

1 **Appendix (Table of content):**

2 **1. Supplemental Figure S1 to S11 and Legends**

3 **2. Supplemental Table S1**

4

5 **Follistatin is a novel therapeutic target and biomarker in FLT3/ITD acute myeloid**
6 **leukemia**

7 Running Title: FST as biomarker and therapeutic target

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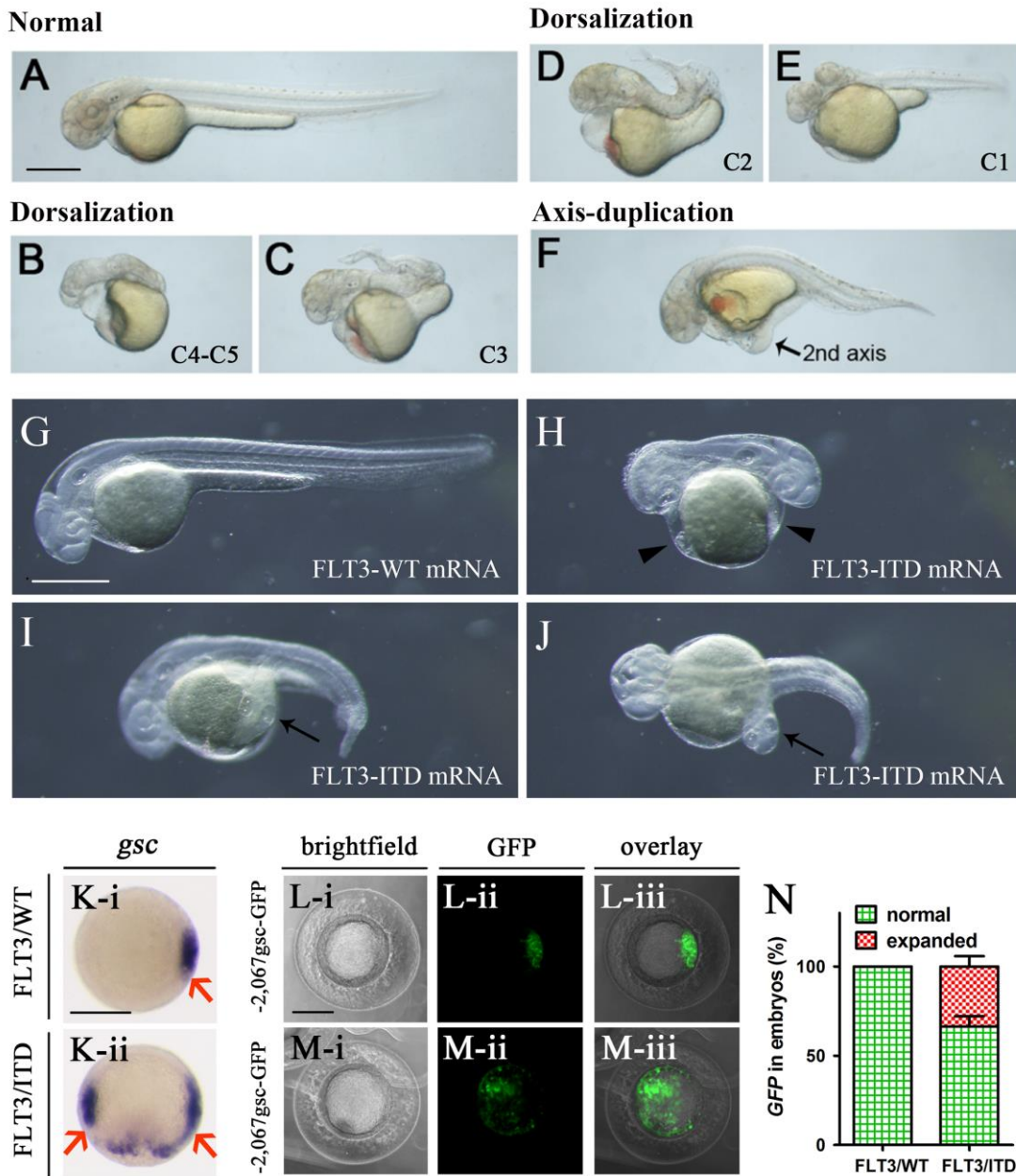
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27

28 **Figure S1**



29

30 **Figure S1. *FLT3/ITD* induced axis-duplication and dorsalization in zebrafish**
 31 **embryos.**

32 **A-F** Definition of dorsalization (C5-C1) and axis-duplication phenotype in early
 33 zebrafish embryogenesis.

34 **G-J** The double-head and axis-duplication phenotype in *FLT3/ITD* mRNA-injected

35 zebrafish embryos at 2dpf. *FLT3*/WT mRNA-injected embryo was used as control.
36 The arrows indicated the second head and the arrowheads indicated the duplicated
37 hearts in the embryos.

