

# **EWS-FLI1 regulates and cooperates with core regulatory circuitry in**

## **Ewing sarcoma**

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29 **Abstract**

30 Core regulatory circuitry (CRC)-dependent transcriptional network is critical for  
31 developmental tumors in children and young adults carrying few gene mutations.  
32 However, whether and how CRC contributes to transcription regulation in Ewing  
33 sarcoma is unknown. Here, we identify and functionally validate a CRC "trio"  
34 constituted by three transcription factors (TFs): KLF15, TCF4 and NKX2-2, in  
35 Ewing sarcoma cells. Epigenomic analyses demonstrate that EWS-FLI1, the  
36 primary fusion driver for this cancer, directly establishes super-enhancers of each  
37 of these three TFs to activate their transcription. In turn, KLF15, TCF4 and  
38 NKX2-2 co-bind to their own and each other's super-enhancers and promoters,  
39 forming an inter-connected auto-regulatory loop. Functionally, CRC factors  
40 contribute significantly to cell proliferation of Ewing sarcoma both *in vitro* and  
41 *in vivo*, and are all overexpressed in this cancer. Mechanistically, CRC factors  
42 exhibit prominent capacity of co-regulating the epigenome in cooperation with  
43 EWS-FLI1, occupying 77.2% of promoters and 55.6% of enhancers genome-  
44 wide. Downstream, CRC TFs coordinately regulate gene expression networks in  
45 Ewing sarcoma, directly controlling important signaling pathways for cancer,  
46 such as lipid metabolism pathway, PI3K/AKT and MAPK signaling pathways.  
47 Together, molecular characterization of the oncogenic CRC model advances our  
48 understanding of the biology of Ewing sarcoma. Moreover, this study identifies  
49 CRC-downstream genes and signaling pathways, which may contain potential  
50 targets for therapeutic intervention for this malignancy.

## 51 **Introduction**

52 As a developmental cancer carrying few genetic alterations, Ewing sarcoma is  
53 the second most common malignancy of bone and soft tissue predominantly  
54 occurring in adolescents and young adults (1). EWS-FLI1, the primary fusion  
55 driver, rewires fundamentally the transcriptome of Ewing sarcoma cells (2-5).  
56 Nevertheless, in many cases, EWS-FLI1 requires the cooperation of other  
57 transcriptional cofactors, such as WDR5 and CBP/p300, to regulate chromatin  
58 modification and gene expression (6-8). Moreover, EWS-FLI1-targeting  
59 transcription factors (TFs), such as MEIS1, NKX2-2, SOX2, and OTX2 (7, 9-11),  
60 are required for the fusion driver to fulfil its oncogenic function in Ewing sarcoma  
61 cells. Thus, despite having immense capacity in chromatin regulation, EWS-FLI1  
62 still relies on additional cooperators and mediators to orchestrate gene expression  
63 programs in Ewing sarcoma. Hence, comprehensive and unbiased  
64 characterization of such partners and mediators is critical for further  
65 understanding the biology of Ewing sarcoma.

66 TFs coordinate cell type-specific transcriptional programs typically through  
67 regulating distal cis-regulatory elements, including enhancers and super-  
68 enhancers. Although hundreds of TFs are expressed to some extent in any given  
69 cell type (12), only a handful appear to be critical for establishing and maintaining  
70 cell type-specific gene expression network (13-15). Notably, this small set of TFs  
71 (sometimes called master TFs) often form a “Core Regulatory Circuitry (CRC)”  
72 (16-18), wherein each TF not only self-regulates but also co-regulates each other

73 by directly co-binding to their super-enhancers. Although such CRC  
74 transcriptional paradigm has been identified in both normal and neoplastic cell  
75 types (19, 20), its functionality seems to be particularly critical for developmental  
76 tumors in children and young adults who carry few somatic genomic alterations.  
77 Indeed, some of the best characterized CRC models are established from  
78 neuroblastoma, medulloblastoma, as well as PAX3-FOXO1<sup>+</sup> rhabdomyosarcoma,  
79 all of which have “quiet” genomic landscapes compared with adulthood tumors  
80 (21-23). For example, in MYCN-amplified neuroblastoma, a set of master TFs  
81 (HAND2, ISL1, PHOX2B, GATA3 and TBX2) assemble a functional CRC to  
82 orchestrate the unique gene expression program in this cancer type (22).  
83 Moreover, in PAX3-FOXO1-driven rhabdomyosarcoma, the fusion protein  
84 exploits super-enhancers to set up a CRC machinery in collaboration with the  
85 master TFs (MYOG, MYOD and MYCN); this CRC is addicted by  
86 rhabdomyosarcoma cells for survival and proliferation (23). Considering that  
87 Ewing sarcoma resembles these developmental cancers in terms of having both  
88 few genomic alterations and a single epigenomic driver, we postulated that such  
89 oncogenic CRC model might exist in Ewing sarcoma, to cooperate with the  
90 transcriptional function of EWS-FLI1. The current study was aimed to identify  
91 and characterize this CRC apparatus in Ewing sarcoma, and to elucidate its  
92 functional significance in this cancer.

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94

## 95 **Results**

### 96 **Identification of CRC under the regulation of EWS-FLI1 in Ewing sarcoma**

97 We recently characterized the super-enhancer landscape in Ewing sarcoma and  
98 confirmed the essential role of EWS-FLI1 in regulating the epigenome of this  
99 cancer (11). As introduced earlier, considering that CRC is particularly important  
100 for childhood developmental cancers having few genomic lesions, we postulated  
101 that such CRC model might also contribute to regulating Ewing sarcoma  
102 transcriptome through either cooperating with or mediating the function of EWS-  
103 FLI1. To test this hypothesis, we first sought to identify mathematically master  
104 TFs with high inter-connectivity through binding to their super-enhancers by  
105 motif scanning using our established method (18, 24, 25). Because of the central  
106 role of EWS-FLI1 in establishing the enhancer landscape in Ewing sarcoma, we  
107 made significant modifications of the method by requiring that all candidate TFs  
108 have both EWS-FLI1 binding motif and binding peaks in their assigned super-  
109 enhancers. We initially focused on the A673 cell line, since it is a well-  
110 characterized Ewing sarcoma line with available H3K27ac and EWS-FLI1 ChIP-  
111 Seq results (7). As a result, a small set (n=9) of CRC candidates were identified  
112 (Fig. 1A), including NKX2-2 and FOS which are known functional cooperators  
113 of EWS-FLI1 (7, 26). Because CRC factors have high and specific expression in  
114 their corresponding cell types, we interrogated the Cancer Cell Line Encyclopedia  
115 (CCLE) dataset and noted that, compared with other 5 CRC candidates (NFATC2,  
116 FOS, IRF2, ZBTB7B and MEF2D, Supplement Fig. 1), the expression of 4

117 candidate TFs (KLF15, NKX2-2, TCF4 and RREB1) showed restricted  
118 expression pattern in Ewing sarcoma cell lines (defined as top 5 among all cell  
119 types, Fig. 1B). However, it should be noted that the specificity of RREB1  
120 expression was overall poor, as most cancer types had comparable mRNA levels.

121 As a parallel analysis, Pearson correlation coefficient of the mRNA levels of  
122 these 9 candidates was determined, considering the co-regulatory relationship  
123 between CRC members. Notably, the same set of 4 factors (KLF15, NKX2-2,  
124 TCF4 and RREB1) displayed strong positive correlations with each other (Fig.  
125 1C). In contrast, this correlation pattern was much weaker in other non-Ewing  
126 sarcoma bone cancer cell lines (Fig. 1C, right panel).

127 Based on these 4 candidates, we next sought to reconstruct functionally the  
128 CRC model and to determine their regulatory relationship with EWS-FLI1, by  
129 silencing of either EWS-FLI1 or each candidate individually. Knockdown of  
130 EWS-FLI1 strongly decreased mRNA expression of KLF15, NKX2-2 and TCF4,  
131 but not RREB1 (Fig. 1D). In contrast, the opposite effect was not observed, i.e.,  
132 knockdown of candidate TFs had no impact on the level of EWS-FLI1,  
133 suggesting that the CRC is under the control of EWS-FLI1. Within the 4 CRC  
134 candidates, knockdown of either KLF15, NKX2-2 or TCF4 decreased the  
135 expression of the other two (Fig. 1D), but not RREB1. On the other hand,  
136 RREB1-silencing did not produce these co-regulatory effects, demonstrating that  
137 in Ewing sarcoma cells, KLF15, TCF4 and NKX2-2 together constitute an inter-  
138 connected circuitry. Importantly, all of these regulatory effects were validated at

139 the protein levels with either additional shRNA or siRNAs in both A673 and EW8  
140 cell lines (Figs. 1E, 1F). In a univariate analysis, high expression of either KLF15  
141 or TCF4 in Ewing sarcoma was associated with significantly poor survival (Fig.  
142 1G).

### 143 **EWS-FLI1 directly activates three CRC factors**

144 To elucidate the co-regulatory mechanisms between CRC TFs and how they  
145 are controlled by EWS-FLI1, ChIP-Seq was performed using specific antibodies  
146 against either KLF15, TCF4 or NKX2-2 in A673 cells. Importantly, these 3 TFs  
147 trio-occupied both super-enhancers and promoters of each other, as well as  
148 themselves (Fig. 2A, Supplement Fig. 2, 3), forming an interconnected circuitry  
149 as predicted by our method. Enrichment of H3K27ac signals at these super-  
150 enhancers was observed across all Ewing sarcoma primary tumor samples (n=3)  
151 and cell lines (n=4). All three distal super-enhancers, but not promoters, were  
152 occupied by EWS-FLI1 in both A673 and SKNMC cells (Fig. 2A, Supplement  
153 Fig. 2), again validating our method. Furthermore, by re-analyzing the publicly-  
154 available Hi-C data of SKNMC cells, we confirmed interactions between super-  
155 enhancers with promoters in every gene locus, suggesting direct transcriptional  
156 co-regulation between KLF15, TCF4 and NKX2-2 by binding to each other's  
157 super-enhancers. These data also highlight that EWS-FLI1 directly controls the  
158 expression of all three CRC members by occupying their super-enhancers (Fig.  
159 2A).

160 Previous *de novo* motif analyses elucidate that cis-regulatory elements

161 activated by EWS-FLI1 are strongly enriched for GGAA repeats (7). Consistently,  
162 prominent enrichment of GGAA repeats were observed in EWS-FLI1 binding  
163 sites at all three CRC TFs (Figs. 2A-B, Supplement Fig. 2). To further examine  
164 whether and how EWS-FLI1 establishes the super-enhancers of CRC factors, we  
165 first interrogated chromatin accessibility of normal primary MSCs infected with  
166 an EWS-FLI1 expression vector. In the absence of ectopic EWS-FLI1 expression,  
167 these genomic regions had negligible ATAC-Seq peaks or H3K27ac signals,  
168 indicating little transcriptional activity. Importantly, ectopic expression of EWS-  
169 FLI1 converted these regions to super-enhancers with much higher H3K27ac  
170 intensity and chromatin accessibility (top 4 tracks in Fig. 2B, Supplement Fig. 2).  
171 On the other hand, in A673 cells, knockdown of EWS-FLI1 drastically decreased  
172 H3K27ac signal in EWS-FLI1-occupied super-enhancers (bottom 4 tracks in Fig.  
173 2B, Supplement Fig. 2). These results strongly suggest that EWS-FLI1 directly  
174 initiates and maintains the super-enhancers of CRC members.

