

1 SUPPLEMENTAL INFORMATION

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3 **ROR1-Specific Cirmtuzumab Blocks ROR1-dependent Activation Of NF-κB And**
4 **Thereby Suppresses STAT3 Stimulation In Chronic Lymphocytic Leukemia**
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23 **Running title:** Targeting WNT5A/ROR1-Activation Of NF-κB In CLL

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25 **Supplementary methods**

26 ***Materials***

27 Human recombinant IL-6 (7270-IL), IL-8 (208-IL), CCL2 (279-MC), CCL3 (270-LD), CCL4
28 (271-BME), and CXCL1 (453-KC) were from R&D Systems (MN, USA). Anti-human IL6R
29 (561696) and isotype control (555749) were from BD biosciences (CA, USA).

30 ***Tissue culture***

31 MEC1 was maintained in DMSO with 20% fetal bovine serum (FBS) in a humidified
32 atmosphere at 37 °C with 5% CO₂.

33 ***Knockout of IL6R in MEC1-ROR1***

34 Crispr/Cas9-mediated knockout was performed according to our previous publication.¹
35 gRNA sequence was GGAGGAAGCATGCTGGCCGTCGG on exon 1 of human *IL6R*.

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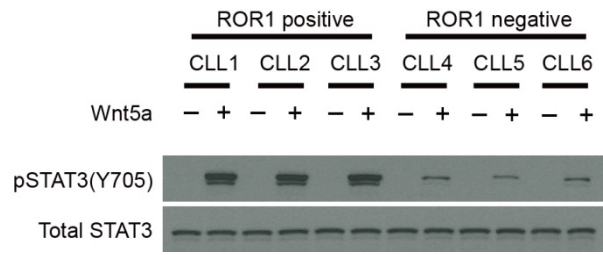
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Supplemental Figure 1

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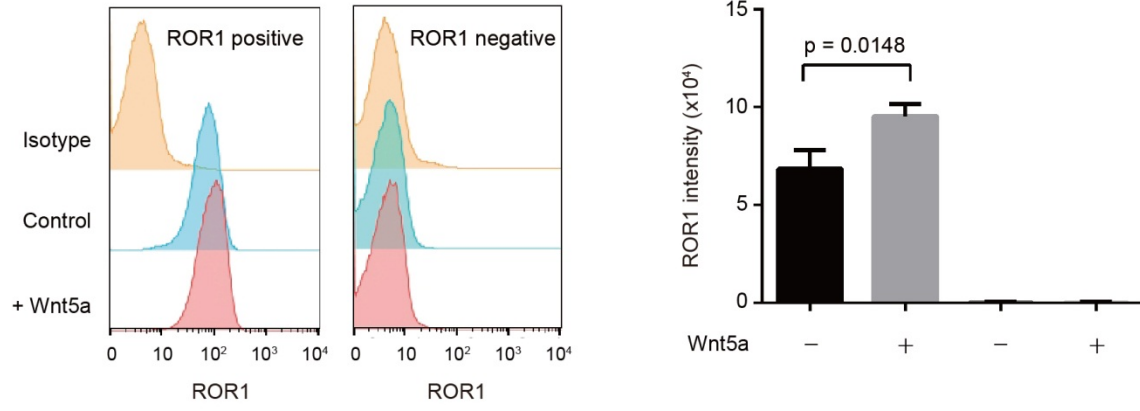
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Supplemental Figure 2

