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1 **SALL1 modulates CBX4 stability, nuclear bodies and regulation of target genes.**

2

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27

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29 **Keywords:** CBX4, SALL1, nuclear bodies, SUMO, ubiquitin

30

31 **ABSTRACT**

32 Development is orchestrated through a complex interplay of multiple
33 transcription factors. The comprehension of this interplay will help us to understand
34 developmental processes. Here we analyze the relationship between two key
35 transcription factors: CBX4, a member of the Polycomb Repressive complex 1
36 (PRC1), and SALL1, a member of the Spalt-like family with important roles in
37 embryogenesis and limb development. Both proteins localize to nuclear bodies and
38 are modified by the Small Ubiquitin-like Modifier, SUMO. Our results show that
39 CBX4 and SALL1 interact in the nucleoplasm and that increased SALL1 expression
40 reduces ubiquitination of CBX4, enhancing its stability. This is accompanied by an
41 increase in the number and size of CBX4-containing Polycomb bodies, and by a
42 greater repression of CBX4 target genes. Thus, our findings uncover a new way of
43 SALL1-mediated regulation of Polycomb bodies through modulation of CBX4
44 stability, with consequences in the regulation of its target genes, which could have an
45 impact in cell differentiation and development.

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48

49 INTRODUCTION

50 Development of higher organisms is orchestrated by a complex interplay of
51 regulatory networks involving multiple signaling pathways and transcriptional
52 regulatory factors. Two key families of transcriptional repressor proteins involved in
53 development are the Polycomb Group (PcG) and the Spalt-like (SALL) proteins.

54 PcG proteins are involved in epigenetic regulation and control cell fate during
55 embryonic development. These proteins accumulate in nuclear foci called Polycomb
56 (Pc) bodies, which are involved in transcriptional repression (Saurin et al., 1998;
57 Cheutin and Cavalli, 2012; Entrevan et al., 2016; Schuettengruber et al., 2017) and
58 form two distinct complexes: Pc Repressive Complex1 and 2 (PRC1 and PRC2),
59 conserved from flies to human. A crucial component of the PRC1 complex is CBX4.
60 CBX4 is required to maintain the transcriptionally repressive state of HOX genes
61 during development, and has an important role in several essential pathways. Thus, it
62 has been described to facilitate differentiation of hematopoietic stem cells (Klauke et
63 al., 2013), counteracting cellular senescence (Ren et al., 2019) and maintaining the
64 epithelial lineage identity via repression of non-epidermal lineage and cell cycle
65 inhibitor genes (Mardaryev et al., 2016). Moreover, CBX4 is recruited rapidly to sites
66 of DNA damage (Ismail et al., 2012) and has emerged as a critical component of the
67 DNA end resection machinery (Soria-Bretones et al., 2017).

68 SALL family members (SALL1 to SALL4), on the other hand, are important
69 regulators of animal development, being crucial for the formation of the limbs,
70 kidneys and the central and peripheral nervous systems, among other organs (de Celis
71 and Barrio, 2009). SALL proteins are characterized by the presence of several
72 precisely spaced copies of the zinc finger domain (de Celis and Barrio, 2009). They

73 also contain a N-terminal glutamine-rich region, which could have a role in
74 dimerization or protein-protein interactions (Kohlhase et al., 1998; Buck et al., 2000;
75 Sweetman et al., 2003; Borozdin et al., 2006), and a conserved N-terminal motif that
76 mediates its interaction with one of the major corepressor complexes in mammalian
77 cells, the Nucleosome Remodeling Deacetylase (NuRD) complex (Kiefer et al., 2002;
78 Lauberth and Rauchman, 2006). Like the PcG proteins, SALL1 and its homologues
79 localize in nuclear bodies, as it has been reported in cultured cells and *in vivo* (Netzer
80 et al., 2001; Kiefer et al., 2002; Sánchez et al., 2010; Abedin et al., 2011). However,
81 the nature and function of these bodies have not been explored.

82 CBX4 and SALL1 play important roles in different aspects of human health.
83 Dysregulation of CBX4 contributes to the occurrence and progression of human
84 tumors, in which it can act as either oncogene or tumor suppressor, depending on the
85 cellular context (Wang et al., 2016). Mutations in *SALL1*, on the other hand cause
86 Townes-Brocks Syndrome (TBS), an autosomal dominant syndrome characterized by
87 renal anomalies, hearing loss, congenital heart defects, and eye anomalies among
88 other symptoms (Kohlhase, 1993). TBS-causing mutations produce truncated SALL1
89 proteins lacking most of the zinc finger pairs, which aberrantly localize to the
90 cytoplasm and interfere with centrosomal components, resulting in the formation of
91 longer and more abundant primary cilia in patient-derived cells (Bozal-Basterra et al.,
92 2018; Bozal-Basterra et al., 2020).

93 As described for many other transcriptional regulatory factors, the localization
94 and activity of CBX4 and SALL1 can be modulated by post-translational
95 modifications, including conjugation to ubiquitin or ubiquitin-like (Ubl) proteins,
96 such as Small Ubiquitin-like Modifier (SUMO). Thus, CBX4 is SUMOylated and it is
97 a substrate of the SUMO-deconjugating enzyme SENP2 (Wotton and Merrill, 2007;

98 Kang et al., 2010). In addition, it was identified as a SUMO substrate in different
99 proteomic analyses (Golebiowski et al., 2009; Galisson et al., 2011; Hendriks et al.,
100 2014; Lamoliatte et al., 2014; Tammsalu et al., 2014; Hendriks et al., 2015; Xiao et
101 al., 2015; Hendriks and Vertegaal, 2016). Interestingly, CBX4 itself is proposed to be
102 a SUMO E3 ligase, and is involved in SUMOylation of the transcriptional corepressor
103 C-terminal-binding protein (CtBP) (Kagey et al., 2003), the nucleocytoplasmic
104 shuttling protein hnRNP (Pelisch et al., 2012), the transcriptional co-activator Prdm16
105 (Chen et al., 2018) and other chromatin-associated factors including CTCF, Dnmt3a
106 or Bmi1 (Li et al., 2007; MacPherson et al., 2009; Ismail et al., 2012). CBX4 has also
107 been found ubiquitinated and its polyubiquitination influences the dynamics of the
108 PRC1 at the chromatin and the regulation of downstream genes (Povlsen et al., 2012;
109 Mertins et al., 2013; Udeshi et al., 2013; Ning et al., 2017; Akimov et al., 2018; Wang
110 et al., 2020).