175 Next, we identified three enhancer constituents (E1, E2, E3) within the super-  
176 enhancer of KLF15 based on the occupancy of TFs (Fig. 2C). Specifically, E1  
177 and E3 were trio-occupied by EWS-FLI1, TCF4 and NKX2-2, while E2 was trio-  
178 occupied by KLF15, TCF4 and NKX2-2 (Figs. 2A, 2C). These enhancer elements,  
179 as well as a control region, were subsequently cloned into the pGL3-promoter  
180 luciferase reporter vector which were transfected into A673 cells. Robust reporter  
181 activities of E1 and E2, but not E3, were observed (Fig. 2D). To determine the  
182 regulation of these enhancers by their occupying TFs, we silenced each TFs, and



183 knockdown of each factor (Fig. 2D) markedly reduced the activity of E1 and E2.  
184 These results support direct regulation of KLF15 super-enhancer by all CRC  
185 members, as well as EWS-FLI1. Moreover, CRC factors, but not the fusion  
186 oncoprotein, co-bound the promoter region of KLF15 (Fig. 2A). We similarly  
187 cloned two promoter constituents of KLF15 (P1 and P2) into the pGL3-basic  
188 luciferase reporter vector, and measured their activities by reporter assays in  
189 A673 cells (Fig. 2E). Both of the two promoter regions expectedly showed strong  
190 reporter activity, and they were significantly inhibited upon silencing each of the  
191 three CRC TFs.

### 192 **CRC factors cooperate with EWS-FLI1 to orchestrate the transcriptional** 193 **network of Ewing sarcoma cells**

194 To gain insights into the mechanistic basis of CRC TFs in regulation of the  
195 transcriptome of Ewing sarcoma, we analyzed the epigenomic characteristics of  
196 their occupancy in A673 cells. To this end, we first annotated the putative  
197 promoter ( $H3K4me3^+/H3K27ac^+/H3K4me1^-$ ) and distal enhancer ( $H3K4me3^-$   
198  $/H3K27ac^+/H3K4me1^+$ ) regions by available histone marks. Genome-wide peaks  
199 of each CRC TFs and EWS-FLI1 were then assigned to these regions. Notably,  
200 the occupancy of these TFs was pervasive throughout the genome, with 77.2% of  
201 all promoters and 55.6% of all enhancers bound by at least one of the factors (Fig.  
202 3A). Although genomic regions quadruple-bound by all 4 TFs were rare (0.6%  
203 promoters and 0.4% enhancers), cooperative occupancy (i.e., trio- and dual-  
204 occupation) were more common than solo-occupancy in both the promoter and

205 enhancer regions (Figs. 3A, 3B, Supplement Fig. 3), suggesting strong  
206 cooperativity between these factors. Specifically, several important co-binding  
207 patterns were observed: i) Validating previous studies (6, 7), EWS-FLI1 peaks  
208 were restricted within enhancer regions; ii) EWS-FLI1 almost always trio-  
209 occupied with TCF4/NKX2-2; iii) KLF15-binding was restricted to promoters,  
210 and it either dual-occupied with TCF4 or trio-occupied with TCF4/NKX2-2.

211 Importantly, regardless of promoter or enhancer elements, trio-binding regions  
212 always harbored much higher H3K27ac intensity compared to either solo- or  
213 dual-binding regions (Fig. 3C), suggesting that regulatory regions trio-occupied  
214 by these TFs may have stronger transcriptional activity. Moreover, tri-occupied  
215 regions were more likely to overlap super-enhancers than either dual- or solo-  
216 occupied regions (Fig. 3D).

217 To determine further the possible transcriptional impact of these binding event,  
218 matched RNA sequencing (RNA-Seq) data of A673 cells were analyzed. Notably,  
219 genes associated with trio-bound promoters exhibited the highest expression  
220 levels compared with either solo- or dual-bound promoters (Fig. 3E, upper panel).  
221 A similar trend was also observed in enhancer elements, albeit without statistical  
222 significance (Fig. 3E, lower panel). Together, these data highlight the cooperative  
223 binding as a key characteristic of CRC TFs. Moreover, CRC factors cooperate not  
224 only among themselves, but also with EWS-FLI1 in regulating the epigenome of  
225 Ewing sarcoma cells.

226 To establish further the regulatory effect of CRC factors on the transcriptome,

227 we performed RNA-Seq of A673 cells in either the presence or absence of  
228 knockdown of each TF. Importantly, gene set enrichment analyses (GSEA)  
229 showed that genes decreased following silencing of KLF15 were strongly and  
230 significantly enriched in those also downregulated upon depletion of either TCF4  
231 or NKX2-2 (Fig. 3F). The same pattern was also observed in RNA-Seq data of  
232 either siTCF4 or siNKX2-2 (Figs. 3G, 3H). Indeed, the downregulated genes in  
233 each dataset substantially and significantly overlapped ( $p < 10^{-6}$ , empirical  
234 distribution test); in fact, the “shared” changes accounted for almost half of all  
235 changes in every case (43.8% for TCF4, 51.3% for KLF15, and 47.0% for NKX2-  
236 2) (Fig. 3I). These results strongly suggest that CRC factors act in concert with  
237 each other to co-regulate the transcriptome of Ewing sarcoma cells.

### 238 **CRC factors have strong tumor-promoting function in Ewing sarcoma cells**

239 Considering the prominent roles of CRC in controlling transcriptional network  
240 (27, 28), we postulated that KLF15, TCF4 and NKX2-2 are required for the  
241 viability and proliferation of Ewing sarcoma cells. Indeed, NKX2-2 has been  
242 established as a tumor-promoting factor in Ewing sarcoma (29), but the functions  
243 of KLF15 and TCF4 remained hitherto unknown in this cancer. We thus  
244 performed loss-of-function assays and showed that knockdown of either KLF15,  
245 TCF4 or NKX2-2 by individual siRNAs (Fig. 4A) markedly inhibited cell  
246 proliferation (Fig. 4B, Supplement Fig. 4A,) and colony growth (Fig. 4C). The  
247 results were verified by doxycycline-inducible expression of multiple  
248 independent shRNAs (Supplement Figs. 4B-4E). Fluorescence-activated cell

249 sorting (FACS) analysis showed that depletion of endogenous expression of CRC  
250 TFs caused cell cycle arrest (Supplement Figs. 4F, 4G). We also detected  
251 increased cell apoptosis after knockdown of TCF4 (Supplement Fig. 4H).  
252 Additionally, silencing of KLF15 decreased cell migration of Ewing sarcoma  
253 cells and its over-expression produced the opposite effect (Supplement Figs. 4I-  
254 4O). A previous study showed that NKX2-2 silencing suppressed xenograft  
255 growth of Ewing sarcoma (30, 31). Here, we tested the dependency of Ewing  
256 sarcoma cells on KLF15 and TCF4 *in vivo*. Expression of doxycycline (DOX)-  
257 inducible shRNAs against either KLF15 or TCF4 potently inhibited xenograft  
258 growth of Ewing sarcoma (Figs. 4D, 4E). Finally, immunoblotting of xenograft  
259 tumors again confirmed the co-regulation of CRC TFs, as shown by the  
260 downregulation of each CRC member upon silencing of either KLF15 or TCF4  
261 (Fig. 4F). Taken together, these results demonstrate that as CRC members, KLF15,  
262 TCF4 and NKX2-2 positively co-regulate each other and promote the growth and  
263 survival of Ewing sarcoma cells.

#### 264 **CRC factors co-regulate lipid metabolism pathways in Ewing sarcoma**

265 Having established the functional significance of CRC TFs in the proliferation  
266 and viability of Ewing sarcoma cells, we next focused on investigating their  
267 downstream pathways. Pathway enrichment analysis was performed using  
268 downregulated genes, defined as  $\log_2$  (fold change)  $< -0.5$  and q value  $< 0.05$ ,  
269 upon knockdown of each TF individually. Multiple top enriched terms were  
270 shared among each analysis, including “Signal transduction”, “Metabolism”,

271 “Metabolism of lipids”, “Gene expression (transcription)” and “RNA Pol-II  
272 transcription” (Fig. 4G), strongly supporting the functional cooperation between  
273 these CRC factors. Among these shared top-ranking pathways, we were  
274 particularly interested in the lipid metabolism signaling, since: i) the biological  
275 significance of lipid metabolism in the biology of Ewing sarcoma is unknown; ii)  
276 the regulatory basis of CRC TFs on lipid metabolism is unclear.

277 To elucidate the mechanisms underlying the regulation of lipid metabolism by  
278 CRC TFs, we analyzed in-depth all the enriched genes (n=242) in the term  
279 “Metabolism of lipids” (Fig. 5A). Again, consistent with the close cooperation  
280 between CRC TFs, these genes were strongly shared between individual RNA-  
281 Seq data upon knockdown of each CRC factors. These downregulated genes were  
282 enriched in key pathways in lipid metabolism, including glycerophospholipid  
283 metabolism, sphingolipid metabolism, steroid biosynthesis, biosynthesis of  
284 unsaturated fatty-acids, as well as fatty-acid elongation (Fig. 5B). To quantify the  
285 functional impact of CRC TFs on these lipid metabolism processes, we performed  
286 liquid chromatography tandem mass spectrometry (LC-MS/MS)-based  
287 lipidomics in A673 cells, considering the immense structural complexity of lipid  
288 species. Specifically, quantitative lipidomics were performed in the presence and  
289 absence of the knockdown of each individual TF. To warrant reproducibility and  
290 robustness, 3-4 replicates of each sample were measured, and the data were  
291 highly comparably and consistent (Supplement Fig. 5). In total, we identified an  
292 average of 1,591 lipid ions in A673 cells, which belonged to 30 lipid classes,

293 suggesting high sensitivity and lipidome coverage of the methodology. The most  
294 abundant lipid classes in A673 cells were phosphatidylcholine (PC),  
295 phosphatidylethanolamine (PE), diacylglycerol (DG), triacylglycerol (TG),  
296 dimethylphosphatidylethanolamine (dMePE) and sphingomyelin (SM). The most  
297 abundant fatty acyl chains were oleate (C18:1), palmitate (C16:0), stearate  
298 (C18:0), palmitoleic (C16:1), arachidonate (C20:4), docosahexaenoic (C22:6). In  
299 agreement with the pathway enrichment analysis, silencing of each TF caused  
300 appreciable changes in the lipid landscape of A673 cells (Figs. 5C-5E).  
301 Specifically, comparing control with knockdown groups, 251 (in siKLF15), 397  
302 (in siTCF4) and 319 (in siNKX2-2) lipid ions were differentially regulated ( $q$   
303 value  $< 0.05$ , absolute fold change  $> 2$ ). Although the alterations in lipid species  
304 were variable between different knockdown experiments, the majority of which  
305 were notably converged to two lipid-associated pathways: glycerophospholipid  
306 and sphingolipid pathways (Fig. 5F). These changes in lipid classes were highly  
307 consistent with and supportive of our pathway analyses of RNA-Seq data, which  
308 identified the top two enriched pathways as glycerophospholipid metabolism and  
309 sphingolipid metabolism (Fig. 5B).

