCRF-CEM cells. *NOTCH3*, *GFI1*, *MYC*,

735   *MYB*, *CD3*, *HES1*, *RUNX3* and *TCF3* tracks are shown.

736

737   **Figure 5.** GFI1 and IKAROS positively regulate a subset of target genes. (**A**) IKAROS Western blot of

738   untransfected CCRF-CEM-GFI1-3×FLAG cells, cells electroporated with ATTO550-conjugated control or

739   IKAROS-specific RNPs. 24 hr post-transfection, ATTO550$^+$ cells were sorted and cultured for 9 additional

740   days prior to preparation of lysates. β-actin is shown as a loading control. (**B**) RNA-Seq analysis was

741   performed using the same control RNP- or IKAROS-specific RNP-transfected cells shown in (A) after

742   treatment with vehicle or doxycycline (Doxy) for 24 hr. The top 500 differentially expressed genes (based

743   on *P*-value) were subjected to hierarchical clustering and are shown as a heatmap. Gene groupings with

744   distinct expression patterns (1-5) are highlighted. (**C**) IGV tracks displaying GFI1 and IKAROS ChIP-Seq

745   and RNA-Seq read distribution for *NOTCH3*.

746

747   **Figure 6.** IKAROS cooperates with GFI1 to regulate cell surface NOTCH3 protein expression. (**A**) Left:

748   example flow cytometry histograms showing NOTCH3 surface expression in CCRF-CEM-GFI1-3×FLAG

749   cells treated with doxycycline (Doxy) for the indicated times. Untreated (0 hr) cells are shown as a control.

750   Right: NOTCH3 MFIs from cells in the regions highlighted in the left panels were averaged from four

751   experiments and plotted as a bar graph. Bar colors match the corresponding histograms. Error bars depict

752   SEM. Ordinary one-way ANOVA was used for statistical analysis. In all bar graphs, values for individual

753   data points are represented by closed shapes. (**B**) Left: CCRF-CEM-IKAROS-3×FLAG cells were treated

754   with Doxy for the indicated times. Example NOTCH3 flow cytometry histograms are shown. Right:

755   NOTCH3 MFIs from four independent experiments were averaged and plotted as in (A). Error bars depict

756   SEM. Ordinary one-way ANOVA was used for statistical analysis. (**C**) CCRF-CEM cells transduced with

757   empty vector (V), wild type GFI1-3×FLAG (GFI1), FLAG-tagged GFI1 lacking the N-terminal SNAG

758   domain (ΔSNAG) or FLAG-tagged GFI1 with a SNAG domain point mutant no longer able to interact with

759   LSD1 (P2A). Cells were treated with vehicle (blue) or Doxy (red) for 48 hr. Left: representative flow

760   cytometry histograms for NOTCH3 cell surface expression. Right: average MFIs from four independent

761   experiments. Error bars depict SEM. Two-way ANOVA was used for statistical analysis. (**D**) Flow

762   cytometry of CCRF-CEM-GFI1-3×FLAG cells pre-treated with DMSO vehicle or LSD1 inhibitor (1 μM

763   SP-2509, MedKoo Biosciences) for 20 hr and subsequently treated with Doxy for 24 or 48 hr. DMSO or

764   SP-2509 was present continuously. Left: representative NOTCH3 flow cytometry. Right: MFIs within the

765   NOTCH3-positive gates were averaged from four independent experiments and plotted as a bar graph. Error

766   bars depict SEM. An unpaired T-test was used for statistical analysis.

767

768   **Figure 7**. (**A**) Flow cytometry for NOTCH3 cell surface expression in SUP-T1cells transduced with vector

769   or GFI1-3×FLAG inducible vectors and treated without or with doxycycline (Doxy), and SP-2509 for 48

770   hr. Left: representative NOTCH3 flow cytometry. Right: NOTCH3 MFIs were averaged from four

771   independent experiments and plotted as a bar graph. Error bars depict SEM. An unpaired T-test was used

772   for statistical analysis. In each bar graph, values for each independent test are represented by closed shapes.

773   (**B**) SP-2509 abolishes the SNAG—LSD1 interaction. Biotinylated SNAG peptide demethylated at K8 was

774   added to CCRF-CEM cell extracts with or without SP-2509 and incubated for 1hr prior to addition of

775   streptavidin-Sepharose beads to collect biotinylated peptide and bound LSD1. LSD1 in the bound and

776   unbound fractions were visualized by western blot using anti-LSD1 antibody. (**C**) Flow cytometry of

777   CCRF-CEM-GFI1-3×FLAG cells pretreated with DMSO or 2 μM lenalidomide (LEN) for 16 hr before

778   treatment with Doxy for 48 hr. DMSO or Lenalidomide was present continuously. Left: example NOTCH3

779   flow cytometry. Right: MFIs within the NOTCH3-positive regions shown at left were averaged from four

780   independent experiments and plotted as a bar graph. Bar color corresponds to histogram shading on the left.