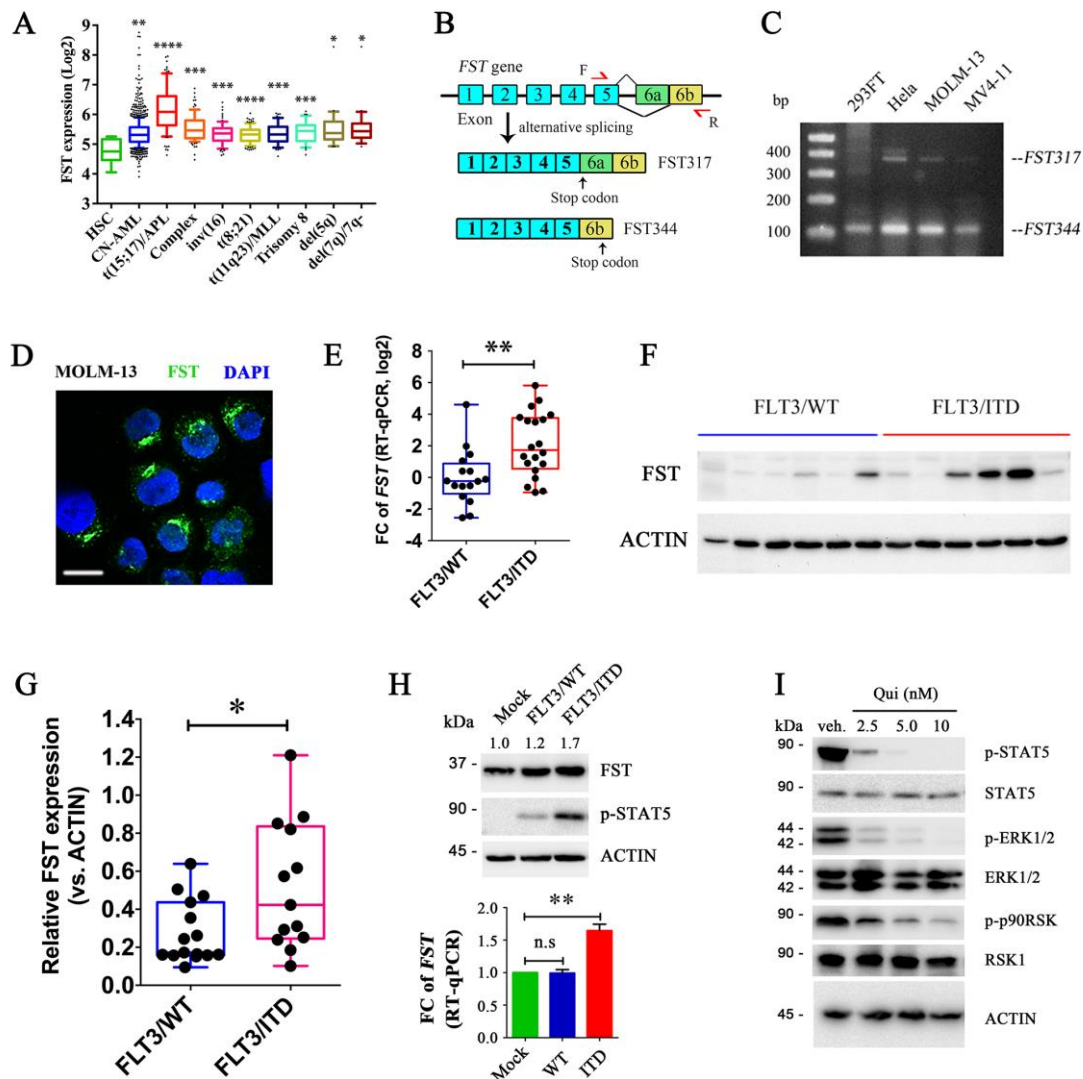
38 **K-N** The expansion of Spemann's organizer after *FLT3*/ITD overexpression was
39 detected by WISH of *gsc* (K, red arrows) and a -2,067*gsc*-GFP reporter assay (L and
40 M) at 6 hpf. 50 pg plasmid vector, in which GFP expression is driven by zebrafish *gsc*
41 promoter (-2,067), was co-injected with *FLT3*/WT or *FLT3*/ITD mRNA (150 pg) in
42 zebrafish embryo at one-cell stage, respectively. The expression of GFP in the
43 *FLT3*/WT- and *FLT3*/ITD-injected embryos (N) was used as a real-time *in vivo*
44 surrogate marker for *gsc* expression and Spemann's organizer at 6 hpf.

45 Data information: Scale bar = 500 μ m. All experiments were performed in triplicates.

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47

48 **Figure S2**



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50 **Figure S2. *FST* was upregulated in *FLT3/ITD*-mutated AML.**

51 **A** *In-silico* analysis of *FST* expression in normal HSC and different subtypes of AML

52 using BloodSpot program. The whiskers, boxes, and central lines represents the 10th-

53 to-90th percentile, 25th-to-75th percentile, and the 50th percentile (median), respectively.

54 **B-C** Schematic representation of alternative splicing of *FST* gene (B) and RT-PCR of

55 *FST317* and *FST344* expression in 293FT, HeLa, MOLM-13 and MV4-11 cells (C).

56 **D** Detection of endogenous FST in MOLM-13 cells by immunofluorescence. Scale bar
57 = 10 μ m.

58 **E-G** FST expression was detected by RT-qPCR (E), and Western blotting (F and G)
59 from *FLT3*/WT and *FLT3*/ITD AML patients (cytogenetic normal, leukemia blast > 80%
60 at diagnosis). Panel F was the representative image showing FST expression from
61 *FLT3*/WT and *FLT3*/ITD AML. β -ACTIN was used for normalization and
62 quantification of FST expression in panel G. The whiskers, boxes, and central lines
63 represents the minimum-to-maximum values, 25th-to-75th percentile, and the 50th
64 percentile (median), respectively.