310 We next examined in detail how lipid metabolism was perturbed by silencing  
311 of CRC TFs via integration of RNA-Seq, ChIP-Seq and lipidomic results. This  
312 systematic approach identified many important lipid enzymes as direct targets of  
313 CRC TFs. For example, rate-limiting enzymes for fatty-acid synthesis and  
314 elongation (FASN, ACLY and SCD) were trio-occupied and directly regulated

315 by KLF15, TCF4 and NKX2-2. Moreover, sphingolipid anabolic enzymes  
316 (including SPTLC1, DEGS1 and UGCG) and glycerophospholipid anabolic  
317 enzyme (LPIN2) were under direct co-regulation by all three CRC TFs (Fig. 5G).  
318 Not surprisingly, we also observed solo- or dual-regulation by only 1 or 2 TFs on  
319 many other enzymes, such as CERS2 (by KLF15), CERS4 (by TCF4), CERS5  
320 (by NKX2-2) and B4GALT6 (by both KLF15 and NKX2-2). These findings  
321 together suggest that CRC factors co-operatively and directly regulate lipid  
322 metabolism pathways in Ewing sarcoma cells.

### 323 **Biological significance of lipid metabolism in Ewing sarcoma cells**

324 Following the characterization of the profound co-regulatory effects of CRC  
325 factors on lipid metabolic processes, we next sought to explore the biological  
326 significance of lipid metabolism in Ewing sarcoma cells. We first tested rate-  
327 limiting enzymes for fatty-acid synthesis (FASN, ACLY and SCD, which were  
328 trio-regulated by three CRC TFs), since this is the initial process upstream of all  
329 lipid metabolism reactions. Verifying our RNA-Seq results, qRT-PCR showed  
330 knockdown of each single TF reduced the expression of these central enzymes  
331 (Fig. 6A). Immunoblotting confirmed downregulation at the protein levels of  
332 FASN, ACLY and SCD (Fig. 6B). In addition, silencing of TCF4 (but not KLF15  
333 or NKX2-2) also reduced the expression of ACC, another key enzyme in fatty-  
334 acid synthesis. Some of the trio-binding peaks were shown in Fig. 6C using *FASN*  
335 and *SCD* promoters as examples.

336 Importantly, silencing of either FASN or SCD markedly reduced cell

337 proliferation and colony growth of both A673 and EW8 cell lines (Figs. 6D, 6E).  
338 Moreover, Orlistat, a specific FASN inhibitor, potently inhibited cell proliferation  
339 both *in vitro* (Fig. 6F) and *in vivo* (Fig. 6G), suggesting the biological importance  
340 of fatty-acid synthesis pathway in Ewing sarcoma cells.

341 In addition to fatty-acid synthesis, we also explored the functional significance  
342 of sphingolipid metabolism, another top-enriched lipid pathway in both RNA-  
343 Seq and lipidomics (Figs. 5B, 5G). Among all the sphingolipid anabolic enzymes  
344 regulated by CRC, SPTLC1 acts as the rate-limiting enzyme for *de novo*  
345 sphingolipid biosynthesis (32). Indeed, knockdown of each single TF inhibited  
346 the expression of SPTLC1 at both transcriptional and protein levels (Figs. 6H, 6I).  
347 Importantly, silencing of SPTLC1 reduced both colony growth and cell  
348 proliferation of Ewing sarcoma cells (Figs. 6J-6L). Moreover, treatment of  
349 Myriocin, a SPTLC1 inhibitor, reduced cell proliferation (Fig. 6M) and colony  
350 growth (Fig. 6N) of both A673 and EW8 cells.

### 351 **CRC TFs co-regulate PI3K/AKT and MAPK signaling pathways**

352 In parallel, we also investigated in-depth the term “Signal transduction” since  
353 it was ranked highest in all three RNA-Seq upon knockdown of CRC TFs (1<sup>st</sup> in  
354 both siKLF15 and siNKX2-2 and 2<sup>nd</sup> in siTCF4, Fig. 4G). To dissect which  
355 specific signaling pathways were coordinately regulated by CRC TFs, KEGG  
356 pathway enrichment was next performed using the genes enriched in “Signal  
357 transduction” term (the union set, n=669) following silencing of each factor (Fig.  
358 7A). Notably, PI3K/AKT and MAPK signaling pathways were identified as the



359 top-ranked pathways (Fig. 7A). Focusing on these two pathways, we found that  
360 genes downregulated upon knockdown of each CRC factors were strongly  
361 overlapped. Specifically, 32/84 (38%) genes in PI3K/AKT signaling and 23/72  
362 (32%) genes in MAPK signaling were decreased in at least two out of three  
363 knockdown groups (Fig. 7B), supportive of the notion that CRC factors  
364 cooperatively regulate these pathways.

365 Further integration with KLF15, TCF4 and NKX2-2 ChIP-Seq data identified  
366 that 40/44 (90.9%) commonly downregulated genes (in at least two knockdown  
367 groups) of these two cascades were directly occupied by at least one of the CRC  
368 TFs (Fig. 7C). Moreover, 23 and 19 of these 40 target genes (57.5% and 47.5%)  
369 were dual- and trio-occupied by CRC TFs, respectively (Fig. 7C). This result  
370 suggests strong cooperation between CRC TFs in the regulation of these two  
371 signaling pathways. These direct co-targets included key mediators or effectors  
372 of PI3K/AKT and MAPK pathways, such as *PDGFRB*, *PIK3R3*, *JAK3*, *VEGFB*,  
373 *RPS6KA5*, *CCND1* and *NFATC1* (Fig. 7C). Indeed, silencing of either KLF15,  
374 TCF4 or NKX2-2 inhibited the expression of these key molecules as validated by  
375 qRT-PCR (Fig. 7D). Some of the trio-binding peaks were shown in Fig. 7E using  
376 the *RPS6KA5* and *PDGFRB* promoters as examples. Moreover, immunoblotting  
377 assays confirmed reduced phosphorylation levels of several central mediators of  
378 PI3K/AKT and MAPK pathways, including P38, ERK, mTOR, P70 and AKT  
379 (Fig. 7F). Taken together, these data demonstrate that KLF15, TCF4 and NKX2-  
380 2 coordinately promote the transcription of chief components of the PI3K/AKT

381 and MAPK signaling pathways, thereby activating these pro-growth and pro-  
382 survival signaling cascades in Ewing sarcoma.

### 383 **Discussion**

384 EWS-FLI1 is a major determinant of genome-wide chromatin states in Ewing  
385 sarcoma (2-5, 33, 34), by functioning as a pioneer factor (7, 9, 10, 35).  
386 Nevertheless, the fusion driver requires other TFs and cofactors to fulfill its  
387 oncogenic activities. Considering the essential role of CRC in other  
388 developmental cancers driven by single transcriptional regulators (such as  
389 NMYC-driven neuroblastoma and PAX3-FOXO1-driven rhabdomyosarcoma)  
390 (22, 23), we postulated that in Ewing sarcoma, a functional CRC apparatus might  
391 cooperate with EWS-FLI1 in the regulation of Ewing sarcoma transcriptome. In  
392 order to address this hypothesis, we modified a computational algorithm given  
393 the central role of EWS-FLI1, and identified a CRC “trio” constituted by KLF15,  
394 TCF4 and NKX2-2 in Ewing sarcoma cells.

395 Integrative epigenomics analyses of ChIP-Seq, ATAC-Seq as well as Hi-C  
396 together demonstrate that EWS-FLI1, as a pioneer factor, directly establishes the  
397 super-enhancers of each of the three CRC TFs to activate their transcription.  
398 Subsequently, KLF15, TCF4 and NKX2-2 co-bind to their own and each other’s  
399 super-enhancers (together with EWS-FLI1 binding) and promoters (without  
400 EWS-FLI1 binding), forming an inter-connected auto-regulatory loop (Fig. 7G).

401 In addition to cooperating with EWS-FLI1 to co-amplify their own  
402 transcription through a CRC model, KLF15, TCF4 and NKX2-2 also exhibit

403 prominent capability of co-regulating the epigenome of Ewing sarcoma cells. In  
404 particular, the cooperative occupancy of CRC TFs is pervasive, covering the  
405 majority (77.2%) of all putative promoters and over half (55.6%) of all putative  
406 enhancers (Fig. 3A). Notably, TCF4 and NKX2-2 have higher capacity in  
407 occupying the genome than either EWS-FLI1 or KLF15, as their binding regions  
408 almost always encompass those of EWS-FLI1 and KLF15. Specifically, TCF4  
409 and NKX2-2 serve as co-binding partners for EWS-FLI1 (in enhancer regions)  
410 and for KLF15 (in promoter regions), in addition to their own binding regions.  
411 Nevertheless, these three TFs still tend to operate collaboratively, such that their  
412 co-bindings events (trio- and dual-) are more common than solo-binding events.  
413 Moreover, cooperative binding by more TFs appears to be associated with higher  
414 transcriptional activity. Indeed, DNA regulatory elements loaded with more TFs  
415 always have higher H3K27ac intensity (Fig. 3C), which are also associated with  
416 higher expression of downstream genes (only significantly in promoter regions,  
417 Fig. 3E). These findings are consistent with the proposed function of  
418 combinatorial binding of multiple factors in close proximity, which is to  
419 overcome with more potency the energetic barrier for nucleosome eviction, thus  
420 facilitating activation of cis-regulatory elements.

421 Not surprisingly, the cooperative binding of CRC TFs results in co-regulation  
422 of gene expression program in Ewing sarcoma cells. Indeed, GSEA of RNA-Seq  
423 data confirms that downregulated genes upon knockdown of each TF strongly  
424 and significantly overlapped with each other. In fact, the shared changes account

425 for almost half of all changes in every RNA-Seq of CRC TFs, strongly suggesting  
426 that KLF15, TCF4 and NKX2-2 coordinate to regulate the transcriptome of  
427 Ewing sarcoma cells (Figs. 3F-3I). Phenotypically, similar to CRC factors in  
428 other cancer types, KLF15, TCF4 and NKX2-2 each shows strong pro-growth  
429 and pro-survival functions in Ewing sarcoma. Indeed, NKX2-2 has been reported  
430 to promoting cell proliferation of Ewing sarcoma (29-31), however, the biological  
431 significance of either KLF15 or TCF4 had hitherto been unknown in this cancer.

432 TCF4 (also known as ITF2) is a basic helix-loop-helix (bHLH) TF that plays  
433 a crucial role in the differentiation and specification of central nervous system  
434 (CNS) (31, 36). Interestingly, depending on different tumor types, TCF4  
435 functions as either an oncogene (diffuse large B-cell lymphoma) (37) or tumor  
436 suppressor (medulloblastoma and colon cancer) (38, 39). KLF15 is a key  
437 regulator of metabolic pathways controlling adipogenesis and gluconeogenesis in  
438 both liver and skeletal muscles (40, 41). However, its involvement in human  
439 malignancies has been poorly understood with mixed findings. For example,  
440 KLF15 has shown anti-proliferative activities in gastric and breast cancer cells  
441 (42, 43); however, KLF15 promotes the proliferation and metastasis of lung  
442 adenocarcinoma cells (44). These reports suggest that the cancer-specific  
443 functions of KLF15 and TCF4 are highly context-dependent. In our study,  
444 consistent with their Ewing sarcoma-promoting functions, all CRC factors are  
445 expressed particularly robustly in Ewing sarcoma relative to most of other cancer  
446 types (Fig. 1B).