781   Error bars depict SEM. Error bars depict SEM. Two-way ANOVA was used for statistical analysis. (**D**)

782   Flow cytometry of CCRF-CEM-GFI1-3×FLAG cells electroporated with control RNP or IKAROS RNPs

783   as in Figure 5A. Cells were treated with Doxy for 0 or 48 hr. Left: example NOTCH3 flow cytometry.

784   Right: MFIs within the NOTCH3-positive regions shown on the left were averaged from four independent

785   experiments and plotted as a bar graph. Error bars depict SEM. Two-way ANOVA was used for statistical

786   analysis.

787

788   **Figure 8. A proposed model of GFI1 mediated-noncanonical transcriptional activation.** In the well-

789   known canonical transcriptional regulation mechanism, GFI1 acts as a transcriptional repressor by

790   recruiting LSD1/CoREST-containing complexes via its SNAG domain. In non-canonical transcriptional

791   activation, GFI1 activates NOTCH3 and other hallmark T cell developmental genes with IKAROS. DNA

792   binding by both proteins is required, as is an intact SNAG domain not bound by LSD1. An LSD1 inhibitor,

793   which blocks SNAG—LSD1 binding enables transactivation of *NOTCH3* and similarly regulated genes via

794   the GFI1—IKAROS partnership**.**

795

796   **Supplementary Figure S1.** ChIP-Seq quality control data and motif analysis. (**A**) Correlation of GFI1 and

797   IKAROS ChIP-Seq (and corresponding input) signaling. Spearman correlation R-values are displayed as a

798   heatmap. (**B**) Principal component analysis (PCA) plot and cluster analysis of all ChIP-Seq samples. (**C**)

799   Motif analysis (HOMER) showing enriched motifs within GFI1 and IKAROS ChIP-Seq peaks. The top 5

800   nonredundant motifs ranked by statistical significance are shown.

801

802   **Supplementary Figure S2.** GFI1 and IKAROS binding and expression data for genes encoding other

803   Notch family members. IGV tracks displaying GFI1 and IKAROS ChIP-Seq and RNA-Seq read

804   distribution similar to Figure 5C, except for *NOTCH1*, *NOTCH2*, and *NOTCH4*.

805

806   **Supplementary Figure S3.** Lenalidomide treatment or CRIPSR RNP transfection rapidly depletes

807   IKAROS protein while maintaining inducible GFI1 expression. (**A**) IKAROS Western blot of

808   Lenalidomide (LEN)-treated CCRF-CEM GFI1-3×FLAG cells. Lysates were prepared from untreated (0

809   hr) cells, and over a 64 hr time course. β-actin is shown as a loading control. (**B**) Lysates from the same

810   cells as in (A) were immunoblotted using GFI1 antibodies. (**C**) IKAROS Western blot using lysates from

811   CCRF-CEM GFI1-3×FLAG cells electroporated with control or IKAROS RNP. Cells were prepared

812   similarly as in Figure 5A. (**D**) Lysates from the same cells as in (C) were immunoblotted using GFI1

813   antibodies.

814

815   **Tables and their legends**

816   **Supplemental Table S1.** BioID mass spectrometry results (average intensity of WT<=VEC and three

817   triplicates T-test (BirA* vs WT) *P*-value >/= 0.05, the data were deleted), 502 proteins.

818

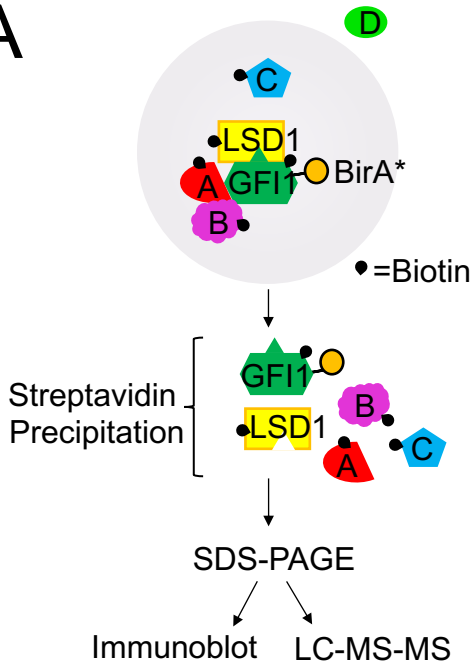819   **Supplemental Table S2.** Gene Ontology (GO) enrichment analysis of GFI1 and IKAROS ChIP-Seq peaks.