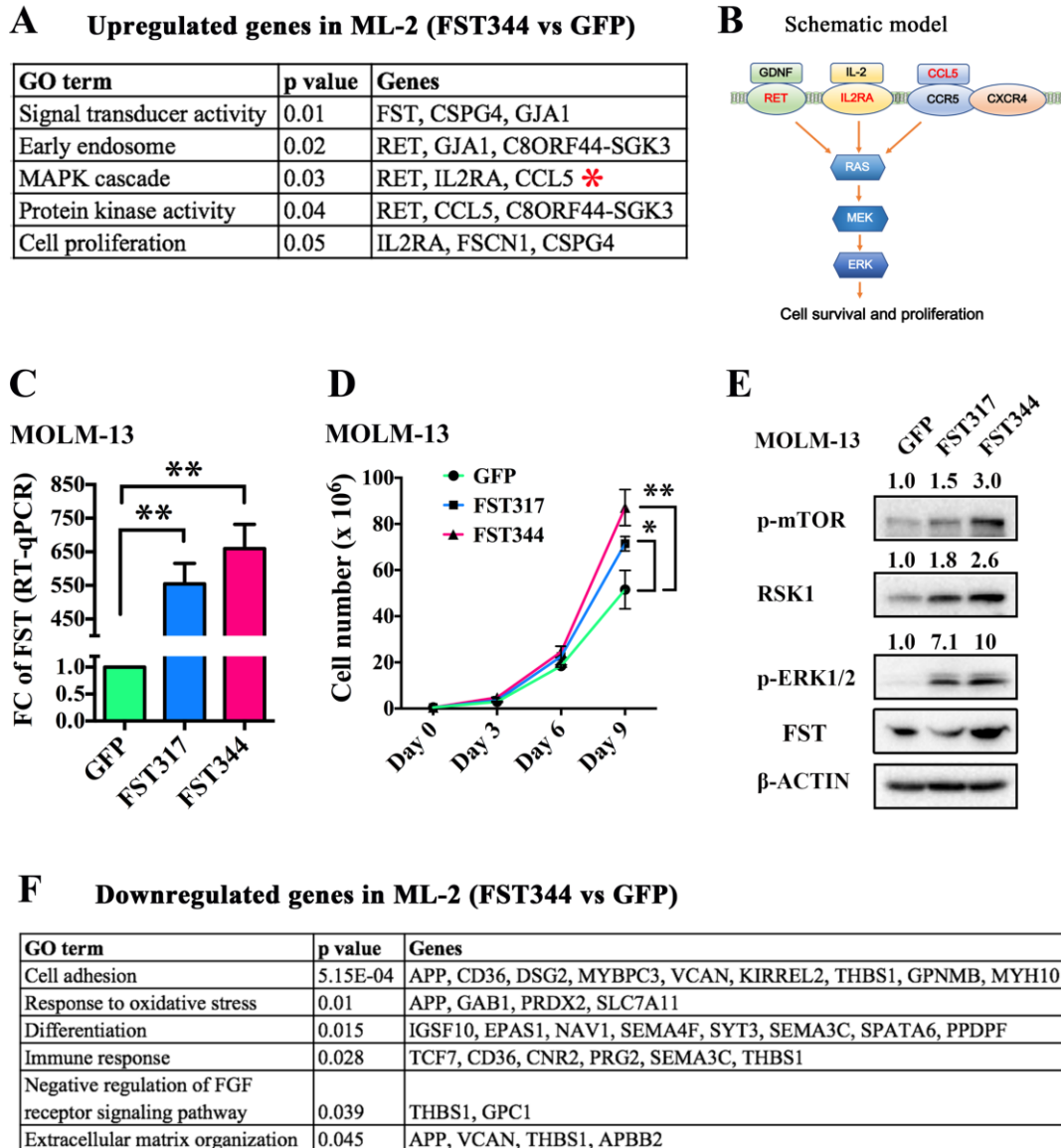
65 **H** Phosphorylation of STAT5 and FST expression were detected by Western Blotting
66 and RT-qPCR in Hela cells-transfected with *FLT3*/ITD. The RT-qPCR experiment were
67 performed in triplicates.

68 **I** FLT3 signaling were detected by Western blot in Ba/F3-*FLT3*/ITD cells-treated with
69 Quizartinib (Qui in short) *in vitro* (0-10 nM) for 1 day.

70 Data information: In (E, G and H), data were presented as mean \pm SEM. *P<0.05,
71 **P<0.01 (Student's t-test), n.s: not significant.

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76 **Figure S3. Effect of *FST* overexpression on MOLM-13 and ML-2 cells.**

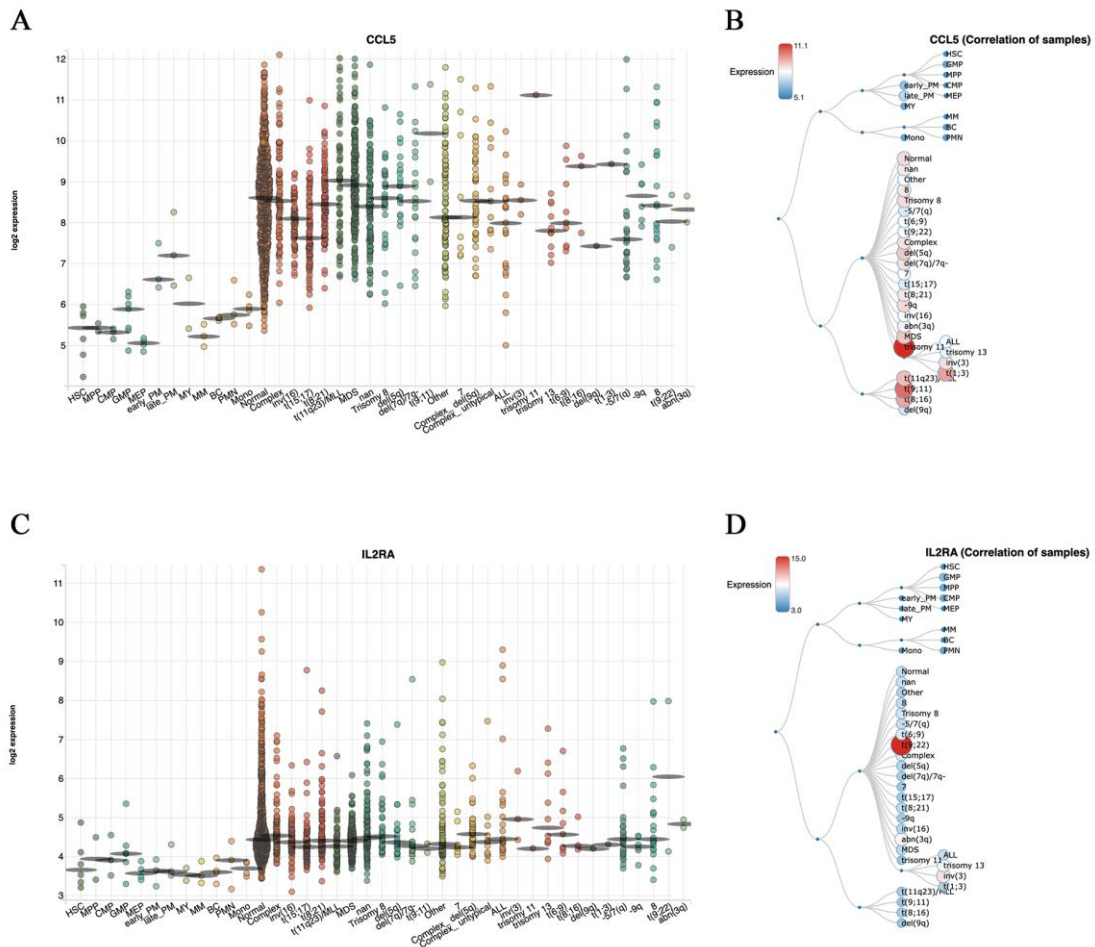
77 **A-B** Gene ontology (GO) analysis of upregulated genes (from RNA-seq) in ML-2 cells-
 78 overexpressing *FST344* comparing to those of *GFP* overexpression (A). The
 79 upregulated genes *RET*, *IL2RA* and *CCL5* (asterisk) were enriched in the MAPK
 80 cascade which was shown in the schematic diagram in panel B.