447 To understand the functional contribution of CRC TFs in Ewing sarcoma, we  
448 performed pathway enrichment analyses of RNA-Seq upon knockdown of each  
449 factors. This approach further substantiates the cooperativity of KLF15, TCF4  
450 and NKX2-2, since multiple top-enriched pathways are shared between three  
451 independent loss-of-function experiments. Two of the overlapped high-ranking  
452 pathways (lipid metabolism and signal transduction) were prioritized for in-depth  
453 investigation given their importance in cancer biology.

454 Dysregulated lipid metabolism is one of the most important metabolic  
455 hallmarks of cancer cells, which is pivotal for synthesis of cellular building blocks  
456 and signaling molecules (45-48). Accumulating evidence suggest that cancer cells  
457 depend on altered lipid metabolism for unrestrained growth and survival (48-50).  
458 However, how lipid metabolism is dysregulated and its functional significance in  
459 Ewing sarcoma remain poorly understood. In the present study, integrative  
460 analyses of LC-MS/MS-based lipidomics, RNA-Seq and ChIP-Seq together  
461 highlight that CRC factors converge on regulating key enzymes responsible for  
462 the biosynthesis of fatty-acids (FASN, SCD and ACLY), sphingolipids (SPTLC1,  
463 DEGS1 and UGCG) as well as glycerophospholipids (LPIN2). These lipid  
464 metabolism processes are required for cell survival of Ewing sarcoma, as  
465 evidenced by the cytotoxicity of their inhibitors (Orlistat against FASN and  
466 Myriocin against SPTLC1). In addition, silencing of either FASN, SCD or  
467 SPTLC1 markedly reduced cell proliferation of Ewing sarcoma, further  
468 corroborating this conclusion. The biochemical finding that KLF15 regulates

469 lipid synthesis in Ewing sarcoma is not completely surprisingly given the  
470 established role of KLF15 in controlling lipid synthesis and fat storage in adipose  
471 tissues (51-54). However, to date, neither TCF4 nor NKX2-2 have been  
472 implicated in such metabolic processes in any cell type. These data together  
473 highlight CRC-lipid metabolism as a novel pro-tumor cascade in Ewing sarcoma.

474 As signature oncogenic pathways, PI3K/AKT and MAPK signaling  
475 pathways are important for almost every cancer type, including Ewing sarcoma  
476 (55-57). Our investigations (Fig. 7) uncover novel epigenomic mechanisms for  
477 the activation of these pathways by CRC factors in Ewing sarcoma. While TCF4  
478 and NKX2-2 have not been involved in these pathways in any cell type, KLF15  
479 is intriguingly reported to inhibit the AKT and MAPK signaling pathways in  
480 normal skeletal muscle, cardiomyocytes and kidney (58-60). It thus appears the  
481 regulatory effects of KLF15 on these signaling pathways are cell-type dependent,  
482 possibly due to the expression pattern of KLF15 target genes are cell-type specific.  
483 This is plausible given that KLF15 almost always cooperates with TCF4 and  
484 NKX2-2 in Ewing sarcoma in terms of genomic binding.

485 In conclusion, we identify and validate an Ewing sarcoma-specific CRC,  
486 which is under control of EWS-FLI1. Formed by KLF15, TCF4 and NKX2-2,  
487 this CRC apparatus coordinates the gene expression programs, including lipid  
488 metabolism, PI3K/AKT and MAPK signaling pathways, in Ewing sarcoma cells.  
489 These data advance the understanding of the mechanistic basis of transcriptional  
490 dysregulation in Ewing sarcoma, and provide potential novel therapeutic

491 strategies against this malignancy.

## 492 **Materials and methods**

### 493 **Cell culture**

494 Ewing sarcoma cell lines (A673, SKNMC, EW8 and TC71) and human  
495 embryonic kidney cells 293T (HEK293T) used in this study were described  
496 previously (61, 62). Cells were grown in Dulbecco's Modified Eagle Medium  
497 (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100  
498 mg/ml streptomycin, and kept in a humidified incubator at 37 °C with 5% CO<sub>2</sub>.  
499 All cell lines were recently authenticated by short tandem repeat analysis.

### 500 **Antibodies and reagents**

501 The following antibodies were used in the current study: Anti-KLF15  
502 (Proteintech, 66185-1-Ig), anti-TCF4 (Abcam, ab223073), anti-NKX2-2  
503 (Proteintech, 13013-1-AP), anti-FLI-1 (Santa Cruz Biotechnology, sc-53826),  
504 anti-ACLY (Abcam, ab40793), anti-FASN (Abcam, ab22759), anti-ACC (Cell  
505 Signaling Technology, 4190S), anti-SPTLC1 (Proteintech, 66899-1-Ig), anti-  
506 SCD (Proteintech, 23393-1-AP), anti-FLAG (Sigma, F1804), anti-GAPDH (Cell  
507 Signaling Technology, 5174), anti-mouse IgG-HRP (Santa Cruz Biotechnology,  
508 sc-2005), anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2004) and rabbit  
509 IgG Isotype Control (Invitrogen, 02-6102), anti-mTOR (Cell Signaling  
510 Technology, 2972S), anti-P-mTOR (S2448) (Cell Signaling Technology, 2971S),  
511 anti-P-p38 MAPK (T180/Y182) (Cell Signaling Technology, 9216S), anti-p38  
512 MAPK (Cell Signaling Technology, 8690S), anti-Akt (Cell Signaling

513 Technology 4691S), anti-P-Akt (S473) (Cell Signaling Technology, 4060S), anti-  
514 p70 S6 Kinase (Cell Signaling Technology, 9202S) and anti-P-p70 S6 Kinase  
515 (Cell Signaling Technology, 9205S).

516 Reagents and kits included: Orlistat (Sigma-Aldrich, 96829-58-2), Myriocin,  
517 (Sigma-Aldrich, 35891-70-4), Propidium iodide (Sigma-Aldrich, 25535-16-4),  
518 FITC Annexin V Apoptosis Detection Kit (BD Biosciences), Dual-Luciferase  
519 Reporter Assay System (Promega), BioT transfection reagent (Bioland  
520 Scientific), Lipofectamine RNAiMAX transfection reagent (Invitrogen), and  
521 siRNA pools targeting *KLF15*, *TCF4*, *NKX2-2*, *FLI-1*, *FASN*, *SCD*, *RREB1* and  
522 *SPTLC1* (Dharmacon). siRNA sequences are provided in Supplementary Table  
523 1.

#### 524 **Construction of expression and lentiviral vectors**

525 The pCDH-CMV-Flag-EF1-puro-KLF15 expression vector was amplified  
526 based on pCDH-CMV-Flag-EF1-puro vector, and a 3xFLAG-tag was added via  
527 PCR. pLKO.1-puro or pLKO-Tet-On vectors expressing shRNAs targeting *FLII*,  
528 *KLF15*, *TCF4* and *NKX2-2* were constructed and confirmed by DNA sequencing.  
529 To produce viral particles, the recombinant viral vectors and packaging vectors  
530 were co-transfected into 293T cells. Supernatants containing viral particles were  
531 harvested at 48 hours and filtered through a 0.45  $\mu$ M filter after transfection.  
532 A673 cells were then infected with the virus in the presence of 10 mg/ml  
533 polybrene.

#### 534 **siRNA knockdown**



535 Control scramble and target siRNAs were purchased from Shanghai Genechem  
536 Co. Cells were transfected with 100 nM siRNA in OPTIMEM-I (Gibco) for 24-  
537 48 hours using Lipofectamine RNAiMAX transfection reagent (Invitrogen). Two  
538 independent siRNA oligonucleotides were used for each gene.

### 539 **Chromatin immunoprecipitation (ChIP) and data analysis**

540 ChIP was performed using our previously-described methods with slight  
541 modifications (11, 63).  $2 \times 10^7$ - $2 \times 10^8$  of A673 cells were harvested and fixed with  
542 1% paraformaldehyde for 10 min at room temperature. The fixation process was  
543 terminated by adding 250 mM of glycine. Chromatin solutions including  
544 lysis/wash buffer, sharing buffer and dilution buffer were prepared following a  
545 standard protocol (18). Samples were washed with PBS and lysed twice with  
546 lysis/wash buffer. After filtering through a 29 G needle, samples were harvested  
547 by spinning at 13,000 rpm for 10 min at 4°C. Sample pellets were then  
548 resuspended in sharing buffer and sonicated to shear genomic DNA to 300~500bp.  
549 Sonicated sample lysates were subsequently spun at 13,000 rpm for 10 min in 4 °C  
550 to remove debris; the supernatants were diluted with dilution buffer. For  
551 immunoprecipitation, solubilized chromatin was incubated and rotated with 5 µg  
552 of anti-KLF15, anti-TCF4, anti-NKX2-2 antibody or IgG control antibody  
553 overnight at 4°C. Antibody-chromatin complexes were pulled down by  
554 Dynabeads Protein G (Life Technologies) for 4 hours at 4°C. The beads were  
555 washed with lysis/wash buffer followed by cold TE buffer. Finally, bound DNAs  
556 were eluted by elution buffer (1% SDS, 100 mM NaHCO<sub>3</sub>) and reverse-

557 crosslinked overnight at 65 °C. DNA molecules were next treated with RNase A  
558 and proteinase K. Immunoprecipitated DNAs were extracted with the Min-Elute  
559 PCR purification kit (Qiagen), followed by either qPCR analysis, or DNA library  
560 preparation and sequencing on the HiSeq 4000 platform (Illumina).

561 ChIP-Seq was analyzed using established pipelines (11, 18). Raw reads were  
562 aligned to hg19 reference genome using bowtie 2 aligner (version 2.3.4.3) (64)  
563 followed by removal of PCR duplicates with Picard MarkDuplicates  
564 (<http://broadinstitute.github.io/picard/>). ChIP-Seq peaks were called using  
565 MACS (Model-Based Analysis of ChIP-Seq, version 2.1.2) (65) with default  
566 parameters. Reads were extended in the 5' to 3' direction by the estimated  
567 fragment length and normalized at  $-\log_{10}$  of the Poisson p-value of IP file  
568 compared to expected background counts by MACS2 `bdgcmp` command. BigWig  
569 files were generated by `bedGraphToBigWig` tool (<https://genome.ucsc.edu/>) and  
570 visualized in Integrative Genomics Viewer  
571 (<http://www.broadinstitute.org/igv/home>). Motif identification and comparison  
572 were performed with HOMER using `findMotifsGenome` program. H3K27ac  
573 ChIP-Seq data generated in 4 Ewing sarcoma cell lines, 3 primary tumors,  
574 primary mesenchymal stem cells (MSCs) and A673 cells were retrieved from  
575 NCBI Gene Expression Omnibus (GSE61953) and processed uniformly.

## 576 **Cell cycle analysis**

577 Cells were harvested and washed with PBS, followed by fixation with 70%  
578 cold ethanol overnight at 4°C. Cells were washed twice with PBS and stained

579 with propidium iodide. Cell cycle distribution was detected by SONY SA3800  
580 spectral cell analyzer. Data were analyzed by FlowJo 7.6 software (Tree Star).