111 In the case of SALL1, interaction with SUMO1 and the SUMO E2 conjugase
112 UBC9 has been reported using yeast two-hybrid and *in vitro* assays, with
113 SUMOylation mapped to lysine 1086 (Netzer et al., 2002). Subsequently, SALL1 as
114 well as other SALL proteins, have been confirmed as targets of SUMOylation by
115 proteomics analyses (Golebiowski et al., 2009; Galisson et al., 2011; Hendriks et al.,
116 2014; Schimmel et al., 2014; Hendriks et al., 2015; Xiao et al., 2015; Hendriks and
117 Vertegaal, 2016). In *Drosophila*, SUMOylation of SALL homologues influences their
118 role in vein pattern formation in the wing and their transcriptional repressor activity
119 (Sánchez et al., 2010; Sánchez et al., 2011).

120 Remarkably, although different functional aspects of CBX4 and SALL1 have
121 been addressed in previous studies, a regulatory interplay between these proteins has
122 not been described so far. Interestingly, we identified CBX4, as well as other PcG

123 proteins, as a possible interactor of SALL1 by proximity proteomics (Bozal-Basterra
124 et al., 2018). In addition, *sall* genes and *Pc* interact genetically in *Drosophila*, as
125 mutations in the homologue *spalt-major* enhanced the phenotypical effects of *Pc*
126 group mutations during embryogenesis (Casanova, 1989; Landecker et al., 1994).
127 These findings, together with the localization of both proteins to nuclear bodies, as
128 well as the regulation by SUMO of both proteins, prompted us to further investigate a
129 potential functional or regulatory interplay between SALL1 and CBX4. We report
130 here a novel interaction between these two transcriptional regulators in the
131 nucleoplasm. Interestingly, SALL1 influences the stability of CBX4 by modulating its
132 ubiquitination, which might be related to changes in the regulatory capacity of CBX4
133 over HOX genes. Overall, we present here a novel mechanism of regulation of a
134 crucial factor in development, which has consequences for the regulation of its target
135 genes.

136

137

138 **MATERIALS AND METHODS**

139

140 **Cell culture and cell transfection**

141 Human U2OS (ATCC HTB-96) and HEK 293FT (Invitrogen) cells, as well as
142 derived cell lines, were cultured at 37°C with 5% of CO₂ in DMEM (Dulbecco's
143 modified Eagle's medium; Gibco) supplemented with 10% FBS and 1%
144 penicillin/streptomycin (Gibco). HEK 293FT cells were transiently transfected using
145 calcium phosphate in 10 cm dishes with 3-10 µg of DNA using different sets of
146 plasmids according to each experiment. Briefly, DNA was mixed with 500 µl of 2.5
147 M CaCl₂ and H₂O (1:10). The was added drop by drop to the same volume of HBS

148 (NaCl 280 mM, KCl 10 mM, Na₂HPO₄ 1.5 mM, Glucose 12 mM, HEPES 50 mM),
 149 incubated for 10-15 minutes and added to the cells. U2OS cells were transiently
 150 transfected using PEI (Sigma Aldrich #408727), or Effectene (Qiagen) according to
 151 the manufacturers' instructions.

152

153 **Generation of plasmids**

154 The following plasmids were used in this study (Table 1). DNA fragments
 155 were amplified from the indicated plasmids by high-fidelity PCR Platinum SuperFi
 156 (Thermo). PCR products were purified using mi-Gel Extraction kit (Metabion),
 157 digested if necessary using the restriction enzymes (Fermentas; NEB) and assembled
 158 by ligation or using NEBuilder HiFi Master Mix (NEB). All resulting plasmids were
 159 checked by sequencing. Cloning details are available upon request.

160

161 **Table 1. Plasmids used in the study**

Name of the vector	Reference	Parental vectors	Cloning sites/notes
<i>CAG-bioSUMO3-T2A-BirA^{opt}-T2A-GFP^{puro}</i>	(Pirone et al., 2017)	-	-
<i>CMV-CBX4-YFP</i>	This work	<i>pEYFP-N1</i>	<i>EcoRI-Sall</i> (KAN); CBX4 generated by high-fidelity PCR
<i>CMV-SALL1-YFP</i>	(Pirone et al., 2017)	<i>pEYFP-N1</i>	<i>EcoRI-Sall</i> (KAN); SALL1 generated by high-fidelity PCR
<i>CMV-SALL1ΔSUMO-YFP</i>	This work	<i>CMV-SALL1-YFP</i>	<i>EcoRI-Sall</i> ; mutants introduced by overlap extension PCR (KAN); K571R; K592R; K982R; K1086R
<i>CMV-SALL1ΔSIM-YFP</i>	This work	<i>CMV-SALL1-YFP</i>	<i>EcoRI-Sall</i> ; mutants introduced by overlap extension PCR (KAN); predicted SIMs mutated to AAAA;