81 **C-E** The effect of *FST* overexpression (C) on cell growth (D) and ERK activation (E)
82 in *FLT3/ITD* MOLM-13 cells *in vitro*. The numbers above the blots indicated the fold
83 change of p-mTOR, RSK1, and p-ERK1/2, respectively (GFP sample was used as
84 control and set as 1). The RT-qPCR experiments were performed in triplicates.

85 **F** Gene ontology (GO) analysis of downregulated genes (from RNA-seq) in ML-2 cells-
86 overexpressing *FST344* comparing to those of *GFP* overexpression.

87 Data information: In (C and D), data were presented as mean \pm SEM. *P<0.05,
88 **P<0.01 (Student's t-test).

89 **Figure S4**



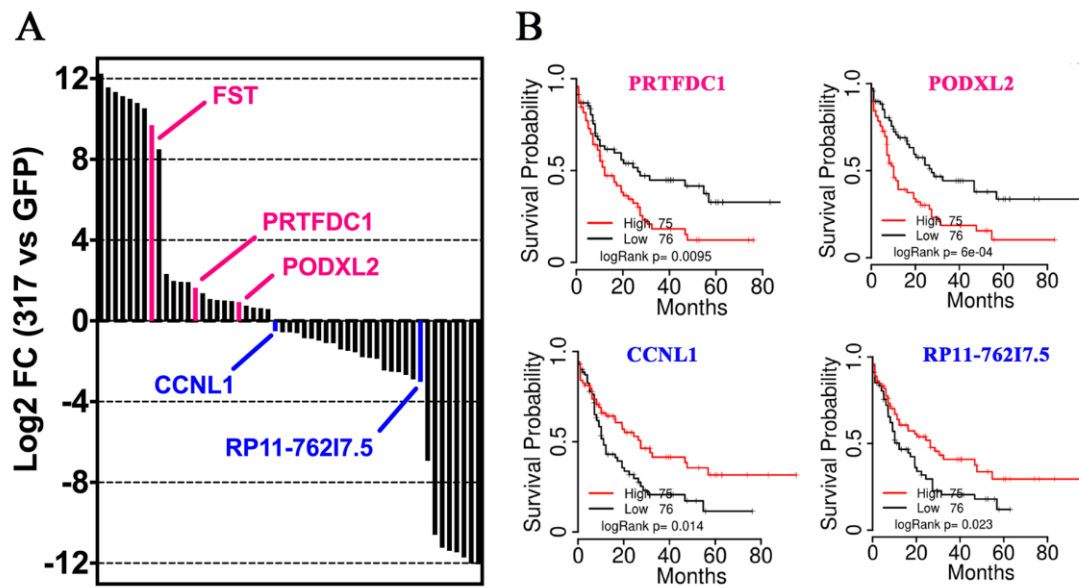
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91 **Figure S4. In silico analysis of CCL5 and IL2RA expression in normal**
 92 **hematopoietic tissues and human AML.**

93 **A-D** The relative expression of CCL5 (A and B) and IL2RA (C and D) in normal
 94 hematopoietic tissues and human AML were analyzed by the public program Bloodspot
 95 (<http://servers.binf.ku.dk/bloodspot/>).