### 581 **Apoptosis assay**

582 Cells were double stained with propidium iodide (PI) and Annexin V by using  
583 FITC Annexin V apoptosis detection kit (BD Biosciences) according to the  
584 manufacturer's instructions. After staining, cells were analyzed using a BD  
585 FACSCanto II flow cytometer. Data were analyzed using FlowJo 7.6 software  
586 (Tree Star).

### 587 **RNA extraction and cDNA expression analysis**

588 Total RNA was extracted using RNeasy mini kit (Qiagen). Purified RNA was  
589 reverse-transcribed using Maxima H Minus cDNA Synthesis Master Mix  
590 (Thermo Fisher). Quantitative real-time qPCR was performed on the AB7300  
591 Detection System (Applied Biosystems, Foster City, CA) using gene-specific  
592 primers and Power SYBR Green PCR Master Mix (Applied Biosystems).  
593 Expression of each gene was normalized to *GAPDH*, and quantified using 2-delta  
594 (ct) method.

### 595 **Luciferase reporter assay**

596 Candidate DNA regions (~500bp) were PCR amplified and cloned into either  
597 pGL3-Promoter firefly luciferase reporter vector or pGL3-Basic luciferase  
598 reporter vector (Promega). Constructs were verified by Sanger sequencing. A673  
599 cells were transfected using BioT transfection reagent. A Renilla luciferase  
600 control vector was co-transfected as a control for normalization. After 48 hours

601 of transfection, the luciferase activity was measured by the Dual-Luciferase  
602 Reporter Assay System (Promega).

### 603 **Immunoblotting analysis**

604 Whole cell lysates were prepared in RIPA buffer (1×PBS, 1% NP-40, 0.5%  
605 sodium deoxycholate, 0.1% SDS) supplemented with 10 mM beta-  
606 glycerophosphate, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM  
607 phenylmethylsulfonyl fluoride (PMSF), and 1×Roche Protease Inhibitor Cocktail  
608 (Roche, Indianapolis, IN). Immunoblotting was performed using specific primary  
609 antibodies as indicated and horseradish peroxidase (HRP)-conjugated secondary  
610 antibodies.

### 611 **Xenograft assays in nude mice**

612 All animal experiments were conducted following protocols approved by the  
613 Institutional Animal Care and Use Committee (IACUC) of Cedars-Sinai Medical  
614 Center. For the study of Orlistat treatment in A673 xenografts, twelve 5-6 weeks  
615 old BALB/c-nu female mice (Taconic Bioscience) were subcutaneously  
616 inoculated in their dorsal flanks with a suspension of A673 cells ( $2.0 \times 10^6$ ). When  
617 the tumors grew to 50 mm<sup>3</sup>, mice were injected intraperitoneally with either  
618 Orlistat (200 mg/kg/day) or equal volume of vehicle (10% DMSO, 20%  
619 cremophor and 70% NaCl). For the study of either KLF15 or TCF4 knockdown,  
620 A673 cells were engineered to stably express either doxycycline (DOX)-  
621 inducible scrambled shRNA or shRNAs against either *KLF15* or *TCF4*, and were  
622 cultured in DMEM supplemented with 10% Tetracycline (Tet)-free FBS

623 (Biological Industries) before implantation. After tumor inoculation, mice were  
624 randomized into two groups, and were fed with 2.5 mg/ml doxycycline containing  
625 water to turn on the expression of shRNAs. Tumor size and body weight were  
626 measured every 2 days. Mice were sacrificed by CO<sub>2</sub> inhalation when the largest  
627 tumors were approximately 1.5 cm in diameter; tumors were dissected, weighted,  
628 and analyzed.

### 629 **RNA-Seq and data analysis**

630 RNAs were isolated using miRNeasy RNA isolation kit (Qiagen). We aligned  
631 150bp paired-end reads to hg19 (UCSC) genome using Kallisto pseudo aligner  
632 (66). Reads were counted with tximport Bioconductor package (67) and  
633 normalized to gene levels by Transcript level abundances (TPM). Differentially  
634 expressed genes were identified by DESeq2 package (68) with adjusted p value  
635  $< 0.05$  and absolute  $\log_2$  (fold change)  $> 0.5$ . Pathway enrichment analysis was  
636 performed using ConsensusPathDB (<http://cpdb.molgen.mpg.de/>) and KEGG.  
637 For GSEA analysis, we used significantly downregulated genes (adjusted p value  
638  $< 0.05$  and  $\log_2$  (fold change)  $< -1$ ) in each knockdown of TF as the annotation  
639 base and performed enrichment analyses in all expressed genes with mean TPM  
640 values  $> 0.5$  in other two TFs.

### 641 **Computational construction of CRC**

642 CRC was computationally constructed using our published methodology (18,  
643 24, 25) with important modifications. Given the central role of EWS-FLI1 in  
644 establishing the transcriptome of Ewing Sarcoma cells, we required that all

645 candidate CRC members have both EWS-FLI1 binding motif and binding peaks  
646 in their super-enhancer regions. Therefore, we focused on A673 cell line, a well-  
647 characterized Ewing Sarcoma line which had matched H3K27ac and EWS-FLI1  
648 ChIP-Seq results (7, 69). Briefly, we first identified 77 super-enhancer-assigned  
649 TFs in A673 cell line as shown in Supplementary Table 2. Next, super-enhancer  
650 regions assigned to these TFs were extended 500 bp both upstream and  
651 downstream, followed by motif scanning with FIMO, for the identification of  
652 super-enhancer-assigned auto-regulated TFs. Finally, motif scanning was applied  
653 to identify further potential binding sites from other TFs of all auto-regulated TFs  
654 in their extended super-enhancer regions which, as mentioned above, must also  
655 have EWS-FLI1 binding motif and peaks. Circuitries were then constructed based  
656 on all possible fully interconnected auto-regulatory loops.

### 657 **Hi-C interactions**

658 The Hi-C data of SKNMC cell line were downloaded from ENCODE database  
659 and processed based on the pipeline published by Dixon et al (69). We extracted  
660 the interactions of the loci of *KLF15*, *TCF4* and *NKX2-2* at 40 kb resolution and  
661 visualized in UCSC genome Browser (<https://genome.ucsc.edu/index.html>).

### 662 **Liquid chromatography tandem mass spectrometry (LC-MS/MS)-based** 663 **lipidomics**

664 Lipidomic analysis was performed as described previously with slight  
665 modifications (70). Briefly, total cellular lipids were extracted with methyl tert-  
666 butyl ether (MTBE) (Sigma Aldrich) from fresh cell pellets and dried in a

667 SpeedVac concentrator (Thermo Scientific). Lipid samples were resuspended in  
668 50% isopropanol, 50% methanol and analyzed by liquid chromatography tandem  
669 mass spectrometry (LC-MS/MS). Twenty microliters of lipid solution were  
670 loaded onto a 15 cm Accucore Vanquish C18 column (1.5  $\mu$ m particle size, 2.1  
671 mm diameter) and separated using an Ultimate 3000 XRS ultraperformance LC  
672 system (Thermo Scientific). The mobile phase consisted of 60% acetonitrile, 10  
673 mM ammonium formate, and 0.1% formic acid (phase A) and 90% isopropanol,  
674 10% acetonitrile, 10 mM ammonium formate, and 0.1% formic acid (phase B).  
675 LC gradient was 35-60% B for 4 min, 60-85% B for 8 min, 85%-100% for 9 min,  
676 100% B for 3 min, 100-35% B for 0.1 min, and 35% B for 4 min at a flow rate of  
677 0.3 ml/min. Mass spectra were acquired by an Orbitrap Fusion Lumos Tribrid  
678 mass spectrometer (Thermo Scientific) operated in a data-dependent manner.  
679 Parameter settings for FTMS1 included orbitrap resolution (120,000), scan range  
680 (m/z 250-1200), AGC ( $2 \times 10^5$ ), maximum injection time (50 ms), RF lens (50%),  
681 data type (profile), dynamic exclusion for 8s using a mass tolerance of 25 ppm,  
682 and cycle time (2 s); FTMS2 included orbitrap resolution (30,000), isolation  
683 window (1.2 m/z), activation type (HCD), collision energy ( $30 \pm 3\%$ ), maximum  
684 injection time (70 ms), AGC ( $5 \times 10^4$ ), and data type (profile). Acquired raw files  
685 were analyzed using LipidSearch (v1.4) (Thermo Scientific) for sample  
686 alignment, MS2 identification, and MS1 peak area calculation. Statistical  
687 analyses were conducted using the Perseus (v1.6.6.0) software (71), wherein the  
688 p values were calculated by two-tailed Student's t-test and corrected for multiple

689 hypothesis testing via the Benjaminin-Hochberg method. Volcano plots were  
690 generated using the ggplot2 in the R environment (R Development Core Team;  
691 <https://www.r-project.org/>) (v3.5.0).

## 692 **Statistical analysis**

693 Two-tailed Student's t-test was used to evaluate the statistical difference between  
694 two groups, while one-way analysis of variance (ANOVA) was applied for multi-  
695 group comparisons. All statistical analyses were performed with SPSS 19.0. Log-  
696 rank test was used for survival analysis. Differences were considered statistically  
697 significant at  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*); n.s., not significant.  
698 Diagrams were created by GraphPad Prism 6.

## 699 **Data availability**

700 ChIP-Seq data of KLF15, TCF4 and NKX2-2, and RNA-Seq data of A673  
701 upon knockdown of each TF have been deposited into the GEO under accession  
702 number GSE141493. For ChIP-Seq data, the matching input file was obtained  
703 from our previous published data (GSM2944109).

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711 **Author contributions**

712 D.-C.L. conceived and devised the study. D.-C.L., X.P.S. and Y.Y.Z. designed  
713 experiments and analysis. X.P.S. and L.L.J., performed the experiments. W.Y.  
714 and B.Z. performed quantitative lipidomic and data analysis. Y.Y.Z. and M.L.H.  
715 performed bioinformatics and statistical analysis. S.G. contributed reagents and  
716 materials. X.P.S., Y.Y.Z., L.L.J., D.-C.L., and H.P.K. analyzed the data. D.-C.L.  
717 X.P.S. and H.P.K. supervised the research and wrote the manuscript.

718 **Acknowledgements**

719 This research is supported by the National Research Foundation Singapore under  
720 its Singapore Translational Research (STaR) Investigator Award  
721 (NMRC/STaR/0021/2014) and administered by the Singapore Ministry of  
722 Health's National Medical Research Council (NMRC), the NMRC Centre Grant  
723 awarded to National University Cancer Institute of Singapore, the National  
724 Research Foundation Singapore and the Singapore Ministry of Education under  
725 its Research Centres of Excellence initiatives (to H.P.K). This work was also  
726 supported by NIH grant (1R01 CA200992) to H.P.K. This work is additionally  
727 supported by NSFC (81670154/H0812,81470355/H1616), Projects  
728 (201707010352) from the Foundation of Guangzhou Science and Technology  
729 Innovation Committee in China (to X.S.). This research was also supported by  
730 Alan B. Slifka Foundation and the Ewing's Sarcoma Research Foundation.

731 **Competing interests:** The authors have declared that no conflict of interest exists.