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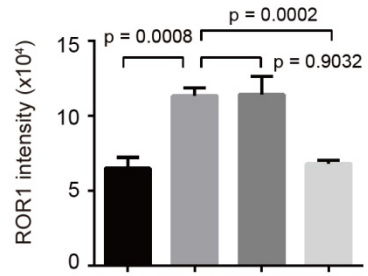
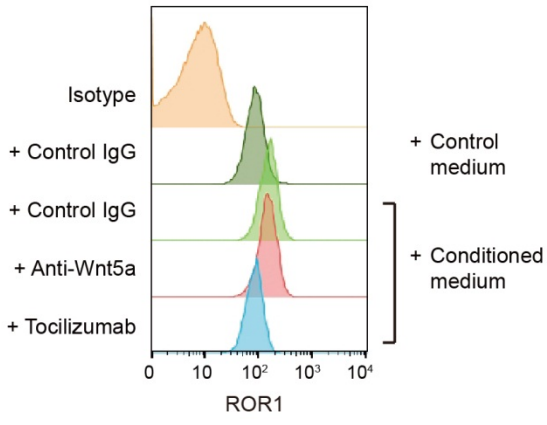
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Supplemental Figure 3

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Control medium	+	-	-	-
Conditioned medium	-	+	+	+
Control IgG	+	+	-	-
Anti-Wnt5a	-	-	+	-
Tocilizumab	-	-	-	+

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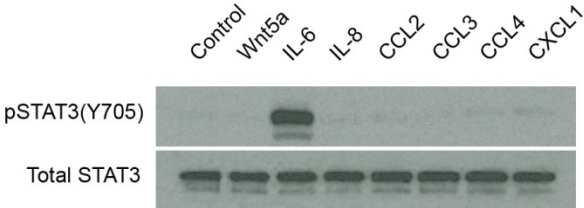
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Supplemental Figure 4



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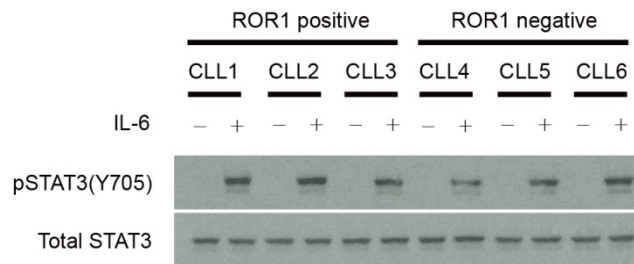
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Supplemental Figure 5

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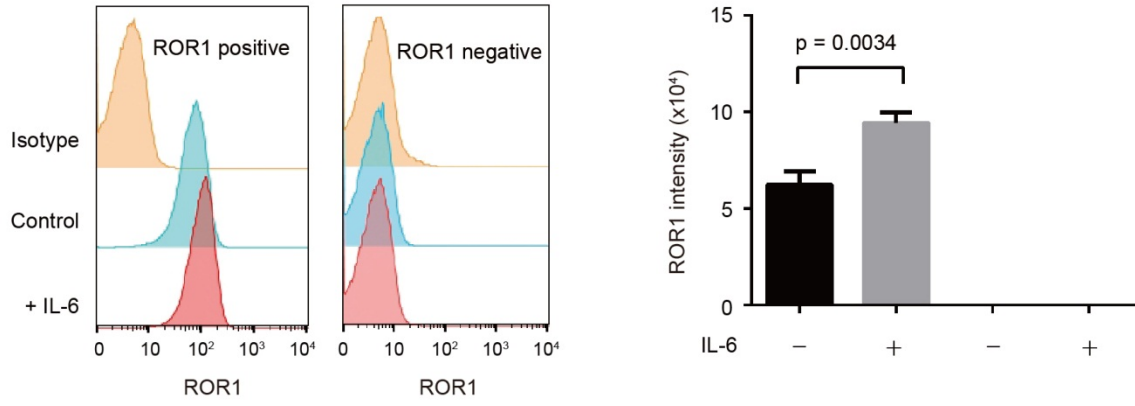
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Supplemental Figure 6