820

821   **Supplemental Table S3.** RNA-Seq differential gene expression.

822

823   **Supplemental Table S4.** List of genes within cluster 4 of Figure 5B and distance from TSS to the nearest
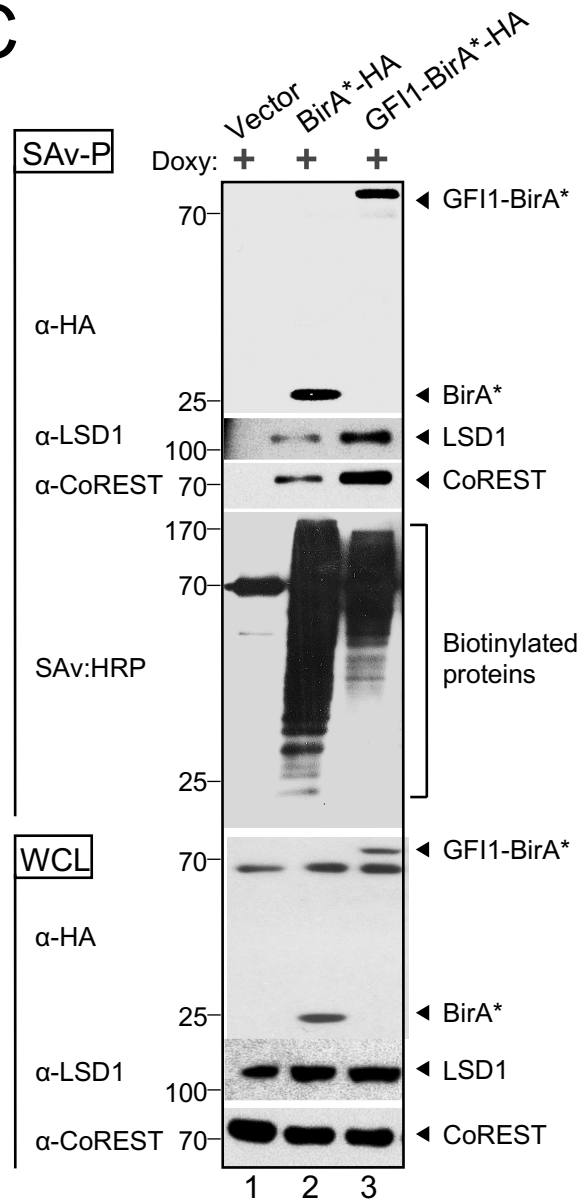
824   GFI1 or IKAROS peak center.
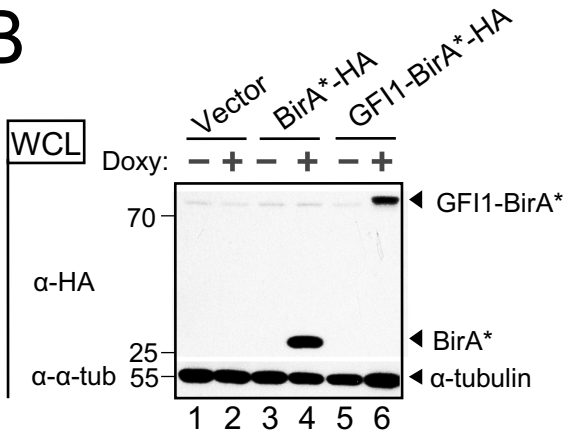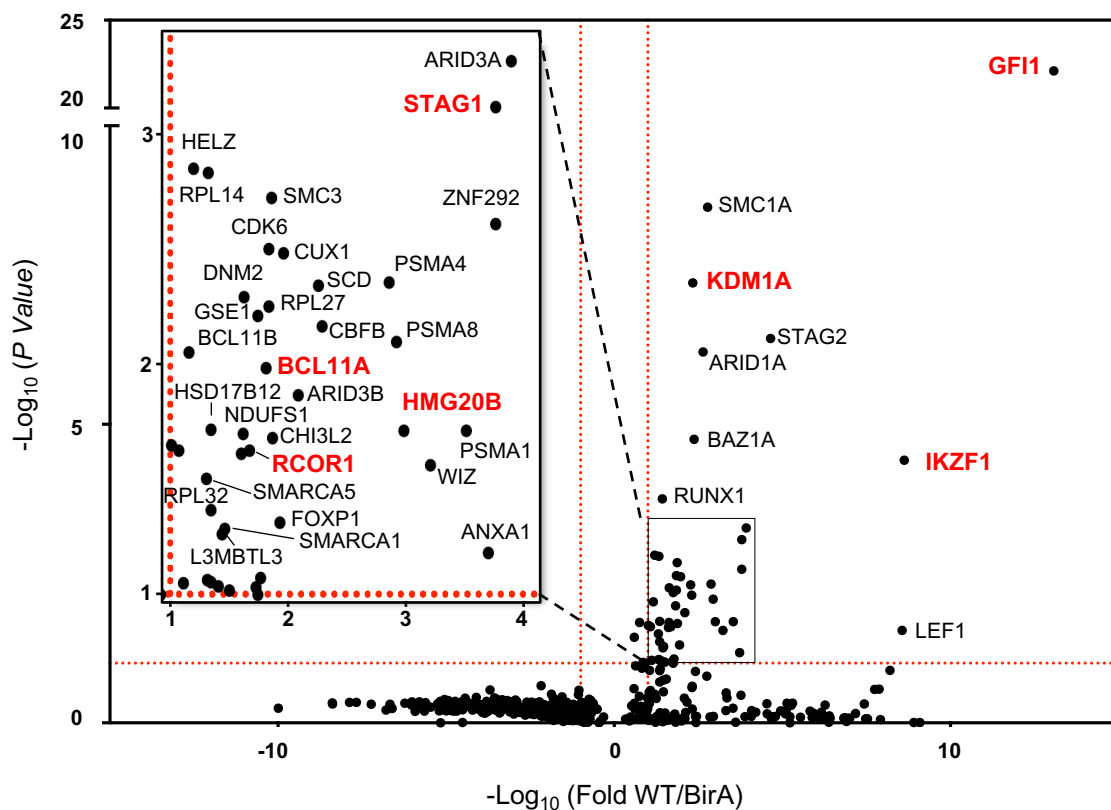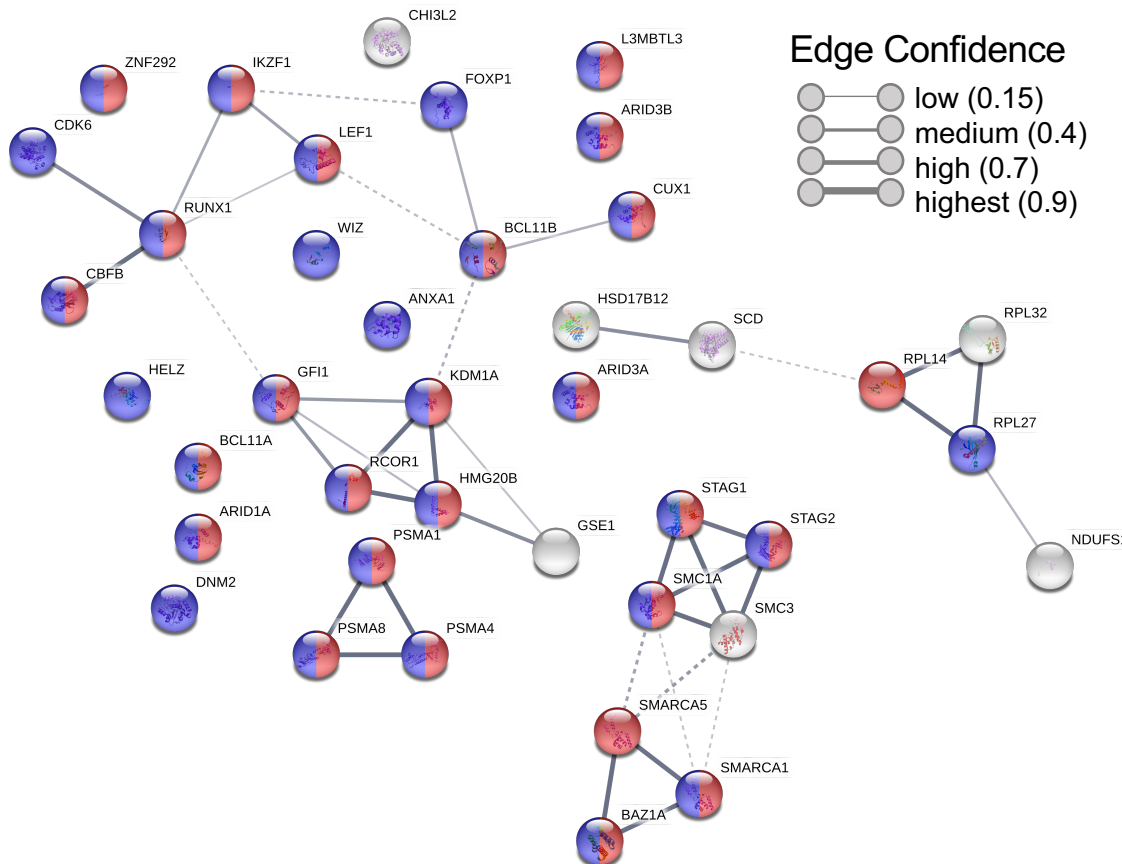
Sun et al. Figure 1