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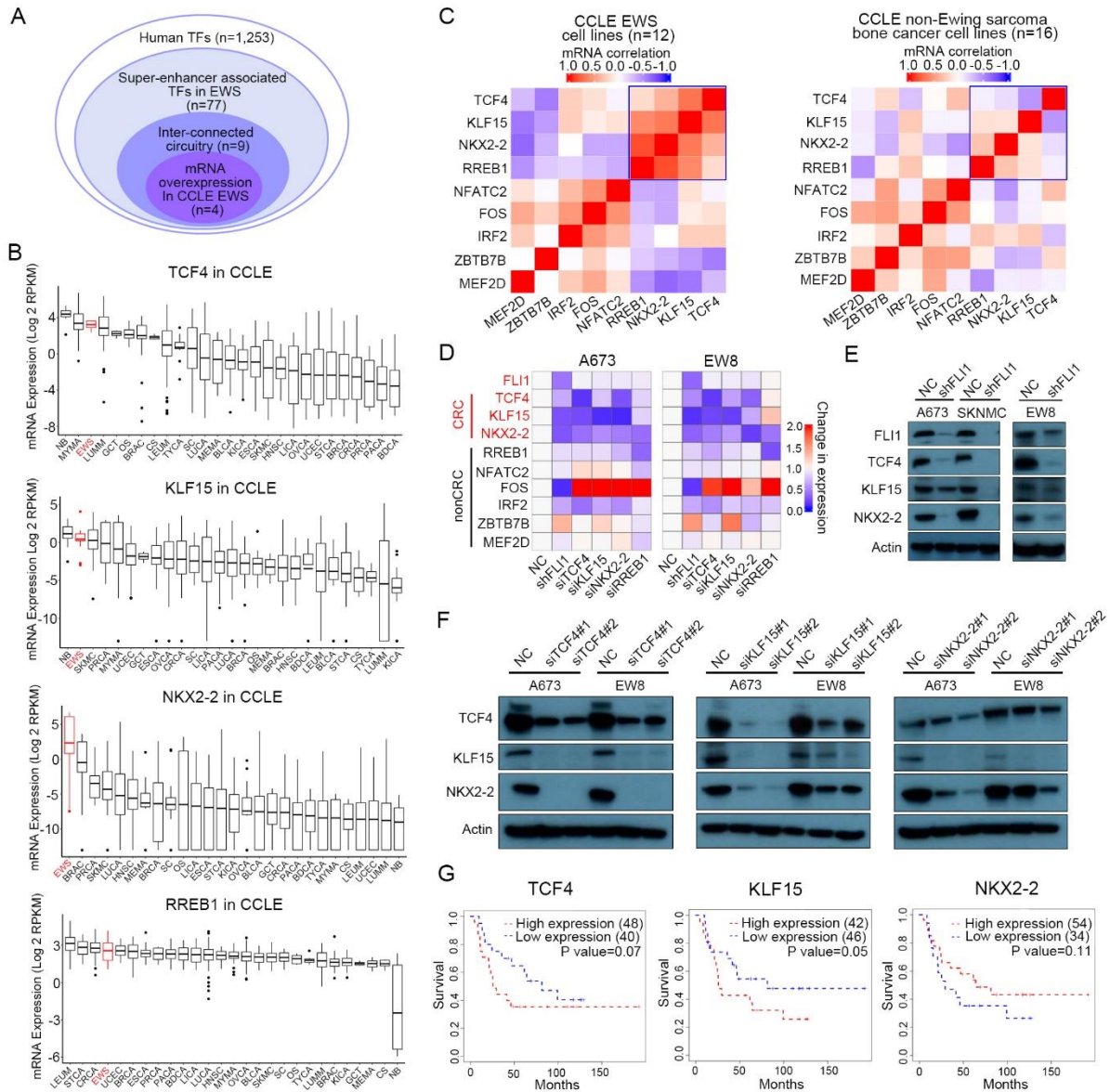
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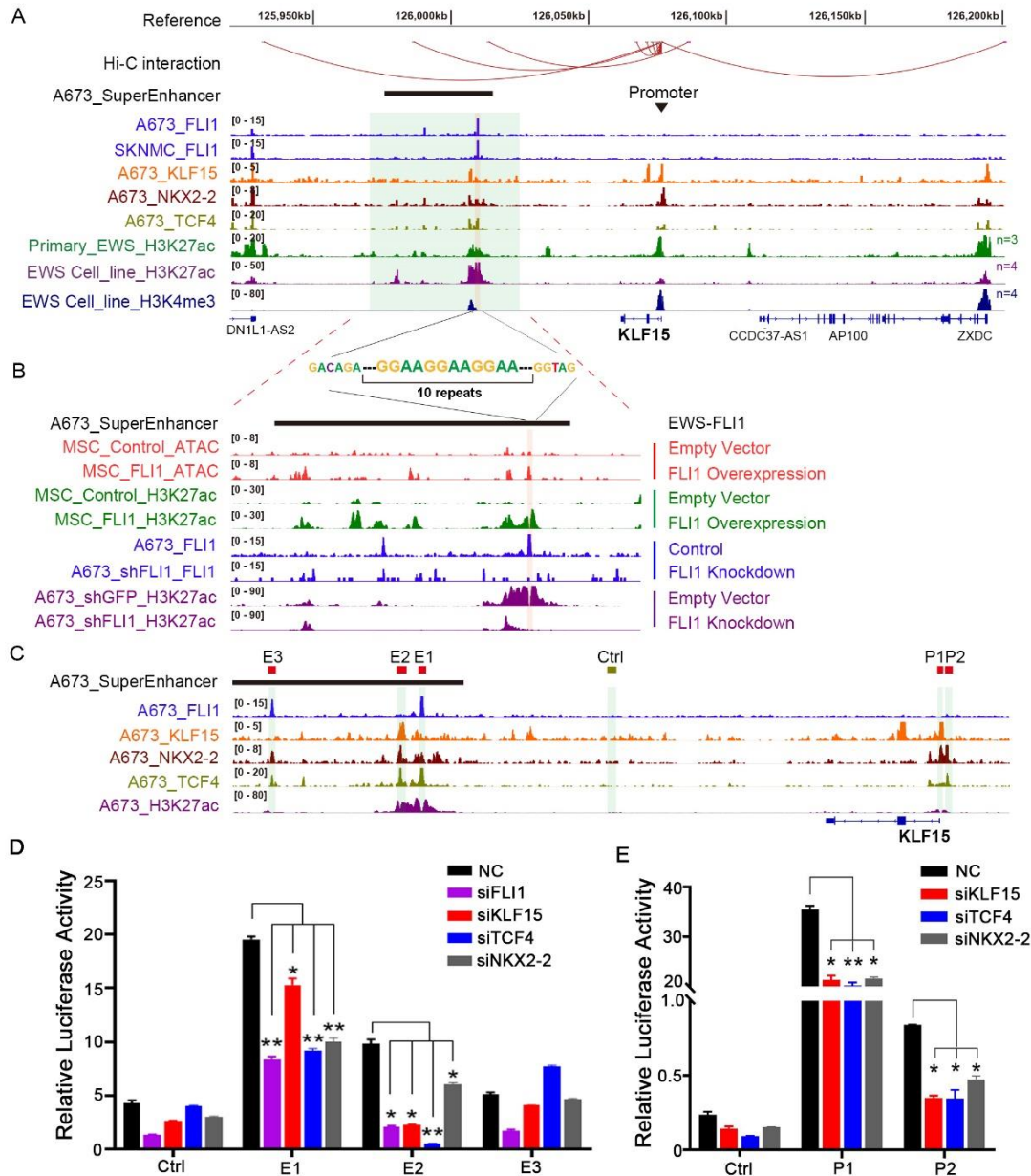
899 **Figure 1. KLF15/TCF4/NKX2-2 form interconnected co-regulatory circuitry in Ewing**  
 900 **sarcoma.**

901 (A) Integrative methods for identification of candidate CRC TFs. (B) The mRNA levels of  
 902 candidate TFs in CCLE database. (C) Heatmap of Pearson correlation coefficient between  
 903 candidate CRC TFs in Ewing sarcoma cell lines (n=12) or other bone cancer, but non-Ewing  
 904 sarcoma, cell lines (n=16). Data were retrieved from CCLE database. (D) Heatmap of fold  
 905 changes of mRNA expression of candidate TFs, following knockdown of either EWS-FLI1 or  
 906 each of candidate TF in A673 and EW8 cells. (E) Immunoblotting of protein expression of  
 907 CRC TFs upon either silencing of EWS-FLI1 or (F) silencing of each individual CRC TFs in  
 908 A673 and EW8 cells. (G) Kaplan-Meier survival plots analyzing the expression of CRC TFs  
 909 in Ewing sarcoma patients.

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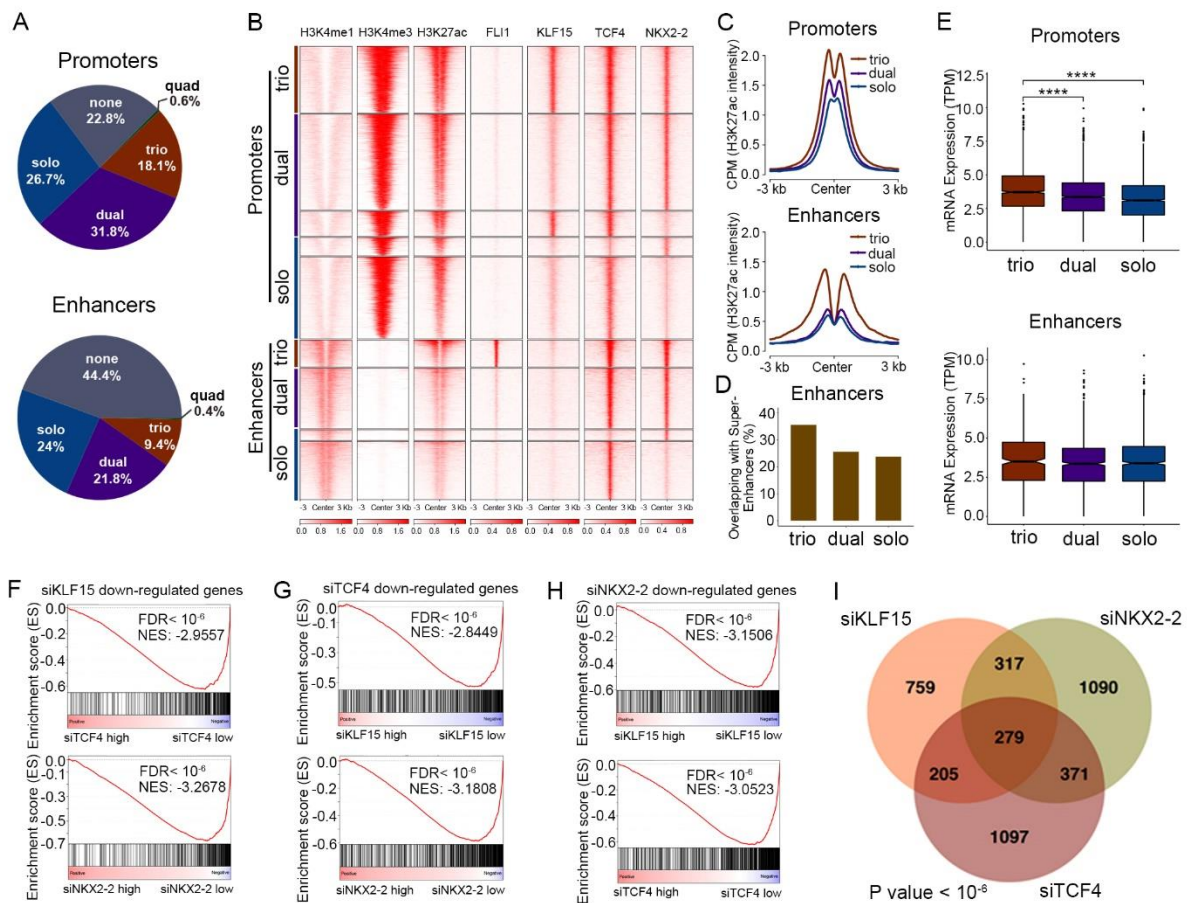
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**Figure 2. EWS-FLI1 and CRC TFs co-operatively activate the transcription of KLF15.**