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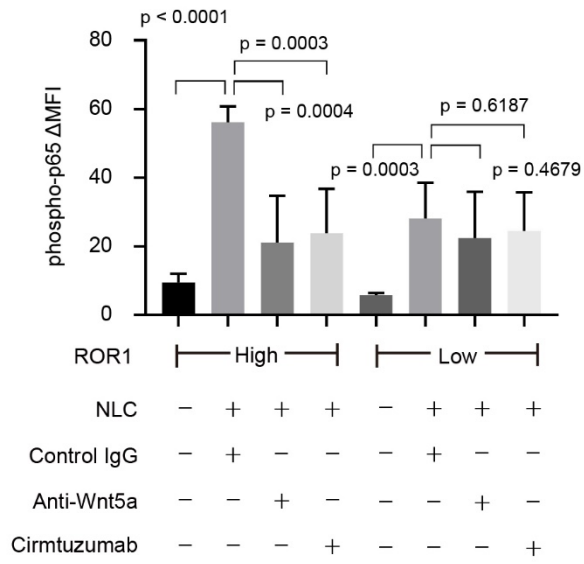
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Supplemental Figure 7

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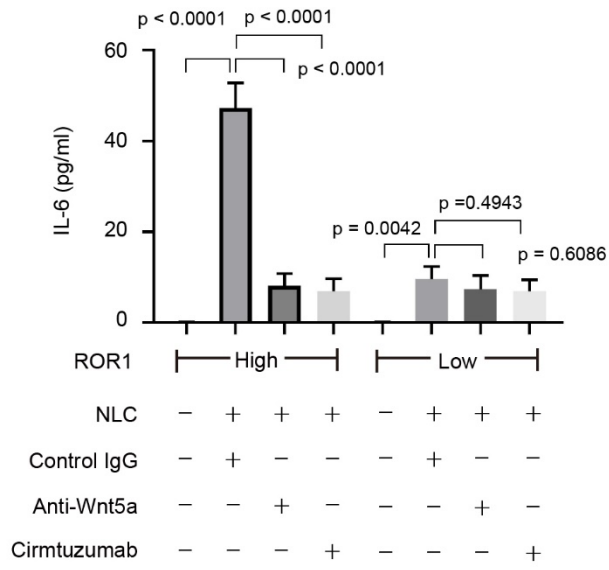
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Supplemental Figure 8

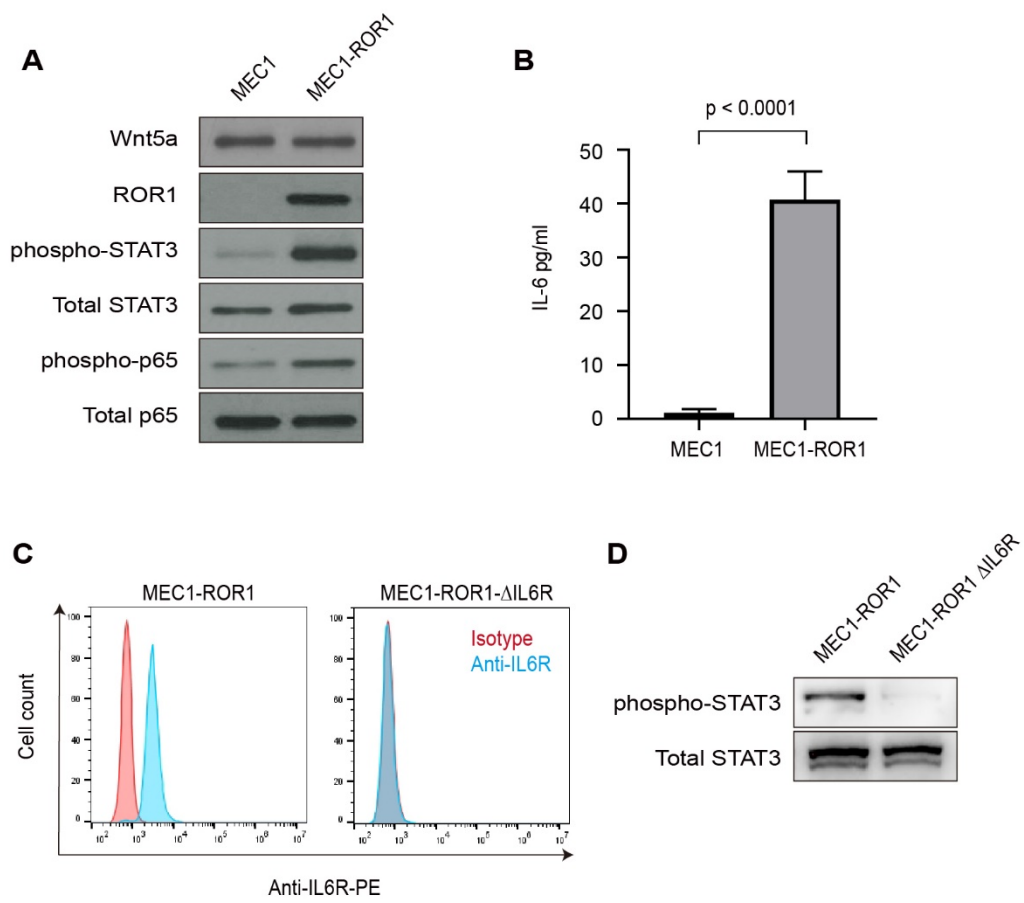
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Supplemental Figure 9