Sun et al. Figure 2

Sun et al. Figure 4

Sun et al. Figure 5

A

GFI1-3X-FLAG

Untransfected, Control RNP, IKAROS RNP

70 — ◄ IKAROS
40 — ◄ β-actin

1 2 3

B

trt

Vehicle/control RNP
Doxy/control RNP
Vehicle/IKAROS RNP
Doxy/IKAROS RNP

1: induced by GFI1, inhibited by IKAROS
2: inhibited by IKAROS
3: inhibited by GFI1, inhibited by IKAROS
4: **induced by GFI1, induced by IKAROS**
5: induced by IKAROS

C

NOTCH3 (46kb)

15,160 kb   15,170 kb   15,180 kb   15,190 kb   15,200 kb

GFI1
IKAROS } ChIP-Seq

Vehicle/control RNP
Doxy/control RNP
Vehicle/IKAROS RNP
Doxy/IKAROS RNP } RNA-Seq

NOTCH3

A

No treatment
Doxy 48h

No treatment
Doxy 48h
SP-2509 48h

B

1. LSD1 binding   2. Complex capture

C

D

Sun et al. Supplemental Figure 1

Sun et al. Supplemental Figure 2
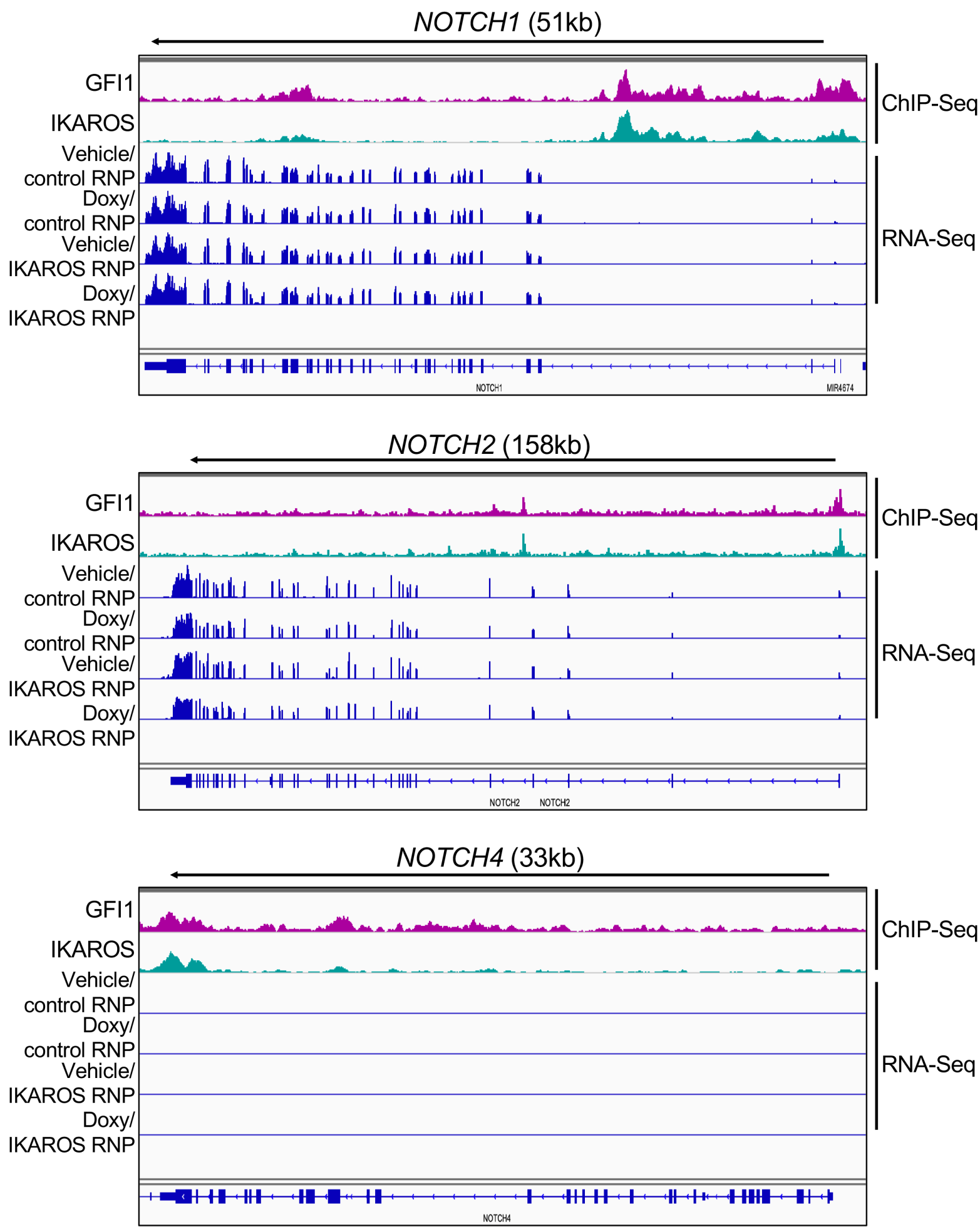
NOTCH1 (51kb)