			SIM71: VLIV; SIM195: VIIE; SIM254: ILLI; SIM1252: ISVI
<i>CMV-SALL1-2xHA</i>	This work	<i>CMV-SALL1-YFP</i>	EYFP exchanged for 2xHA using <i>SalI-NotI</i> (KAN)
<i>CMV-SALL1⁸²⁶-2xHA</i>	This work	<i>CMV-SALL1(826)-YFP</i>	EYFP exchanged for 2xHA using <i>SalI-NotI</i> (KAN)
CB6-HA-N	M. Way lab (CRUK, London)	<i>CB6</i>	CB6 has CMV promoter and confers neo selection; contains N-terminal HA epitope and MCS (AMP)
<i>CMV-EGFP-β-galactosidase</i>	This work	<i>pEGFP-N1</i>	LacZ subcloned from pIND/lacZ (Invitrogen)
<i>CB6-HA-SALL1</i>	This work	<i>CB6-HA-N</i>	<i>SALL1</i> from <i>CMV-SALL1-YFP</i>
<i>CB6-HA-SALL1ΔSUMO</i>	This work	<i>CB6-HA-N</i>	<i>SALL1</i> from <i>CMV-SALL1 ΔSUMO-YFP</i>
<i>CB6-HA-CBX4</i>	This work	<i>CB6-HA-N</i>	CBX4 from <i>CMV-CBX4-YFP</i>
<i>CMV-SALL1-BirA*</i>	This work	<i>CMV-SALL1-YFP</i>	Exchanged YFP for <i>BirA*(BioID)</i> by <i>SalI-NotI</i>
<i>CMV-Pc-BirA*</i>	This work	<i>CMV-SALL1-BirA*</i>	<i>Drosophila Pc</i> (PCR amplified) exchanged for <i>SALL1</i> using <i>EcoR1-Sal1</i> (KAN); Pc source: Addgene #1927
<i>CMV-CBX4-BirA*</i>	This work	<i>CMV-SALL1-BirA*</i>	CBX4 (PCR amplified) exchanged for <i>SALL1</i> using <i>EcoR1-Sal1</i> (KAN)
CMV-BirAopt-2A-puro	(Pirone et al., 2017)	-	-
CMV-bioUB-2A-BirAopt-2A-puro	(Pirone et al., 2017)	-	-
LL-CMV-GFS-SALL1-IRES-puro	This work	LL-CMV-GFS-IRES-puro	<i>SALL1</i> inserted into modified version of Lentilox3.7; expresses N-terminal GFP-FLAG-STREP

			tag
TripZ-SALL1-2xHA-puro	This work	<i>CMV-SALL1-2xHA; TRIPZ</i>	Inserted SALL1-2xHA amplicon into BshT1-Mlu1TRIPZ (Dharmacon)
<i>pcDNA3</i>	Invitrogen	-	-
Lenti-Cas9-blast vector	Addgene #52962	-	-
psPAX2	Addgene #12260	-	-
pMD2.G (VSV-G envelope)	Addgene #12259	-	-
<i>pEYFP-N1, pEYFP-C</i>	Clontech	-	-

162 (KAN) or (AMP) indicate the antibiotic resistant cassette (kanamycin or ampicillin,
163 respectively) in the vector for bacterial transformation.

164

165 **Lentiviral transduction**

166 Lentiviral expression constructs were packaged using psPAX2 and pMD2.G in
167 HEK 293FT cells, and cell culture supernatants were used to transduce HEK 293FT
168 cells to generate stable cell populations expressing SALL1 (constitutive: LL-GFS-
169 SALL1-IRES-puro; or inducible: TripZ-SALL1-2xHA-puro). Selection was
170 performed using 1 µg/ml of puromycin.

171

172 **Bioinformatics analyses**

173 SUMOylation sites and SUMO-interacting motif predictions were searched
174 using SUMOplot (<http://www.abgent.com/sumoplot>), GPS-SUMO
175 (<http://sumosp.biocuckoo.org>; (Zhao et al., 2014)), and JASSA programs (Beauchair
176 et al., 2015). Sequence search and comparison was performed using BLAST
177 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Alignments were performed using Clustal
178 (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

179

180 **SUMOylation and Ubiquitination assays in cultured cells**

181 For the isolation of SUMOylated SALL1, one 10 cm dish of HEK 293FT cells
182 was transfected with 7 µg of *CMV-SALL1-2xHA*, *CMV-SALL1ΔSUMO-2xHA*, and
183 3µg of *CAG-bioSUMO3-T2A-BirA^{opt}-T2A-GFPpuro* or *CAG-BirA^{opt}-T2A-GFPpuro*
184 as control. Isolation of SUMOylated protein was done according to previously
185 reported methodology (Pirone et al., 2016; Pirone et al., 2017).

186 For the ubiquitination assay of CBX4, one 10 cm dish was transfected with 5 µg
187 of *CMV-SALL1-YFP*, *CMV-SALL1ΔSUMO-YFP*, *CMV-GFP-β-Galactosidase*, *CMV-*
188 *BirA-2A-puro*, *CMV-bioUB-2A-BirA-2A-puro*, or *CB6-HA-CBX4*. After transfection,
189 medium was supplemented with biotin at 50 µM. 24 hours after transfection, plates
190 were treated with MG132 (10 µM, 12 hours; Calbiochem). Transfected cells were
191 collected after 48–72 hours, washed 3 times with phosphate buffered saline (PBS) and
192 resuspended in lysis buffer [0.5 ml/10 cm dish; 8 M urea, 1% SDS, 50 mM N-
193 ethylmaleimide, 1x protease inhibitor cocktail (Roche) in 1x PBS]. Sonication was
194 performed to reduce sample viscosity and samples were cleared by centrifugation at
195 room temperature (RT). High-capacity NeutrAvidin-agarose beads (Thermo
196 Scientific) were equilibrated and 30-60 µl suspension was used for incubation with
197 extracts (12-18 hours; RT; gentle agitation). Beads were subjected to stringent washes
198 using the following washing buffers all prepared in 1x PBS (Franco et al., 2011):
199 WB1 (8 M urea, 0.25% SDS); WB2 (6 M Guanidine-HCl); WB3 (6.4 M urea, 1 M
200 NaCl, 0.2% SDS), WB4 (4 M urea, 1 M NaCl, 10% isopropanol, 10% ethanol and
201 0.2% SDS); WB5 (8 M urea, 1% SDS); and WB6 (2% SDS). Samples were eluted in
202 50 µl of Elution Buffer (4x Laemmli sample buffer, 100 mM DTT) by two cycles of
203 heating (5 minutes; 99 °C), with vortexing in between. Beads were separated by
204 centrifugation (18000x g, 5 minutes).