96

97 **Figure S5**



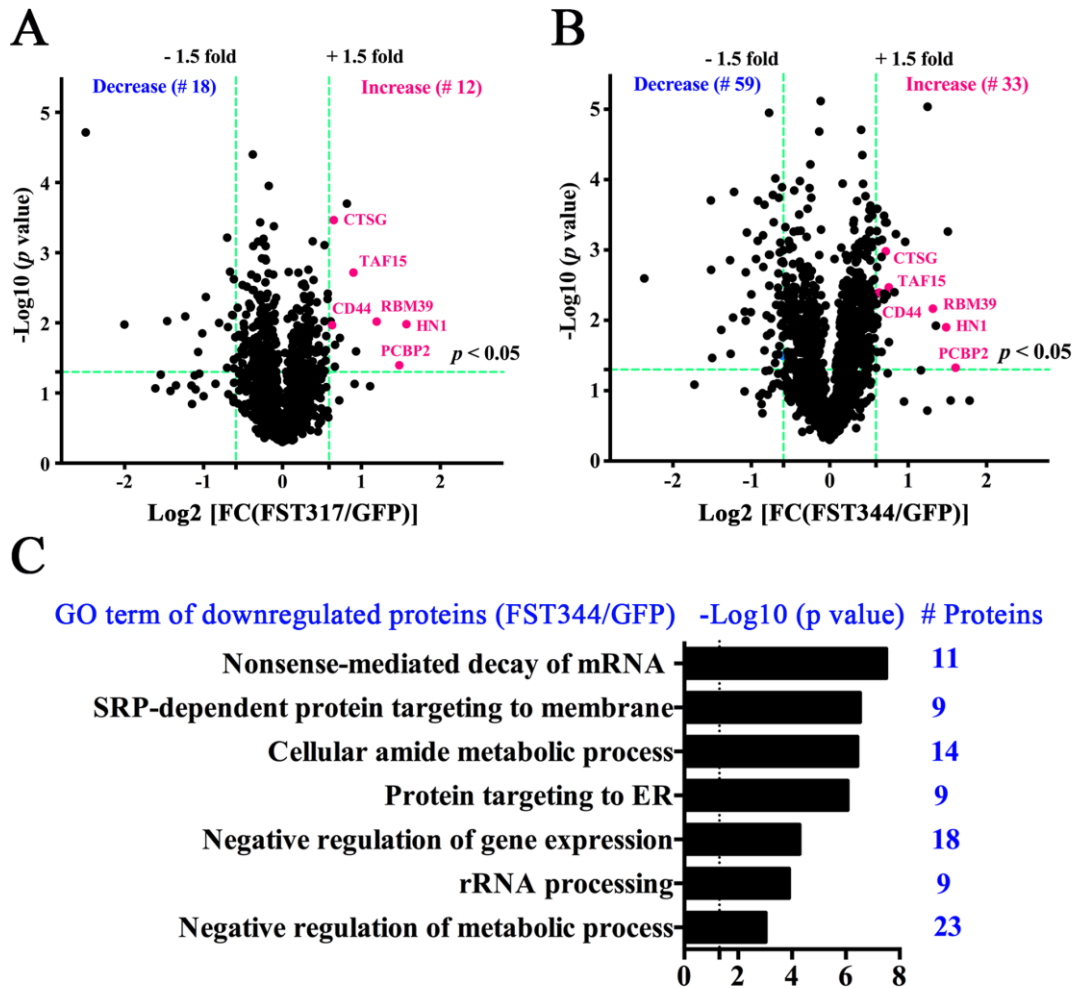
98

99 **Figure S5. RNA-seq showing the differentially expressed gene after FST317**
100 **overexpression in ML-2.**

101 **A** The differentially expressed genes in ML-2 cells (FST317 vs GFP) were shown as
102 waterfall plot.

103 **B** The clinical relevance of these differentially expressed genes were analyzed based
104 on the patients' survival data from TCGA-AML. Upregulation of *PRTFDC1* and
105 *PODXL2*, and downregulation of *CCNL1* and *RP11-762I7.5* after *FST317*
106 overexpression in ML-2 predicted the poor survival of AML patients.

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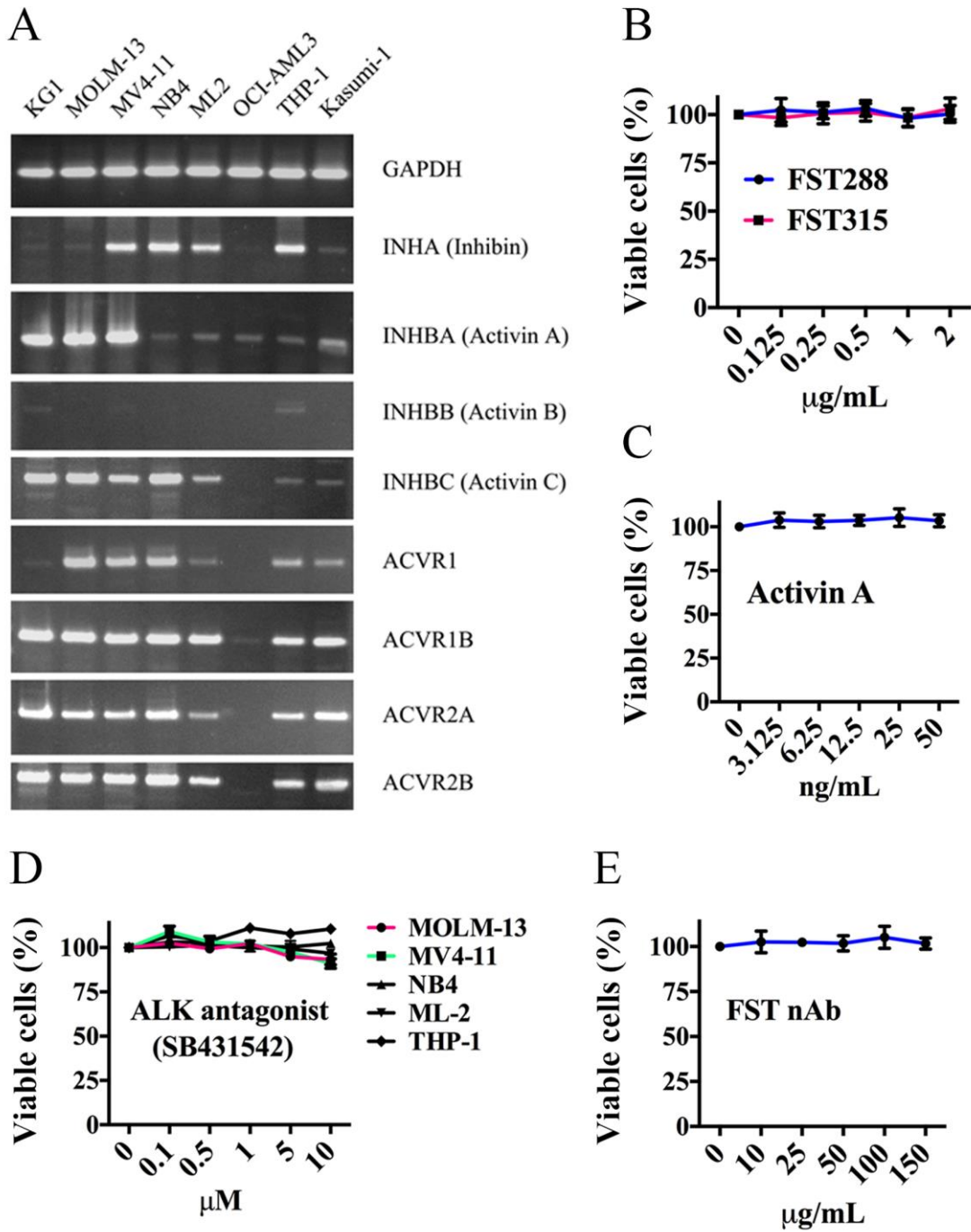
110 **Figure S6. Proteomic analysis of ML-2 cells after *FST* overexpression.**