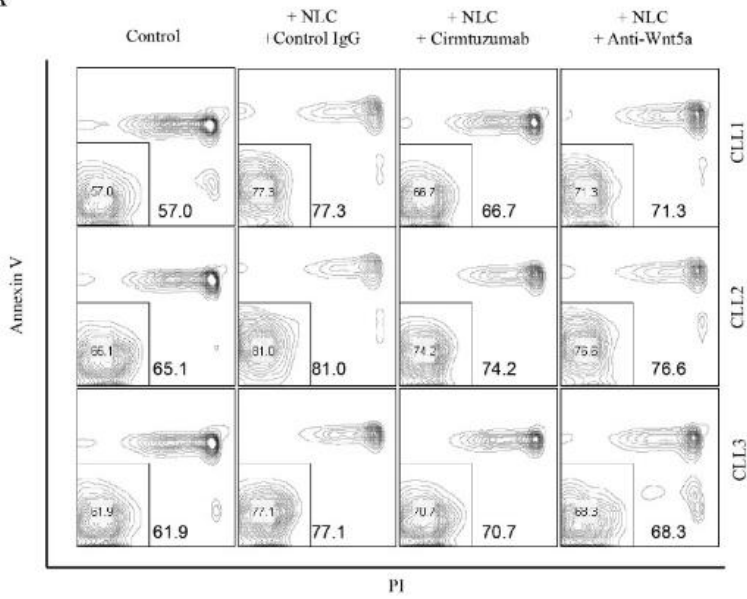


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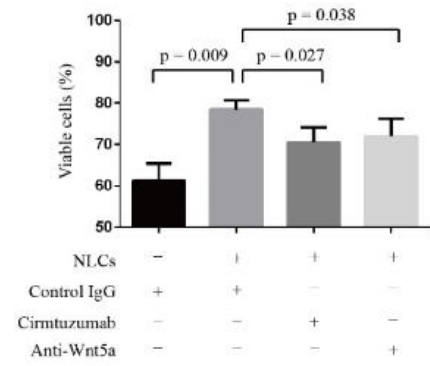
Supplemental Figure 10