205 For the isolation of ubiquitinated endogenous CBX4 from cells lysates, 10 cm
206 dishes were transfected with 5 µg of *CMV-SALL1-2xHA* plasmid or with with
207 pcDNA3 plasmid as control. After 48 hours, cells were washed three times with 1x
208 PBS and lysed in 500 µl of TUBEs buffer [20 mM Phosphate buffer, pH 7.5 (Sigma),
209 2 mM EDTA (Sigma), 50 mM sodium fluoride (Sigma), 5 mM tetra-sodium pyro-
210 phosphate (Sigma), and 10 mM β-glycerol 2-phosphate (Sigma)]. The buffer was
211 filtered through a 0.22 µm membrane and stored at 4°C. 80 µl of the lysate were taken
212 as input. Ubiquitinated material was isolated using Tandem Ubiquitin Binding
213 Entities (TUBEs) based on RAD23 Homolog A (RAD23A) ubiquitin binding
214 domains fused to GST and expressed in bacteria (Hjerpe et al., 2009; Aillet et al.,
215 2012). To eliminate proteins with binding affinity for the beads (Glutathione
216 Sepharose 4B, GE Healthcare), lysates were incubated with 125 µg of GST bound to
217 glutathione-agarose beads for 1 hour at 4°C and centrifugated for 2 minutes at 1000
218 rpm. After washing GST-TUBES beads with cold 1x PBS twice, supernatants were
219 added, incubated for 1 hour at 4°C and centrifugated for 2 minutes at 1000 rpm. The
220 supernatants were then removed and beads were washed 3 times with TUBEs buffer.
221 The beads were washed 3 times with PBS-Tween 0.5% and twice with TUBEs buffer
222 containing NaCl (0.5 M). Finally, the beads were resuspended in 50 µl of Boiling
223 buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, Bromophenol Blue, 10% β-
224 mercaptoethanol) warmed at 60°C before use.

225

226 **In vitro SUMOylation**

227 Using PCR templates with incorporated 5' T7 priming site +/- 3' epitope-tags,
228 SALL1-2xHA and CBX4 were transcribed/translated *in vitro* using the TNT ® Quick
229 Coupled Transcription/Translation System (Promega) according to the manufacturer's

230 instruction and were then incubated in a buffer containing an ATP regenerating
231 system [(50 mM Tris pH 7.5, 10 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate
232 (Sigma), 3.5 U/ml of creatine kinase (Sigma), and 0.6 U/ml of inorganic
233 pyrophosphatase (Sigma)], 10 µg of SUMO1 or a combination of 5 µg of SUMO2
234 and SUMO3, 0.325 µg UBC9 and 0.8 µg of purified SAE1/2 (ENZO Life Sciences).
235 SALL1 SUMOylation was checked adding 0.5 to 2 µl of *in vitro*
236 transcribed/translated protein in the SUMOylation assay. Reactions were incubated at
237 30°C for 2 hours and stopped by addition of SDS sample buffer.

238

239 **GFP-Trap co-pulldown**

240 HEK 293FT cells were plated at 25-30% confluence. Transient transfections
241 were performed using calcium phosphate in a 10 cm dish with 5 µg of *CMV-CBX4-*
242 *YFP*, *CMV-SALL1-YFP*, *CMV-SALL1ΔSUMO-YFP*, *CMV-SALL1ΔSIM-YFP*, *CMV-*
243 *YFP*, *CMV-SALL1-2xHA*, *CMV-SALL1-826-2xHA*, *CB6-HA*, *CB6-HA-SALL1*, *CB6-*
244 *HA-SALL1ΔSUMO* or *CB6-HA-CBX4* in complete medium. All steps after
245 transfection were performed at 4°C. Two days after transfection, cells were washed 3
246 times with cold 1x PBS and detached from the dish with a scraper. Cells of 10 cm
247 dishes were lysed by adding 1 ml of Lysis Buffer [25 mM Tris-HCl pH 7.5, 150 mM
248 NaCl, 1 mM EDTA, 1% NP-40, 0.5% Triton X-100, 5% glycerol, protease inhibitors
249 (Roche)] followed by incubation on a rotating wheel for 30 minutes at 4°C. Lysates
250 were sonicated and spun down at 25000x g for 20 minutes. After saving 40 µl of
251 supernatant (input), the rest of the lysate was incubated overnight with 30 µl of
252 equilibrated GFP-Trap resin (Chromotek) in a rotating wheel. Beads were washed 5
253 times for 5 minutes each with washing buffer (25 mM Tris-HCl pH 7.5, 300 mM
254 NaCl, 1 mM EDTA, 1% NP-40, 0.5% Triton X-100, 5% glycerol). Beads were

255 centrifuged at 2000x g for 2 minutes after each wash. For elution, samples were
256 boiled for 5 minutes at 95°C in 2x Laemmli buffer.

257

258 **BioID analysis of interactions**

259 Proximity interaction between CBX4 or Pc proteins to SALL1 was verified by
260 the BioID method (Roux et al., 2013), consistent on fusing them to a promiscuous
261 form of the enzyme BirA (BirA*) and to isolate the biotinylated material by
262 streptavidin-beads pulldowns. HEK 293FT cells were transfected with 5 µg of CMV-
263 CBX4-BirA* or CMV-Pc-BirA* in combination with *CMV-SALL1-2xHA* or *CMV-*
264 *SALL1826-2xHA*. After 24 hours, the medium was supplemented with 50 mM of
265 biotin. At 48 hours, cells were washed three times in cold 1x PBS and collected in 1
266 ml of lysis buffer [8 M urea, 1% SDS, protease inhibitor cocktail (Roche) in 1x PBS].
267 Lysates were sonicated and cleared by centrifugation, incubated overnight with 40 µl
268 of equilibrated NeutrAvidin-agarose beads (Thermo Scientific) and washed with WB1
269 to 6 as indicated in the ubiquitination protocol above. Elution was done as previously
270 described using 50 µl of Elution Buffer (4x Laemmli sample buffer, 100 mM DTT)
271 by two cycles of heating (5 minutes, 99 °C), with vortexing in between. Beads were
272 separated by centrifugation (18000x g, 5 minutes).