111 **A-B** Volcano plot showing differentially expressed proteins after *FST317* and *FST344*
 112 overexpression in ML-2 comparing to those of *GFP* overexpression. CTSG, TAF15,
 113 CD44, RBM39, HN1, and PCBP1 were significantly upregulated in both *FST317* and
 114 *FST344*-overexpressing ML-2 cells.

115 **C** Gene ontology (GO) analysis of downregulated proteins in ML-2 cells-
 116 overexpressing *FST344* comparing to those of *GFP*. The downregulated proteins

117 associated with nonsense-mediated decay of mRNA were most significant.

118



120

121 **Figure S7. Exogenous FST and Activin treatment on AML cell growth.**

122 **A** Gene expression of Activin and its receptors in AML cell lines by semi-quantitative

123 PCR. *GAPDH* was used as house-keeping gene.

124 **B-C** The effect of exogenous FST and Activin A on MOLM-13 cell growth after 3 days
125 treatment *in vitro*.

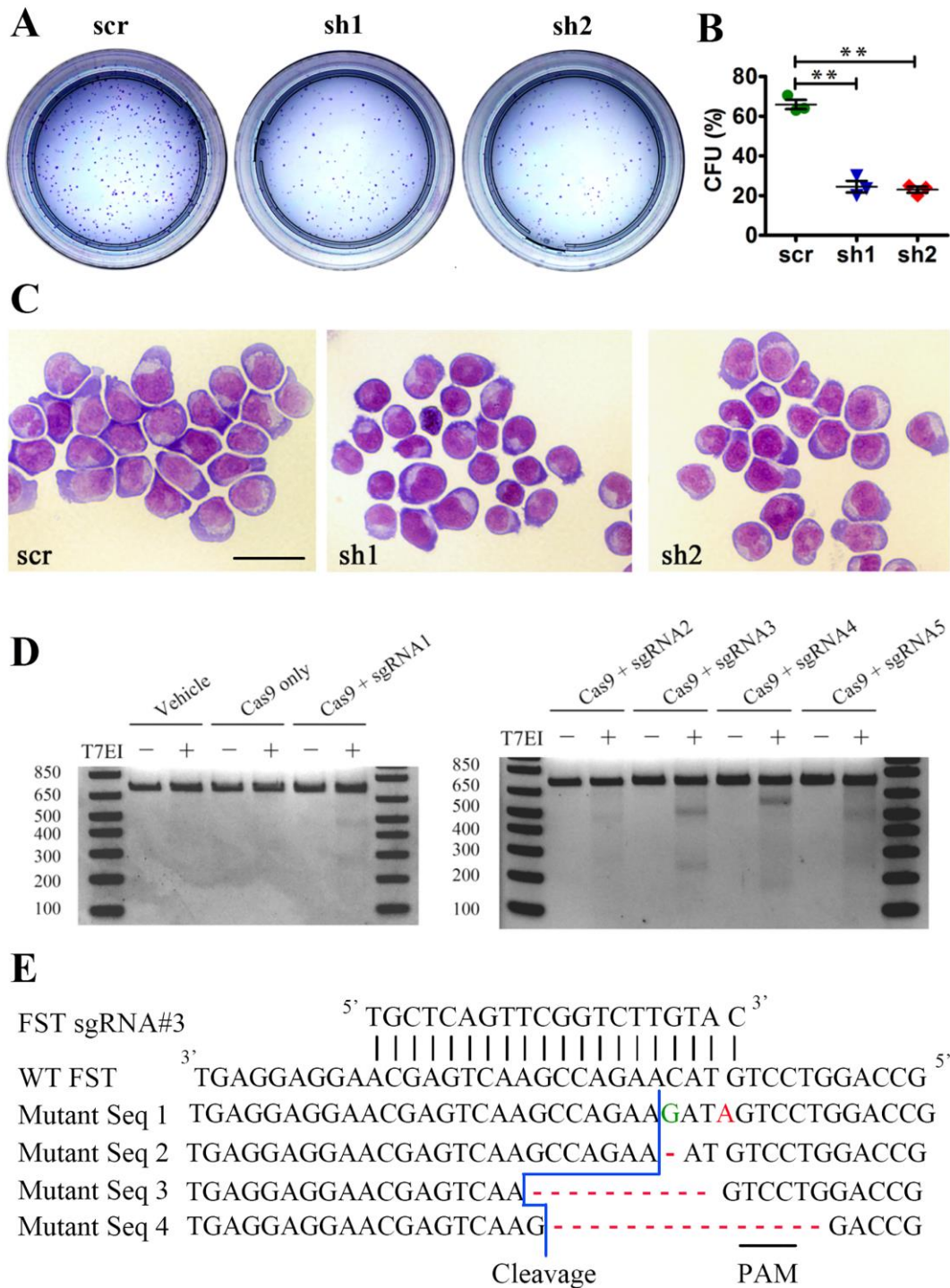
126 **D** The effect of Activin receptor antagonist (SB431542) on cell growth of different
127 AML cell lines after 3 days treatment *in vitro*.