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Supplemental Table S1

		ROR1(Δ MFI)		phospho-stat3 (Δ MFI)		phospho-p65 (Δ MFI)		
Sample ID	IGHV status	CD5highCXCR4dim	CD5dim CXCR4high	CD5highCXCR4dim	CD5dim CXCR4high	CD5highCXCR4dim	CD5dimCXCR4high	
ROR1^{high}	Patient 1	Unmutated	40	25	79	52	16	21
	Patient 2	Unmutated	49	33	15	12	70	47
	Patient 3	Unmutated	59	47	39	28	83	54
	Patient 4	Mutated	51	35	25	19	88	68
	Patient 5	Unmutated	51	35	27	18	70	57
	Patient 6	Mutated	54	42	31	17	68	42
	Patient 7	Mutated	69	56	27	16	33	19
	Patient 8	Mutated	65	43	45	28	23	12
	Patient 9	Unmutated	42	33	25	14	70	17
	Patient 10	Mutated	61	44	29	19	95	66
	Patient 11	Mutated	66	52	42	22	72	59
	Patient 12	Mutated	64	38	29	14	78	54
ROR1^{low}	Patient 13	Unmutated	26	24	14	11	43	32
	Patient 14	Unmutated	7	8	14	7	40	26
	Patient 15	Unmutated	8	9	14	8	52	46
	Patient 16	Unmutated	9	6	17	9	35	21
	Patient 17	Unmutated	8	8	10	8	39	23
	Patient 18	Unmutated	22	10	15	8	24	22
	Patient 19	Unmutated	8	10	17	8	50	28
	Patient 20	Mutated	5	8	11	5	32	17
	Patient 21	Mutated	8	8	17	8	47	22
	Patient 22	Mutated	26	12	8	5	21	15
	Patient 23	Mutated	12	9	15	9	33	22
	Patient 24	Mutated	22	11	16	10	31	22

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Supplemental Table S2

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The plasma levels of IL-6 in patients treated by cirmtuzumab

Patient	Pre-treatment of IL-6 (pg/ml)	Post-treatment of IL-6 (pg/ml)	Treatment	sampling
Patient #08	7.7	0.2	60 – 240 µg/kg	Two weeks after the 3 rd infusion
Patient #11	32.3	26.2	2 mg/kg	One week after the only infusion
Patient #04	5.7	0.1	2 mg/kg	One week after the 2 nd infusion
Patient #24	8.2	1.1	20 mg/kg	Two weeks after the 3 rd infusion
Patient #28	27.3	8.0	20 mg/kg	Two weeks after the 3 rd infusion

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111 **Supplementary Figure Legends**

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113 **Supplemental Figure 1** Wnt5a Induces Phosphorylation Of STAT3 Via ROR1 In CLL
114 Cells. The lysates of ROR1-positive or ROR1-negative CLL cells cultured overnight
115 without or with Wnt5a (200 ng/ml) were examined by immunoblot analysis probed with
116 anti-pSTAT3 (Y705) or anti-STAT3.

117

118 **Supplemental Figure 2** Wnt5a Induces Upregulation Of ROR1 In ROR1-positive CLL
119 Cells. The expression level of ROR1 was analyzed by flow cytometry in ROR1-positive
120 or ROR1-negative CLL cells with or without treatment of Wnt5a (200 ng/ml) for 16 hours.
121 Bar histograms represent ROR1 intensity. Error bars indicate standard deviation about
122 the mean (n = 3).

123

124 **Supplemental Figure 3** The Conditioned Medium Harvested From CLL-cell Cultured
125 With Wnt5a Induced Upregulation Of ROR1 Via Wnt5a/ROR1 In CLL Cells. The
126 expression level of ROR1 was analyzed by flow cytometry in CLL cells cultured with or
127 without NLC in the presence of control IgG, anti-Wnt5a, or tocilizumab for 16 hours. Bars
128 depict ROR1 intensities on each of the treated cell populations. Error bars provide the
129 standard deviation about the mean (n = 3).

130

131 **Supplemental Figure 4** Exogenous Factors Can Induce STAT3 Activation In CLL Cells.
132 CLL cells were treated for 30 minutes with Wnt5a (200 ng/ml), IL-6, IL-8, CCL2, CCL3,
133 CCL4, or CXCL1 (each at 20 ng/ml). Phosphorylation of STAT3 was analyzed by

134 immunoblot analyses of lysates from treated CLL cells and probed with anti-pSTAT3 (top
135 row) (Y705) or anti-STAT3 (bottom).