273

274 **Cycloheximide assay**

275 3 x 10⁵ HEK 293FT cells per well were plated in 6-well plates. Four hours later,
276 cells were transfected with 2 µg of *CMV-SALL1-YFP*, *CMV-SALL1ΔSUMO-YFP* or
277 *CMV-GFP-β-Galactosidase* plasmid per well using the calcium phosphate method. 24
278 hours after transfection, cells were treated with 50 µg of cycloheximide (CHX, 50
279 µg/ml) in combination or not with MG132 (10 µM) for different time points (0, 4, 8

280 or 16 hours). Cells were lysed in RIPA buffer [150 mM NaCl, 1.0% NP-40, 0.5%
281 sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, protease inhibitors (Roche)]
282 and analyzed by Western blot.

283

284 **Western blot**

285 Samples were boiled at 95° for 5 minutes. Proteins were separated by SDS-
286 PAGE (BioRad) and blotted using wet transfer to nitrocellulose membranes (0.45 µm
287 pore; Cytiva). Membranes were blocked in 1x PBS with 0.1% Tween-20 (PBS-T) and
288 5% non-fat dry milk (blocking buffer) for 1 hour and, for biotin detection, Casein
289 Blocking Buffer 1x (Sigma #B6429). After that, membranes were incubated in
290 blocking buffer for 1 hour at RT or overnight at 4°C with the following primary
291 antibodies: mouse monoclonal anti-HA (Sigma, 1:1000, #H3663), mouse monoclonal
292 anti-β-Actin (Sigma, 1:1000, #A2228), mouse monoclonal anti-GFP (Roche, 1:1000,
293 #11814460001), mouse monoclonal anti-SALL1 (R&D, 1:1000, #PP-K9814-00),
294 rabbit polyclonal anti-CBX4 (Proteintech, 1:1000, #18544-1-AP), rabbit polyclonal
295 anti-Avitag (GeneScript, 1:1000, #A00674), or rabbit monoclonal Vinculin (Cell
296 Signaling, 1:1000, #13901S).

297 After three washes with PBS-T, the blots were incubated for 1 hour with
298 secondary antibodies: HRP-conjugated anti-mouse or anti-rabbit (1:5000, Jackson
299 ImmunoResearch # 115-035-062 or # 111-035-045, respectively), HRP-conjugated
300 anti-biotin (1:1000, Cell Signaling Technology #7075), HRP-conjugated anti-tubulin
301 (1:5000, Proteintech #66031), or HRP-conjugated anti-GAPDH (1:5000, Proteintech
302 #60004). Membranes were washed three times in PBS-T, developed using Clarity
303 Western ECL substrate (Biorad) or Super Signal West Femto (Pierce), and
304 chemiluminescent signals detected using a ChemiDoc camera system (Biorad).

305 Quantification of bands was performed using Fiji software and normalized to Actin,
306 GAPDH or Vinculin levels, unless otherwise indicated. At least three independent
307 blots were quantified per experiment.

308

309 **Immunostaining and microscopy analysis**

310 For immunostaining and microscopy analysis, 50000 cells per well were seeded
311 in a 24 well-plate on 12 mm diameter round acid-washed sterile coverslips. U2OS
312 cells were transfected with 2 µg of *CMV-SALL1-YFP*, *CMV-SALL1ΔSUMO-YFP* or
313 *pEYFP-C1*, 1.5 µg of *CMV-SALL1-YFP* or HEK 293FT_TripZ-SALL1-2xHA were
314 used.

315 After 2 days cells were washed 3 times with cold 1x PBS, fixed in 4%
316 paraformaldehyde (Santa Cruz) supplemented with 0.1% Triton X-100 in 1x PBS for
317 20 minutes at RT. Then, coverslips were washed 3 times with 1x PBS to remove the
318 fixative. Blocking was performed in blocking buffer (1% BSA, 0.3% Triton X-100,
319 25 mM NaCl in 1x PBS) for 1 hour at RT. Incubation with primary antibodies diluted
320 in blocking solution was performed during 1 h at 37°C in a humidity chamber or
321 overnight at 4°C. The following primary antibodies were used: rabbit polyclonal anti-
322 SALL1 (1:200, Abcam #31905), mouse monoclonal anti-GFP (1:500, Roche
323 #11814460001), mouse monoclonal anti-PML (Promyelocytic Leukemia Protein)
324 (1:100, Santacruz #sc-966), mouse monoclonal anti-SC35 (Splicing Component, 35
325 KDa, also known as Serine And Arginine Rich Splicing Factor 2) (1:200, BD
326 Pharmingen #556363), rabbit polyclonal anti-CBX4 (1:100, Proteintech #18544-1-
327 AP), rabbit polyclonal anti-SUMO2/3 (1:100, Eurogentec #AV-SM23-0100), mouse
328 monoclonal anti SUMO1 (1:100, Developmental Studies Hybridoma Bank, DSHB,
329 #21C7) or mouse monoclonal anti-SUMO2 (1:100, DSHB #8A2). Endogenous

330 SALL1 or SALL1-2xHA in HEK 293FT_TripZ-SALL1-2xHA cells were stained by
331 a primary antibody against SALL1 (R&D, 1:100, #PP-K9814-00).

332 After incubation with the primary antibody, cells were gently washed 3 times
333 with 1x PBS and then incubated with the secondary antibody in the dark for 1 hour at
334 RT. The secondary antibodies conjugated to fluorophores used were donkey anti-
335 mouse or anti-rabbit Alexa Fluor 488, Alexa Fluor 568 or Alexa Fluor 647 (1:200,
336 Molecular Probes). To visualize the nuclei, we incubated the cells with DAPI
337 (1:15000, Roche #10236276001) for 5 minutes at RT. Another 3 washes were
338 performed to remove unbound secondary antibody. Finally, coverslips were mounted
339 using Prolong Gold antifade reagent (Molecular Probes #P36930) and stored in the
340 dark at 4°C.

341 Stained cells were visualized using an Upright Fluorescent Microscope
342 Axioimager D1 or a Leica SP2 or SP8 confocal microscope with 63x objective. For
343 the quantification of Pc bodies, Fiji software was used.