NOTCH2 (158kb)

NOTCH4 (33kb)

Sun et al. Supplemental Figure 3

A

GFI1-3✕FLAG

LEN: 0 16 40 64 (h)

kDa

70 — IKAROS

40 — ◄ β-actin

1 2 3 4

B

GFI1-3✕FLAG

DMSO LEN

Doxy: 0 48 0 48 (h)

kDa

55 — ◄GFI1

40 — ◄ β-actin

1 2 3 4

C

control IKAROS
RNP RNP

kDa

70 — IKAROS

40 — ◄ β-actin

1 2

D

GFI1-3✕FLAG

control IKAROS
RNP RNP

Doxy: 0 48 0 48 (h)

kDa

55 — ◄GFI1

40 — ◄ β-actin

1 2 3 4
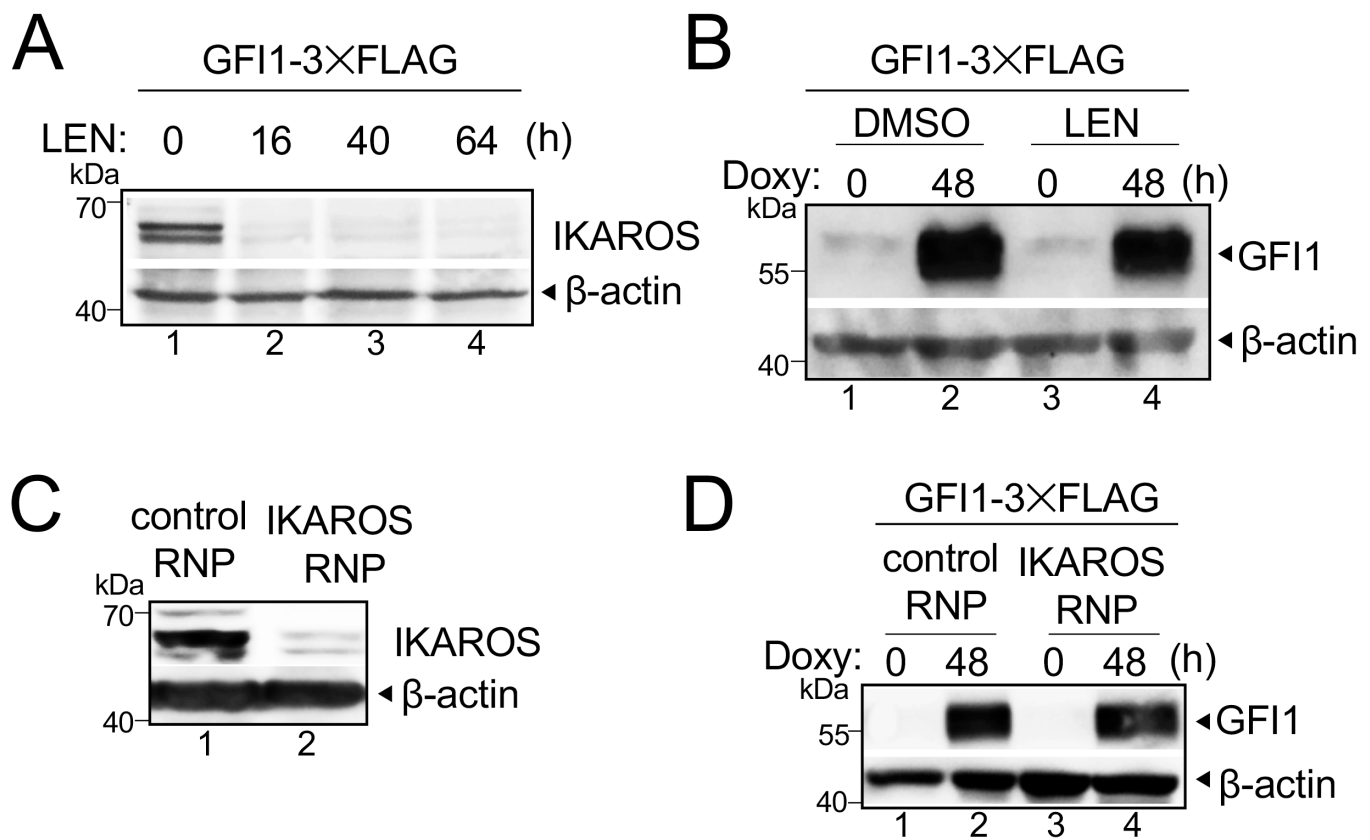
Sun et al. Supplemental Table S2: Gene Ontology (GO) enrichment analysis