(A) Integrative Genomics Viewer (IGV) plots of ChIP-Seq showing co-occupancy of EWS-FLI1 and CRC TFs at the super-enhancer and promoter of the KLF15 gene locus. Hi-C interactions were re-analyzed from the Hi-C data of SKNMC cell line downloaded from ENCODE database; H3K27ac, H3K4me3 and EWS-FLI1 ChIP-Seq data were retrieved from GEO (GSE61953). (B) ATAC-Seq and ChIP-Seq profiles at KLF15 super-enhancer region in either the presence or absence of EWS-FLI1 overexpression (top 4 tracks) or knockdown (bottom 4 tracks). Data were retrieved from GEO (GSE61953). (C) Zoom in view of ChIP-Seq signals in KLF15 locus. Three putative enhancer elements (E1-E3), two promoter elements (P1, P2) and one negative control region were separately cloned into luciferase reporter vectors. (D) Enhancer and (E) promoter activities were measured by luciferase reporter assays in A673 cells in either the presence or absence of knockdown of indicated TFs. Mean  $\pm$  s.d. are shown, n = 3. \*, P < 0.05; \*\*, P < 0.01.



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928 **Figure 3. Transcriptional cooperativity between EWS-FLI1 and CRC TFs.**

929 (A) Pie charts of the fractions of combinatorial binding patterns of EWS-FLI1 and three CRC  
 930 TFs. (B) Heatmaps of ChIP-Seq signals of indicated factors in A673 cells, stratified by different  
 931 combinatorial binding patterns. (C) Line plots of H3K27ac ChIP-Seq signals from indicated  
 932 groups of peaks in A673 cells. (D) The overlapping of indicated groups of peaks with super-  
 933 enhancers in A673 cells. (E) Box plots of mRNA expression of genes associated with indicated  
 934 groups of peaks in A673 cells. (F) GSEA plots showing the enrichment of downregulated genes  
 935 upon knockdown of KLF15 in either siTCF4 or siNKX2-2 RNA-Seq data. Reciprocal GSEA  
 936 plots of RNA-Seq following knockdown of TCF4 (G) and NKX2-2 (H) are similarly provided.  
 937 NES, normalized enrichment score; FDR, false discovery rate. (I) Venn diagram of  
 938 downregulated genes following silencing of each single TFs (p value <  $10^{-6}$ ; empirical  
 939 distribution test).

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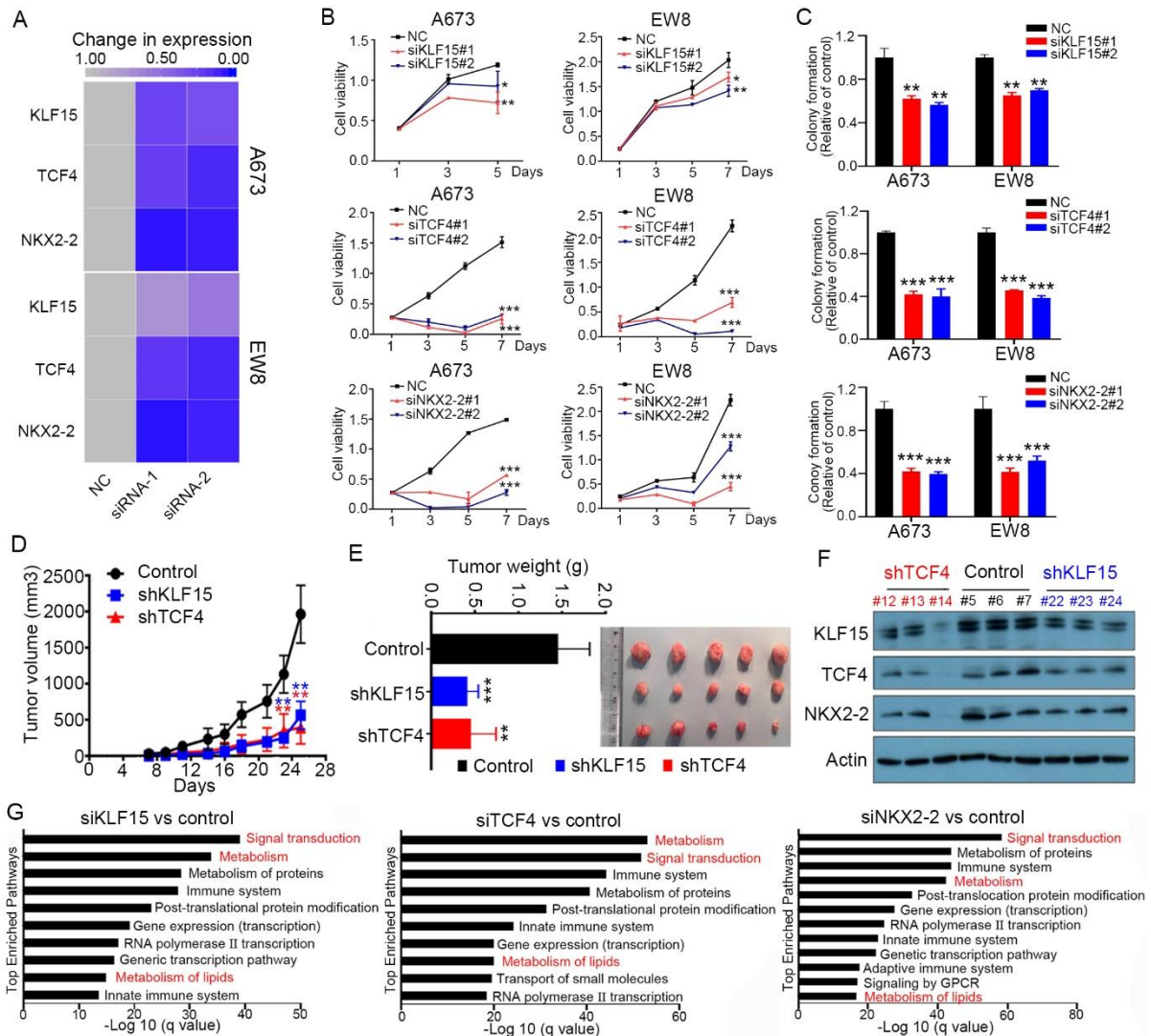
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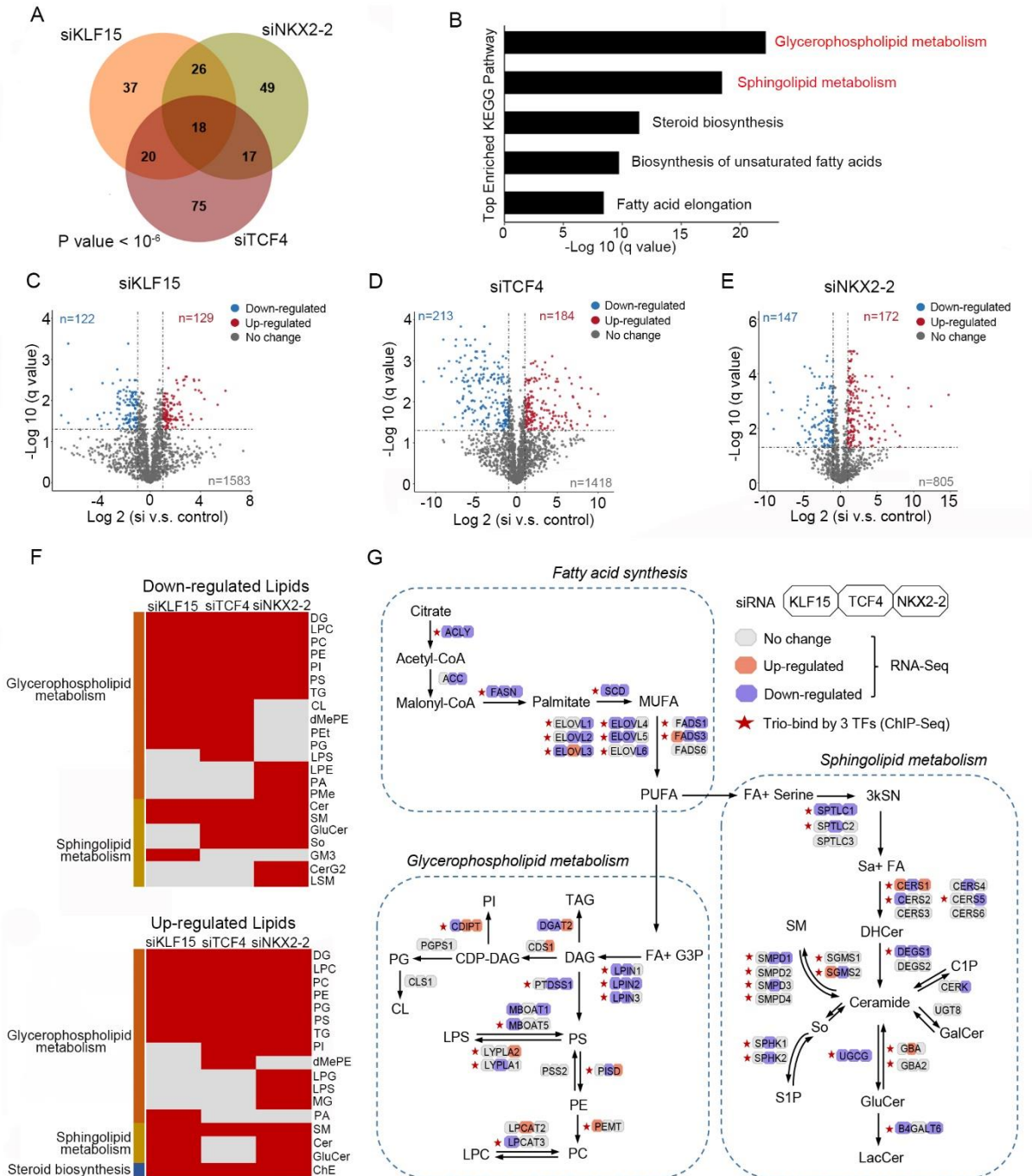




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**Figure 4. Cancer-promoting functions of CRC TFs in Ewing sarcoma cells**

(A) Heatmap of mRNA levels of three TFs before and after knockdown by individual siRNAs in A673 and EW8 cells. (B-C) Silencing of three TFs by individual siRNAs decreased cell proliferation (B) and colony growth (C) in Ewing sarcoma cells. (D-F) Silencing of either KLF15 or TCF4 by inducible shRNAs decreased xenograft growth *in vivo*. Growth curves (D), tumor weights and images (E), and co-regulation between CRC TFs (F) in resected tumors. Mean  $\pm$  s.d. are shown, n = 6. \*\*, P < 0.01; \*\*\*, P < 0.001. (G) Pathway enrichment analysis of downregulated genes after knockdown of each of three CRC TFs in A673 cells.