344

345 **Proximity Ligation Assays**

346 U2OS cells were plated and transfected by PEI in 6-well plates with 2 µg of
347 *CMV-SALL1-2xHA* or *pcDNA3*. After 2 days, cells were transferred to an 8-well
348 chamber slide (LabTek #177410) and allowed to attach for 12 hours. Proximity
349 Ligation Assay (PLA) was performed using the Duolink In Situ Red kit (Olink
350 Biosciences; (Gullberg et al., 2004; Söderberg et al., 2006) according to the
351 manufacturer's instructions. Primary antibodies used: mouse monoclonal anti-SALL1
352 (1:250, R&D Systems #PP-K9814-00); rabbit polyclonal anti CBX4 (1:100,
353 Proteintech #18544-1-AP). Images were recorded on a Leica SP8 confocal
354 microscope system using 488 nm and 561 nm wavelengths for excitation and a 63x

355 lens for magnification, and were analyzed with the Leica confocal software, Adobe
356 Photoshop and ImageJ softwares.

357

358 **Reverse transcription-quantitative PCR (RT-qPCR) analysis**

359 HEK 293FT cells transfected with 5 μ g of *CMV-SALL1-YFP*, *CMV-*
360 *SALL1 Δ SUMO-YFP* or *CMV-GFP- β -Galactosidase* plasmids, or HEK 293-TripZ-
361 SALL1-2xHA_puro cells induced with different concentrations of doxycycline (dox),
362 were used for RT-qPCR analysis. 48 hours after transfection, or 72 hours after
363 induction, total RNA was obtained by using EZNA Total RNA Kit (Omega) and
364 quantified using a Nanodrop spectrophotometry. cDNAs were prepared using the
365 SuperScript III First-Strand Synthesis System (Invitrogen) using 1 μ g of total RNA in
366 20 μ l volume per reaction. qPCR was done using PerfeCTa SYBR Green SuperMix
367 Low Rox (Quantabio). Reactions were performed in 20 μ l, adding 5 μ l of cDNA and
368 0.5 μ l of each primer (10 μ M), in a CFX96 thermocycler (BioRad) using the
369 following protocol: 95°C for 5 minutes and 40 cycles of 95°C for 15 seconds, 56°C or
370 62°C for 30 seconds and 72°C 20 seconds. Melting curve analysis was performed for
371 each pair of primers between 65°C and 95°C, with 0.5°C temperature increments
372 every 5 seconds. Relative gene expression data were analyzed using the $\Delta\Delta$ Ct method
373 (Livak and Schmittgen, 2001). Reactions were carried out in duplicate and results
374 were derived from at least three independent experiments, normalized to GAPDH and
375 presented as relative expression levels. Primer sequences are listed in Table 2.

376 **Table 2. Oligonucleotide sequences used for RT-qPCR.**

Name	Sequence
hHoxa11_for	5'-AACGGGAGTTCTTCTTCAGCGTCT-3'
hHoxa11_rev	5'-ACTTGACGATCAGTGAGGTTGAGC-3'
hHoxb4_for	5'-AGGTCTTGGAGCTGGAGAAGGAAT-3'
hHoxb4_rev	5'-GGTGTTGGGCAACTTGTGGTCTTT-3'

hHoxb7_for	5'-AGACCCTGGAGCTGGAGAAAGAAT-3'
hHoxb7_rev	5'-ATGCGCCGGTTCTGAAACCAAATC-3'
hHoxb13_for	5'-TACGCTGATGCCTGCTGTCAACTA-3'
hHoxb13_rev	5'-AGTACCCGCCTCCAAAGTAACCAT-3'
hHoxc6_for	5'-AGGACCAGAAAGCCAGTATCCAGA-3'
hHoxc6_rev	5'-ATTCCTTCTCCAGTTCCAGGGTCT-3'
hHoxc10_for	5'-TGAAATCAAGACGGAGCAGAGCCT-3'
hHoxc10_rev	5'-TTGCTGTCAGCCAATTTCTGTGG-3'
hHoxc12_for	5'-AGGGAAGTCTCAGACCGCTTGAAT-3'
hHoxc12_rev	5'-AGAGCTTGCTCCCTCAACAGAAGT-3'
hHoxd13_for	5'-ATGTGGCTCTAAATCAGCCGGACA-3'
hHoxd13_rev	5'-AGATAGGTTCGTAGCAGCCGAGAT-3'
hGata4_for	5'-TCTCAGAAGGCAGAGAGTGTGTCA-3'
hGata4_rev	5'-GGTTGATGCCGTTTCATCTTGTGGT-3'
hGAPDH_for	5'-CATGTTTCGTCATGGGTGTGAACCA-3'
hGAPDH_rev	5'-AGTGATGGCATGGACTGTGGTCAT-3'
hSALL1_for	5'-GCTTGCACTATTTGTGGAAGAGC-3'
hSALL1_rev	5'-GAACTTGACGGGATTGCCTCCT-3'
hCBX4_for	5'-CATCGAGAAGAAGCGGATCCGCAAG-3'
hCBX4_rev	5'-CTGTTCTGGAAGGCGATCAGCAGCC-3'

377

378

379 **Statistical analysis**

380 Statistical analysis was performed using GraphPad 7.0 software. Data were
381 analyzed by Shapiro-Wilk normality test and Levene's test of variance. We used
382 Mann Whitney-U test or Unpaired T-test for comparing two groups and One-way
383 ANOVA for more than two groups. P values were represented by asterisks as follows:
384 (*) P-value < 0.05; (**) P-value < 0.01; (***) P-value < 0.001; (****) P-value <
385 0.0001. Differences were considered significant when P < 0.05.

386

387

388 **RESULTS**

389

390 **SALL1 does not colocalize with CBX4 in nuclear bodies**

391 In agreement with previous reports (Netzer et al., 2001; Kiefer et al., 2002;
392 Sánchez et al., 2010; Abedin et al., 2011), we detected endogenous SALL1 in discrete
393 domains in the nucleus of U2OS human osteosarcoma cells (Figure S1A). Similar
394 results were obtained in U2OS cells transfected with human SALL1-YFP (Figure
395 S1B, C). These SALL1 foci were reminiscent of Pc bodies, where PRC proteins, such
396 as CBX4 accumulate. Thus, we hypothesized that SALL1 and CBX4 could colocalize
397 in nuclear bodies.