Gene Ontology (GO) enrichment analysis with GF		
Rank	Term Name	P-Value
1	regulation of gene expression, epigenetic	7.0764E-85
2	regulation of hematopoietic progenitor cell differentiation	3.4017E-68
3	regulation of hematopoietic stem cell differentiation	4.4564E-62
4	negative regualtion of gene expression, epigenetic	4.3610E-52
5	somatic diversification of immune receptors via germiline recombination within a single locus	1.8269E-40
6	V(D)J recombination	2.1433E-39
7	somatic diversification of immune receptors	5.8618E-39
8	regulation of megakaryocyte differentiation	3.3875E-38
9	DNA replication-dependent nucleosome assembly	7.4252E-38
10	positive regulation of viral life cycle	2.0917E-33
11	positive regulation of gene expression, epigenetic	3.2093E-32
12	chromatin silencing	6.7381E-31
13	positive regualtion of myeloid leukocyte differentiation	6.9143E-31
14	beta-catenin-TCF complex assembly	1.9700E-28
15	chromatin silencing at rDNA	3.6319E-28
16	intrinsic apoptotic signaling pathway in response to DNA damage	4.8943E-27
17	positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway	7.0662E-22

Gene Ontology (GO) enrichment analysis with IKA		
Rank	Term Name	P-Value
1	regulation of hematopoietic progenitor cell differentiation	1.1371E-61
2	regulation of hematopoietic stem cell differentiation	1.8733E-52
3	B cell receptor signaling pathway	4.4539E-40
4	positive regulaiton of mitochondrial outer membrane permeabilizaiton involved in apoptotic signaling pathway	7.3392E-34
5	somatic diversification of immune receptors	1.1375E-32
6	regulation of T cell receptor signaling pathway	3.9032E-26

*The GO analysis was performed using GREAT tool (v 4.0.4) following its default settings.

I1 binding peaks

Fold Enrichment	Observed Gene Hits	Total Genes
2.1208	209	248
2.7850	92	113
2.8136	86	103
2.5319	93	114
2.9966	31	32
3.6440	14	14
2.7361	36	38
2.2967	65	76
7.2147	29	32
2.0164	78	98
2.4171	70	80
2.4221	78	97
2.0172	45	50
2.1595	39	43
4.0207	35	36
2.1653	57	69
2.3287	27	29

AROS binding peaks

Fold Enrichment	Observed Gene Hits	Total Genes
2.1139	107	113
2.0869	97	103
2.0451	35	35
2.079	37	37
2.0353	38	38
2.0342	36	36

Sun et al. Supplemental Table S4: List of genes within cluster 4 of Figure 5B and distance from TSS to th