136

137 **Supplemental Figure 5** IL-6 Induces Phosphorylation Of STAT3 Independent Of ROR1
138 In CLL Cells. Cell lysates of ROR1-positive or ROR1-negative CLL cells with or without
139 treatment of IL-6 (20 ng/ml) for 30 minutes were examined by immunoblot analysis using
140 anti-pSTAT3 (Y705) or anti-STAT3.

141

142 **Supplemental Figure 6** IL-6 Induced Upregulation Of ROR1 In ROR1-positive CLL Cells.
143 The expression level of ROR1 was analyzed by flow cytometry in ROR1-positive or
144 ROR1-negative CLL cells with or without treatment of IL-6 (20 ng/ml) for 16 hours. Bars
145 represent the intensity of ROR1 staining for each of the treatment groups. Error bars
146 indicate the standard deviation (n = 3).

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148 **Supplemental Figure 7** NF- κ B was activated in CLL cells co-cultured with NLC through
149 Wnt5a/ROR1. Bar figures represent the intensity of phospho-p65 staining of CLL cells
150 from ROR1^{high} (n = 6) and ROR1^{low} (n = 6) patients co-cultured with or without NLC for
151 16 hours in the presence of control IgG, anti-Wnt5a, or cirmtuzumab. Error bars denote
152 standard deviation.

153

154 **Supplemental Figure 8** The production of IL-6 was upregulated in CLL cells co-cultured
155 with NLC through Wnt5a/ROR1. Bar figures represent the concentration of IL-6 in media
156 of CLL cells from ROR1^{high} (n = 6) and ROR1^{low} (n = 6) patients co-cultured with or without

157 NLC for 16 hours in the presence of control IgG, anti-Wnt5a, or cirmtuzumab. Error bars
158 denote standard deviation.

159
160 **Supplemental Figure 9** STAT3 was activated in MEC1-ROR1 by the autocrine of IL-6.
161 A. Immunoblot of lysates of MEC1 and MEC1-ROR1 with anti-Wnt5a, ROR1, pSTAT3,
162 tSTAT3, pp65 or tp65 antibodies. B. ELISA of IL-6 in medium culturing MEC1 and MEC1-
163 ROR1. Error bars denote standard deviation (n = 3). C. Flow cytometry analysis of MEC1-
164 ROR1 and MEC1-ROR1- Δ IL6R with anti-IL6R-PE antibody. D. Immunoblot of lysates of
165 MEC1-ROR1 and MEC1-ROR1- Δ IL6R with anti-pSTAT3 and tSTAT3.

166
167 **Supplemental Figure 10** Wnt5a-induced ROR1-signaling Contributes To The Protective
168 Effects Of NLC For CLL Cells *In Vitro*. A. Contour plots of CD5+CD19+ CLL cells stained
169 with Annexin V and propidium iodide (PI) following culture with or without NLC control
170 IgG, cirmtuzumab, or neutralizing antibody to Wnt5a, as indicated on top of the panel for
171 data on CLL cells from 3 patients. The proportion of viable CLL cells that do not stain
172 with Annexin V or PI is indicated in each panel. B. The mean percentages of viable CLL
173 cells for all patient samples (N=3) cultured in each condition are provided by the histogram.
174 The conditions are indicated at the bottom of the histograms. Error bars indicate the
175 standard deviation about the mean. Statistical differences using Student T test with
176 Bonferroni correction are indicated by the brackets.

177

178 **References**

- 179 1 Yu, J. et al. Wnt5a induces ROR1 to associate with 14-3-3zeta for enhanced
180 chemotaxis and proliferation of chronic lymphocytic leukemia cells. *Leukemia* **31**,
181 2608-2614, doi:10.1038/leu.2017.132 (2017).