398 To test this hypothesis, *SALL1-YFP* plasmid was transfected into U2OS cells,
399 where endogenous CBX4 was visualized by immunofluorescence using anti-CBX4
400 specific antibodies. However, SALL1 and CBX4 were found to localize to different
401 subsets of nuclear bodies (Figure 1A).

402 In order to further characterize the nature of CBX4 and SALL1 bodies, we
403 explored their possible colocalization with SUMO. We transfected U2OS cells with
404 SALL1-YFP and examined its localization, and that of endogenous CBX4, with
405 SUMO using immunofluorescence. While CBX4 did not colocalize with SUMO1 or
406 SUMO2/3 (Supplementary Figure S2A, B), a partial colocalization between SALL1
407 and SUMO proteins was observed: some of the SALL1 bodies clearly colocalized
408 with SUMO1 and SUMO2/3, while other SALL1 bodies did not (Figure 1 and
409 Supplementary Figure S2E). Conversely, some SUMO1 and SUMO2/3 bodies
410 colocalized with SALL1, while others did not. These results fit with the well-known
411 heterogenic nature of nuclear bodies (Zidovska, 2020). Neither CBX4, nor SALL1
412 colocalize with other nuclear factors, such as PML (Supplementary Figure S2C, H) or
413 SC35 (Supplementary Figure S2D, I)

414 As shown previously, SALL1 undergoes SUMOylation in cells (Pirone et al.,
415 2017), which might modulate its localization. To test this possibility, we generated a
416 SALL1 SUMO mutant (SALL1 Δ SUMO) by mutating four lysine residues (K571,
417 K592, K982 and K1086) to arginine (Supplementary Figure S3A). These residues
418 correspond to the four SUMOylation motifs conserved in vertebrates, predicted by
419 SUMOplot and GPS-SUMO programs with highest scores (Supplementary Figure
420 S3C, S3D) and the motif IKED (K982) being previously identified by proteomic
421 analysis (Xiao et al., 2015; Hendriks and Vertegaal, 2016). As predicted, the
422 SALL1 Δ SUMO mutant lost the capacity to be SUMOylated in cells (Supplementary
423 Figure S3B). Therefore, we considered SALL1 Δ SUMO a SUMO-deficient mutant of
424 SALL1. Interestingly, neither the lack of colocalization with CBX4, nor the partial
425 colocalization with endogenous SUMO1 and SUMO2/3 were visibly altered when
426 SALL1 Δ SUMO-YFP was analyzed (Figure 1B, E and Supplementary Figure S2F).
427 These results, indicating that the localization of SALL1 to a subset of SUMO bodies
428 does not depend on its SUMOylation status, raised the possibility that SALL1
429 localization to these foci might be mediated by the presence of SUMO-interacting
430 motifs (SIMs) in this protein.

431 By analyzing the amino acid sequence of SALL1, we noted the presence of four
432 high-scored SIMs (Supplementary Figure S3A, D). To investigate the role of these
433 putative SIMs, we generated a SALL1 Δ SIM version in which the four motifs were
434 mutated to alanines. Remarkably, localization of SALL1 was unaffected by these
435 mutations. Thus, SALL1 Δ SIM-YFP readily localized to nuclear bodies, and partially
436 colocalized with SUMO1 and SUMO2/3, but not with CBX4 (Figure 1C, F, and
437 Supplementary Figure S2G). The lack of colocalization between these two proteins in

438 nuclear bodies prompted us to re-examine the interaction results obtained previously
439 by mass spectrometry (MS).

440

441 **SALL1 interacts with CBX4 in a SUMOylation-independent manner**

442 Previous MS results suggested that CBX4 could interact with full length SALL1
443 (Bozal-Basterra et al., 2018). We checked whether we could detect the CBX4-SALL1
444 interaction using CBX4-BioID. HEK 293FT cells were transfected with CBX4 fused
445 to a promiscuous variant of the BirA biotin ligase (CBX4-BirA*) together with either
446 full length SALL1-2xHA or the truncated form of SALL1⁸²⁶-2xHA, causative of
447 TBS. After pulldown using NeutrAvidin beads, the eluates were analyzed by Western
448 blot. As shown in Figure 2A, CBX4 was in close proximity to both the full length and
449 the truncated SALL1 forms (elution panel, lanes 1 and 2). *Drosophila* Pc (DmPc-
450 BirA, the fly CBX4 homologue; lane 3) is also able to interact with full-length
451 SALL1-HA.

452 We further confirmed the interaction between SALL1 and CBX4 by using
453 pulldown experiments. CBX4-YFP was transiently overexpressed in HEK 293FT
454 together with SALL1-2xHA or SALL1⁸²⁶-2xHA, and GFP-Trap-based pulldown
455 assays were carried out. SALL1-YFP was used as a positive control, since it is known
456 to bind to the truncated mutant. As shown in Figure 2B, CBX4-YFP interacted both
457 with full length and truncated SALL1 (elution panel, lanes 1 and 2).

458 SALL1 post-translational modifications could affect its interaction with other
459 proteins. In this regard, SALL1 SUMOylation might be particularly relevant for its
460 interaction with CBX4, which contain SIM domains (Merrill et al., 2010). In order to
461 test whether SUMOylation could have a role in SALL1 binding to CBX4, we
462 analyzed the SALL1 Δ SUMO capability to interact with CBX4 (Figure 2C). WT

463 SALL1-YFP and SALL1 Δ SUMO-YFP were transiently transfected in HEK 293FT
464 cells together with CBX4-HA (lanes 4 and 5, respectively). A GFP-Trap pulldown
465 was performed and analyzed by Western blot. Our results show that the
466 SUMOylation-deficient SALL1 mutant was still able to interact with CBX4 (elution
467 panel, compare lanes 4 and 5). No appreciable differences were noted between WT
468 SALL1 and SALL1 Δ SUMO in their ability to interact with CBX4.