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### Figure 5. CRC TFs regulate lipid metabolism in Ewing sarcoma

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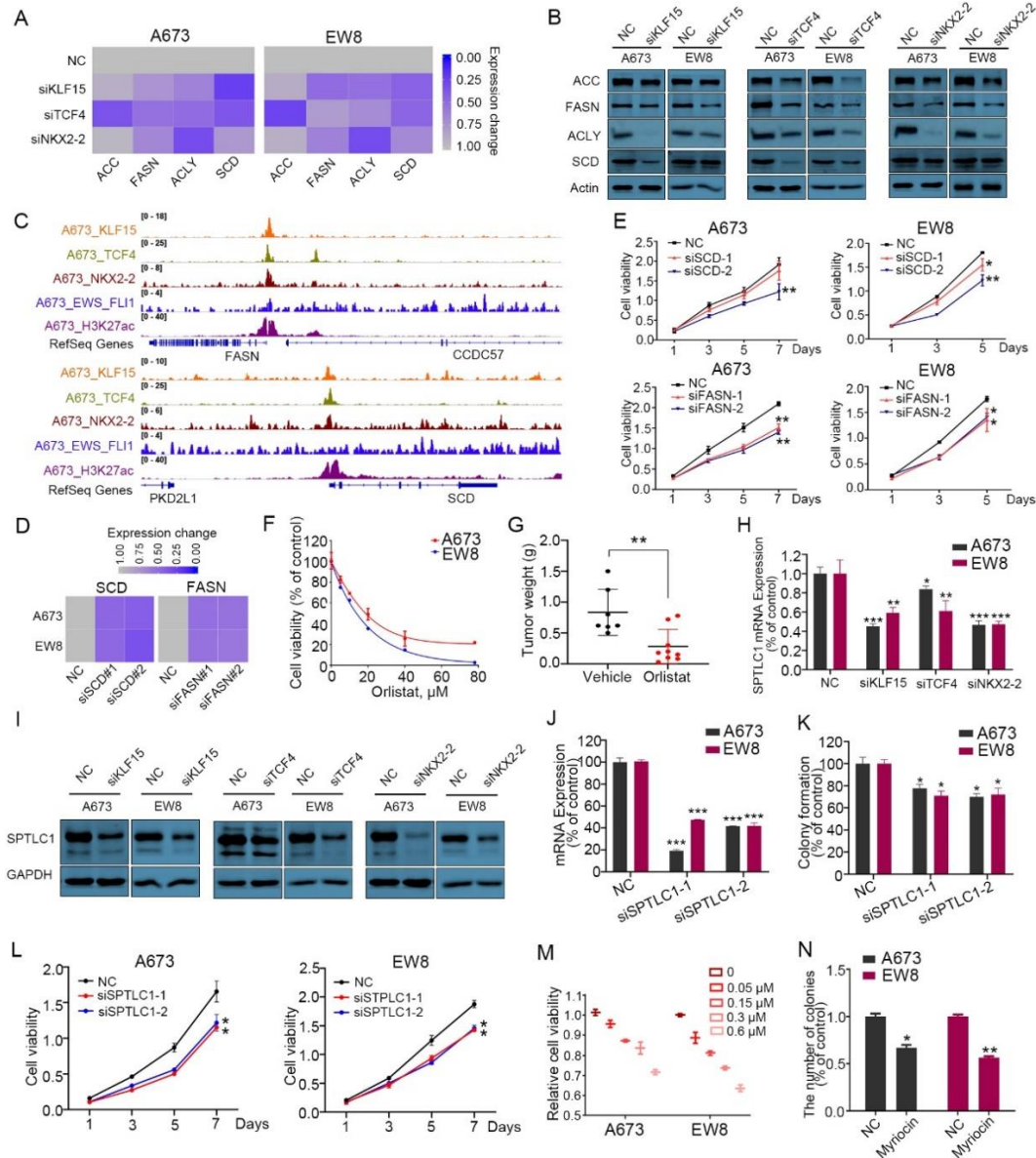
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(A) Venn diagram and (B) KEGG pathway enrichment of downregulated lipid metabolism-associated genes following silencing of each single CRC TFs (p value <math>< 10^{-6}</math>; empirical distribution test). (C-E) Volcano plots of lipidomic analyses showing differentially regulated lipid ions upon silencing of either (C) KLF15, (D) TCF4 or (E) NKX2-2. Each dot is a lipid ion. (F) Heatmaps of alterations in lipid classes upon silencing of each CRC TFs. Glycerophospholipid metabolism associated lipid classes: DG, LPC, PC, PE, TG, PG, PS, PI, PA, LPG, LPS, MG, dMePE, LPE, PMe, CL and PEt; Sphingolipid metabolism associated lipid classes: SM, GluCer, Cer, So, GM3, CerG2 and LSM; Steroid biosynthesis associated lipid class: ChE. (G) A diagram showing lipid metabolism which was perturbed by silencing of

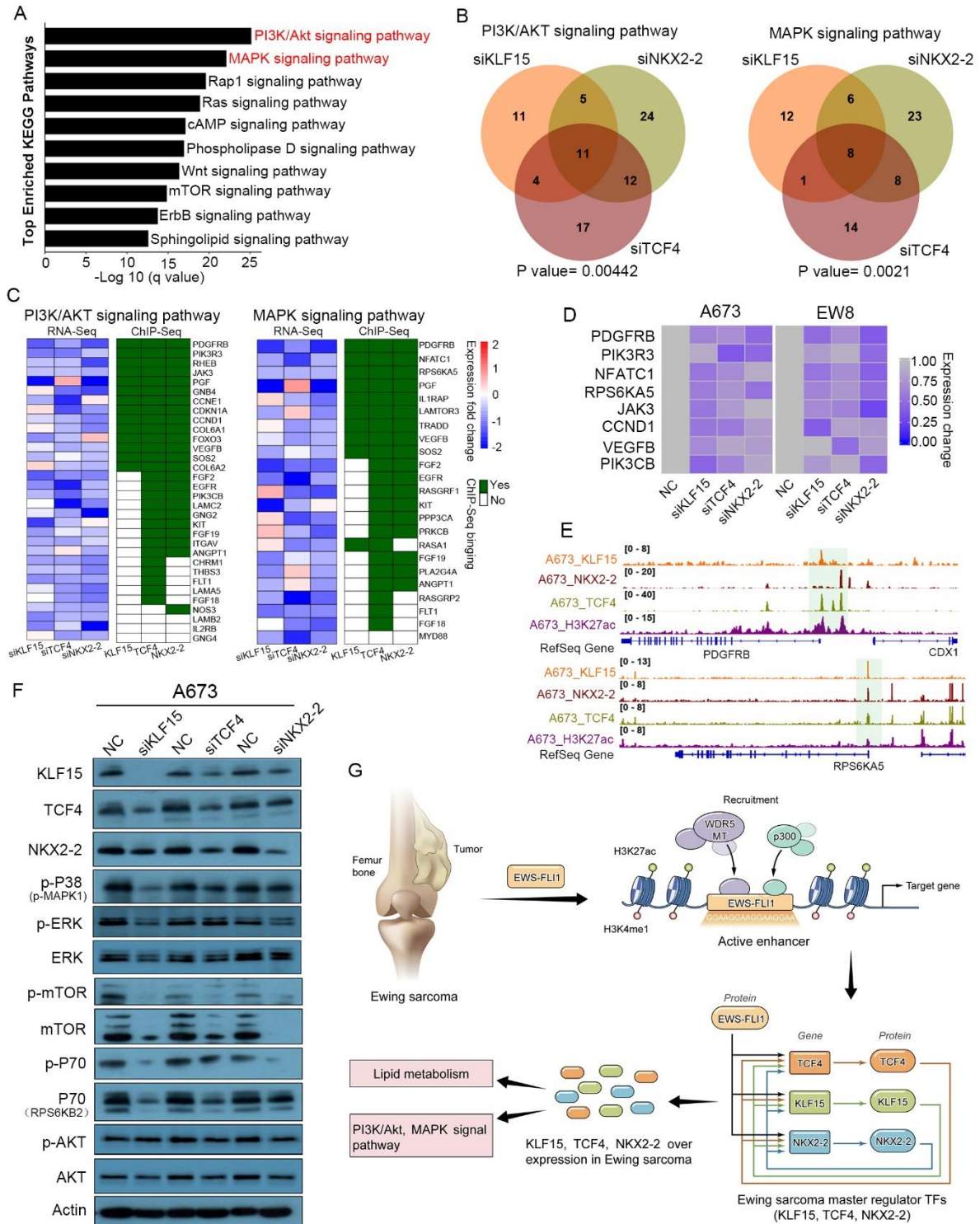
975 CRC TFs via integration of RNA-Seq, ChIP-Seq and lipidomic results. Color-coded bars  
976 indicate relative expression changes in RNA-Seq after silencing of each of the three TFs.

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**Figure 6. Biological functions of lipid synthesis pathway in Ewing sarcoma cells**

(A, B) Silencing of each of three CRC TFs decreased the expression of lipid biosynthetic enzymes at both the mRNA (A) and protein levels (B) in Ewing sarcoma cell lines. (C) IGV plots showing *FASN* and *SCD* promoters which were trio-occupied by three CRC TFs. (D) Silencing of *FASN* and *SCD* by siRNAs (E) decreased Ewing sarcoma cell proliferation. (F) *In vitro* MTT proliferation assay of Ewing sarcoma cells in the presence of different doses of orlistat (*FASN* inhibitor). (G) Orlistat treatment suppressed xenograft growth *in vivo*. Weights of resected tumors from both groups are shown. (H, I) Silencing of each CRC TFs decreased *SPTLC1* expression at both the mRNA (H) and protein levels (I) in Ewing sarcoma cell lines. (J-L) Silencing of *SPTLC1* by siRNA (J) decreased colony growth (K) and cell proliferation (L) in Ewing sarcoma cell lines. (M, N) *SPTLC* inhibitor (Myriocin) decreased cell proliferation (M) and colony growth (N) in both A673 and EW8 cells. Data are presented as mean±SD of three replicates. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001.



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**Figure 7. PI3K/AKT and MAPK signal pathways are regulated by CRC TFs in Ewing sarcoma cells**

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(A) KEGG pathway enrichment of downregulated genes after silencing each of the three CRC TFs. (B) Venn diagrams of downregulated genes in PI3K/AKT (left panel) and MAPK (right panel) pathways following silencing of each single CRC TFs. (C) Heatmaps showing the mRNA expression and binding patterns of the downregulated genes of these two pathways in at least two knockdown groups. (D) Silencing of each single CRC TFs inhibited

1027 the mRNA expression of key molecules of PI3K/AKT and MAPK signal pathways. (E) IGV  
1028 plots showing *RPS6KA5* and *PDGFRB* promoters which were trio-occupied by three CRC  
1029 TFs. (F) Immunoblotting assay showing the total and phosphorylation levels of key mediators  
1030 of PI3K/AKT and MAPK pathways upon silencing of each of the three CRC TFs. (G) A  
1031 proposed model of transcriptional dysregulation mediated by EWS-FLI1 and CRC TFs in the  
1032 biology of Ewing sarcoma.