128 **E** The effect of FST neutralizing antibody on MOLM-13 cell growth after 3 days
129 treatment *in vitro*.

130 Data information: In (B-E), the data were presented as mean \pm SEM.

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135 **Figure S8. *FST* targeting by shRNA and CRISPR/Cas9 in *FLT3*/ITD AML cell**
 136 **lines.**

137 **A-C** The clonogenicity (A and B) and morphology (C) of MV4-11 cells after *FST*

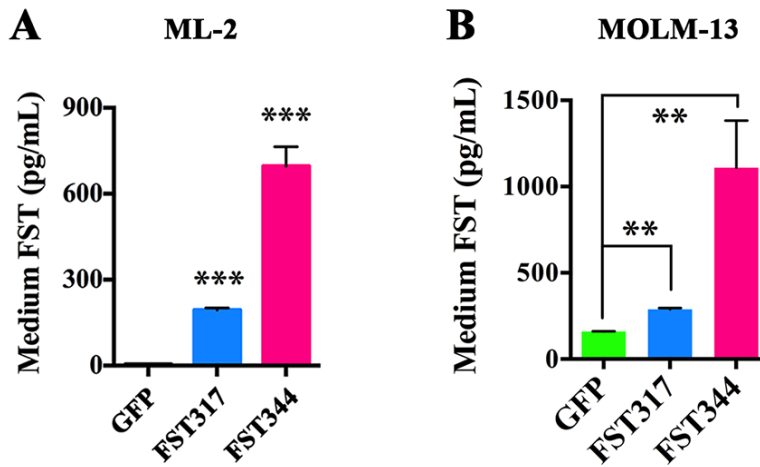
138 knockdown *in vitro*. Scale bar = 20 μ m (C). In (B), the data were presented as mean \pm
139 SEM. **P<0.01 (Student's t-test)

140 **D-E** T7EI assay (D) and sanger sequencing (E, for sgRNA#3) detecting the genome
141 editing efficiency of different sgRNAs for human *FST* in 293FT cells. Genomic DNA
142 was extracted from 293FT cells at 3 days post transfection. Fragments containing the
143 sgRNA target sites were PCR amplified, purified, denatured, annealed to form the
144 heteroduplex, and digested with or w/o T7 Endonuclease I (T7EI) (D). To detect these
145 insertion and deletion, fragments containing the sgRNA#3 target site were ligated into
146 pGEM-T-easy vector for sanger sequencing (E). Green: mismatch; Red: insertion and
147 deletion; PAM: protospacer adjacent motif.

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150 **Figure S9**



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152 **Figure S9. *FST* overexpression resulted in increased FST level in culture medium.**

153 FST in the culture medium was measured by ELISA from ML-2 (A) and MOLM-13

154 (B) cells stably expressing *GFP*, *FST317*, and *FST344*, respectively. The ELISA

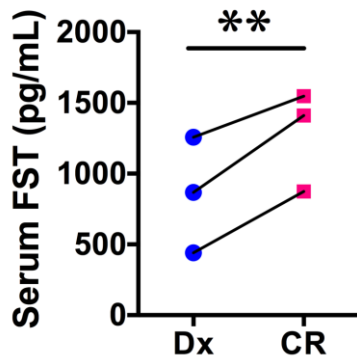
155 experiments were performed in triplicates and the data were presented as mean \pm SEM.

156 **P<0.01, ***P<0.001 (Student's t-test)

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158

159 **Figure S10**



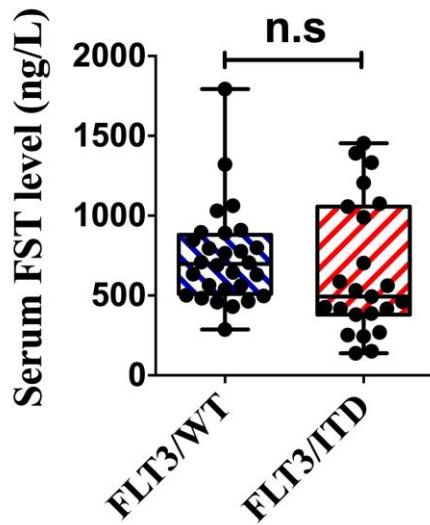
160

161 **Figure S10. Serum FST level from FLT3/WT AML patients treated with**
162 **conventional chemotherapy.**

163 Serum FST levels were measured by ELISA from FLT3/WT AML patients treated with
164 conventional “7+3” chemotherapy at diagnosis (Dx) and complete remission (CR). The
165 data were presented as scatter dot plot. **P<0.01 (Student’s t-test).

166

167 **Figure S11**



168

169 **Figure S11. Serum FST levels from FLT3/WT and FLT3/ITD AML patients from**
170 **our achieved samples.**

171 Serum FST levels were measured by ELISA from FLT3/WT and FLT3/ITD AML
172 patients at diagnosis. The data were presented in box plot. The whiskers, boxes, and
173 central lines represented the minimum-to-maximum values, 25th-to-75th percentile, and
174 the 50th percentile (median), respectively. n.s: not significant (Student's t-test).