Gene name
Plectin
SH3 Domain And Tetratricopeptide Repeats 1
Sphingomyelin Phosphodiesterase 3
LIM Zinc Finger Domain Containing 2
BAH Domain And Coiled-Coil Containing 1
Inositol-3-Phosphate Synthase 1
Proline-Rich Protein 36
Tescalcin
ADAMTS-Like Protein 2
Tumor Protein P73
AT-Rich Interactive Domain-Containing Protein 3A
Protein Tyrosine Phosphatase Non-Receptor Type 6
RAS Guanyl Releasing Protein 2
Myosin VIIB
Proteasome 20S Subunit Beta 8
Transporter 1, ATP Binding Cassette Subfamily B Member
RAS P21 Protein Activator 3
MX Dynamin Like GTPase 1
Adhesion G Protein-Coupled Receptor B2
CAMP Responsive Element Binding Protein 3 Like 3
Meteorin, Glial Cell Differentiation Regulator
Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate-Associated Protein 1 (Glutamate Binding
Y-Box Binding Protein 2
Semaphorin 7A
Recombination Activating 1
IL2 Inducible T Cell Kinase
Palladin, Cytoskeletal Associated Protein
Mindbomb E3 Ubiquitin Protein Ligase 1
Notch Receptor 3
Dematin Actin Binding Protein
Calcium And Integrin Binding Family Member 2
Collagen Type XVIII Alpha 1 Chain
Cadherin EGF LAG Seven-Pass G-Type Receptor 2
Hydroxyacylglutathione Hydrolase-Like Protein
Glutamate-Ammonia Ligase
Adenine Nucleotide Translocase Lysine N-Methyltransferase
Insulin Receptor Substrate 1
Protein Kinase CAMP-Activated Catalytic Subunit Beta

e neareast GFI1 or IKAROS peak center.

Gene symbol	TSS (Hg38)	distance to nearest GFI1 peak center (kb)
PLEC	chr8:143,976,745	4.1
SH3TC1	chr4:8,199,244	0
SMPD3	chr16:68,448,506	4.1
LIMS2	chr8:143,976,745	1.8
BAHCC1	chr17:81,399,721	0
ISYNA1	chr19:18,438,301	15.6
PRR36	chr19:7,874,441	69.8
TESC	chr12:117,099,446	0
ADAMTSL2	chr9:9,133,532,164	2.6
TP73	chr1:3,652,565	31.2
ARID3A	chr19:926,037	52.4
PTPN6	chr12:6,946,577	1.7
RASGRP2	chr15:3,856,486	0.5
MYO7B	chr2;127,535,802	8.7
PSMB8	chr6:32,844,935	0.2
TAP1	chr6:32,853,971	0.2
RASA3	chr13:114,132,623	1.2
MX1	chr2:41,420,558	58.7
ADGRB2	chr1:31,764,063	40
CREB3L3	chr19:4,153,601	28.9
METRN	chr16:715,173	5.9
GRINA	chr8:143,990,058	11.5
YBX2	chr17:7,294,559	13.8
SEMA7A	chr15:74,433,959	4.8
RAG1	chr11:36,568,013	0.1
ITK	chr5:157,180,896	0
PALLD	chr4:168,497,066	15.8
MIB1	chr18:21,741,329	1.2
NOTCH3	chr19:15,200,981	0.8
DMTN	chr8:22,048,955	0.2
CIB2	chr15:78,131,535	0.1
COL18A1	chr21:45,405,137	73.3
CELSR2	chr1:109,250,019	35.1
HAGHL	chr16:726,936	5.8
GLUL	chr1;182,392,206	0.4
ANTKMT	chr16:721,142	0
IRS1	chr2:226,798,790	3.9
PRKACB	chr1:84,077,975	0.3

distance to nearest IKAROS peak center (kb)	GFI1 and IKAROS peaks <10 kb	GFI1 and IKAROS peaks
4.2	Yes	No
0.5	Yes	Yes
4.2	Yes	No
1.7	Yes	No
0	Yes	Yes
8.3	No	No
5.8	No	No
0.1	Yes	Yes
1.4	Yes	No
24.7	No	No
0.3	No	No
2.2	Yes	No
0.2	Yes	Yes
9.9	Yes	No
0.2	Yes	Yes
0.2	Yes	Yes
0.8	Yes	No
0.1	No	No
6.9	No	No
13.1	No	No
0.1	Yes	No
3.7	No	No
0.4	No	No
0.2	Yes	No
0.1	Yes	Yes
0	Yes	Yes
12	No	No
0.3	Yes	No
0.6	Yes	No
0.1	Yes	Yes
0.1	Yes	Yes
8.6	No	No
33.2	No	No
0.2	Yes	No
0.2	Yes	Yes
0	Yes	Yes
0.9	Yes	No
0.2	Yes	Yes

<500 bp