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178 **Supplemental Table S1**

Figure and panel	comparing groups	p-value	Symbol
Fig 1, panel K	DMSO vs Qui 5.0 μ M (dorsalization)	1.51E-04	***
	DMSO vs Qui 5.0 μ M (axis duplication)	1.15E-04	***
Fig 1, panel L	FLT3/WT vs FLT3/ITD (fst)	5.74E-03	**
	FLT3/WT vs FLT3/ITD (gsc)	7.95E-03	**
	FLT3/WT vs FLT3/ITD (fzd4)	3.23E-02	*
Fig 2, panel N	WT vs ITD	1.17E-04	***
Fig 2, panel O	WT vs ITD	9.23E-03	**
Fig 3, panel C	IgG vs p-CREB (c-Fos)	1.09E-04	***
	IgG vs p-CREB (FST)	3.28E-03	**
Fig 3, panel D	Red vs purple	5.99E-05	***
	Green vs purple	9.72E-04	***
Fig 3, panel G	Ba/F3-P vs Ba/F3-ITD (DMSO)	7.37E-05	***
	DMSO vs Qui-2.5 nM (Ba/F3-ITD)	9.54E-05	***
	DMSO vs Qui-5.0 nM (Ba/F3-ITD)	1.17E-05	***
	DMSO vs Qui-10 nM (Ba/F3-ITD)	1.11E-05	***
Fig 3, panel H	DMSO vs Qui-10 nM	2.42E-04	***
Fig 3, panel J	DMSO vs BRD7389 (Ba/F3-ITD)	8.76E-04	***
	DMSO vs BRD7389 (MOLM-13)	1.39E-03	**
	DMSO vs BRD7389 (MV4-11)	1.18E-03	**
Fig 4, panel B	Green vs blue	2.42E-03	**
	Green vs red	4.26E-03	**
Fig 4, panel C	Green vs blue	7.91E-03	**
	Green vs red	7.40E-03	**
Fig 4, panel E	Green vs blue	2.06E-02	*
	Green vs red	5.20E-03	**
Fig 4, panel G	Green vs red	2.90E-03	**
Fig 4, panel H	GFP vs FST317	3.40E-02	*
	GFP vs FST344	2.40E-03	**
Fig 4, panel J	GFP vs FST344 (RET)	8.33E-03	**
	GFP vs FST344 (IL2RA)	2.08E-02	*
	GFP vs FST344 (CCL5)	9.33E-04	***

Fig 5, panel C	scr vs sh1	8.42E-03	**
	scr vs sh2	3.42E-03	**
Fig 5, panel E	scr vs sh1	1.90E-02	*
	scr vs sh2	5.70E-03	**
Fig 5, panel G	Green vs blue	4.58E-02	*
	Green vs red	3.16E-02	*
Fig 5, panel H	scr vs sh1	5.34E-03	**
	scr vs sh2	3.29E-03	**
Fig 6, panel B	Cas9 vs sgRNA#3	6.22E-03	**
	Cas9 vs sgRNA#4	5.32E-03	**
Fig 6, panel D	Cas9 vs sgRNA#3	4.45E-02	*
	Cas9 vs sgRNA#4	2.89E-02	*
Fig 6, panel F	Neg-ASO vs FST-ASO3	7.13E-03	**
Fig 6, panel G	Neg-ASO vs FST-ASO3	9.41E-03	**
Fig 7, panel C	Flt3 ^{+/+} vs Flt3 ^{ITD/+}	9.70E-03	**
Fig 7, panel D	Flt3 ^{+/+} vs Flt3 ^{ITD/+}	9.80E-03	**
Fig 7, panel G	pre-injection vs week 2	1.55E-02	*
Fig 7, panel H	scr vs sh1	3.64E-02	*
	scr vs sh2	3.54E-02	*
Fig 7, panel K	Pre vs week 6	8.89E-03	**
Fig 7, panel N	Pre vs 1	6.72E-03	**
	Pre vs 2	3.74E-03	**
Fig S2, panel A	HSC vs CN-AML	2.70E-03	**
	HSC vs t(15;17)/APL	6.00E-05	****
	HSC vs Complex	1.00E-04	***
	HSC vs inv(16)	2.00E-04	***
	HSC vs t(8;21)	7.00E-05	****
	HSC vs t(11q23)/MLL	3.00E-04	***
	HSC vs Trisomy 8	5.00E-04	***
	HSC vs del(5q)	1.70E-02	*
	del(7q)/7q-	1.26E-02	*
Fig S2, panel E	FLT3/WT vs FLT3/ITD	2.60E-03	**
Fig S2, panel G	FLT3/WT vs FLT3/ITD	3.76E-02	*

Fig S2, panel H	Mock vs ITD	7.32E-03	**
Fig S3, panel C	GFP vs FST317	5.96E-03	**
	GFP vs FST344	4.78E-03	**
Fig S3, panel D	FST317 vs GFP (Day 9)	2.12E-02	*
	FST344 vs GFP (Day 9)	4.64E-03	**
Fig S8, panel B	scr vs sh1	5.22E-03	**
	scr vs sh2	3.96E-03	**
Fig S9, panel A	FST317 vs GFP	2.84E-04	***
	FST344 vs GFP	6.15E-04	***
Fig S9, panel B	FST317 vs GFP	2.04E-03	**
	FST344 vs GFP	9.25E-03	**
Fig S10	Dx vs CR	9.79E-03	**

179 **Supplemental Table S1**

180 The p-values in the main figures and Appendix were summarized. *P<0.05, **P<0.01,

181 ***P<0.001.

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