469 On the other hand, since CBX4 is known to be SUMOylated *in vitro* (Kagey et
470 al., 2003; Merrill et al., 2010), we tested whether the predicted SIMs in SALL1 could
471 have a role in its interaction with CBX4. As shown in Figure 2C (elution panel,
472 compare lanes 4 and 6) SALL1 WT and SALL1 Δ SIM showed similar capacity to
473 bind CBX4. While differences in the intensity of CBX4 signals between SALL1 WT,
474 SALL1 Δ SUMO and SALL1 Δ SIM can be observed, these differences were mostly
475 due to the expression levels of the YFP-tagged SALL1 proteins. For example, the
476 higher expression levels of SALL1 Δ SUMO compared to SALL1 WT are most likely
477 directly related to the higher levels of CBX4-HA detected in the pulldown.

478 In summary, these results confirm SALL1/CBX4 interaction, and show that
479 neither SALL1 SUMOylation, nor its predicted SIM motifs are necessary for binding
480 to CBX4 in our experimental setting.

481

482 **SALL1 and CBX4 interact in the nucleoplasm**

483 Both proteins localize to the nucleus, with non-overlapping enrichment in
484 nuclear bodies, so we thought that the SALL1-CBX4 interaction might occur in the
485 nucleoplasm where weaker immunofluorescence signals can be observed
486 (Supplementary Figure S4). In order to explore this possibility, we decided to apply

487 the Proximity Ligation Assay (PLA), a technique that allows the detection of protein-
488 protein interactions *in situ*.

489 U2OS cells were transfected with *CMV-SALL1-2xHA* or with an empty *pcDNA3*
490 vector as negative control, and anti-SALL1 and anti-CBX4 antibodies were used to
491 perform PLA (Figure 3A-E) (Söderberg et al., 2006; Matic et al., 2010). The signal
492 from each detected pair of PLA probes is visualized as a fluorescent spot. Our
493 analysis of the number of spots revealed an interaction between SALL1 and CBX4 in
494 the nucleus (Figure 3A, E). Combined with the SALL1/CBX4 localization analyses
495 described above, these results suggest that the interaction between SALL1 and CBX4
496 takes place most probably in the nucleoplasm instead of in nuclear bodies.

497

498 **SALL1 post-transcriptionally increases the levels of CBX4**

499 Considering previous evidence that SALL1 can be SUMOylated and that CBX4
500 can act as an E3 ligase to increase SUMOylation of several substrates (Kagey et al.,
501 2003; Li et al., 2007; MacPherson et al., 2009; Ismail et al., 2012; Pelisch et al., 2012;
502 Chen et al., 2018), we hypothesized that the SALL1/CBX4 interaction could drive
503 SALL1 SUMOylation. However, our *in vitro* SUMOylation assays in the presence of
504 SUMO1 or SUMO2/3 showed that the SUMOylated form of SALL1 did not vary in a
505 statistically significant manner when different amounts of CBX4 were added to the
506 reaction (Supplementary Figure S5).

507 These results suggested that CBX4 does not function as a SUMO E3 ligase for
508 SALL1 in this experimental settings, leaving the question of what could be the
509 biological outcome of the interaction between these proteins unanswered.
510 Intriguingly, while performing the experiments to validate the SALL1-CBX4
511 interaction, we had noticed that the levels of CBX4 were higher in cells co-transfected

512 with SALL1 proteins (SALL1 WT, SALL1 Δ SUMO or SALL1 Δ SIM) than in control
513 cells co-expressing YFP (Figure 2B, lanes 1 vs 5; Figure 2C, lanes 4, 5, and 6 vs 8
514 and 9). This observation was supported by a quantitative analysis of the immunoblot
515 results (Figure 2D), and was further confirmed using a transient co-expression
516 experiment in HEK 293FT cells. In this experiment, Western blot analysis revealed
517 higher levels of CBX4-HA in cells co-expressing SALL1-YFP than in cells co-
518 expressing YFP alone (Figure 4A).

519 In order to discard any potential artefact due to the transient overexpression
520 conditions, we generated two HEK 293FT-derived cell lines stably expressing
521 SALL1. On one hand, we generated a HEK 293FT cell line constitutively expressing
522 a GFS (GFP-Flag-Strep)-tagged version of SALL1 at levels moderately increased
523 over the endogenous SALL1. Western blot analysis showed increased levels of
524 endogenous CBX4 in HEK 293FT_GFS-SALL1 cells compared with parental HEK
525 293FT cells (Figure 4B). On the other hand, we used the inducible lentiviral vector
526 TripZ to generate the HEK 293FT_TripZ-SALL1-2xHA cell line (see Materials and
527 Methods). This vector, based on the Tet-On system, allowed us to induce the
528 expression of SALL1-2xHA in a doxycycline dependent manner, while preserving the
529 expression of endogenous SALL1. As verified by immunofluorescence analysis
530 (Figure 4C), increasing concentrations of doxycycline (1 ng/ml, 10 ng/ml, 0.1 μ g/ml
531 or 1 μ g/ml), lead to a progressive increment of the SALL1 expression in HEK
532 293FT_TripZ-SALL1-2xHA cells. The levels of endogenous CBX4 protein were
533 analyzed in these cells using Western blot (Figure 4D, E). Quantification of three
534 independent experiments showed that CBX4 levels were significantly increased when
535 the cells were treated with 1 μ g/ml of doxycycline compared to untreated cells
536 (Figure 4E).

537 Since SALL1 is a transcription factor, we wondered whether the increased
538 CBX4 levels described above could be due to SALL1-mediated transcriptional
539 activation of CBX4 expression, potentially in an indirect way, as SALL1 is mostly
540 described as a transcriptional repressor. We tested this possibility using the inducible
541 HEK 293FT_TripZ-SALL1-2xHA cell model. SALL1 and CBX4 mRNA expression
542 was analyzed by RT-qPCR) in control or doxycycline-treated cells. As expected,
543 SALL1 mRNA expression increased in a doxycycline-dependent manner (Figure 4F).
544 However, CBX4 mRNA expression levels did not vary significantly.

545 Altogether these results demonstrate that increasing levels of SALL1 are
546 correlated with increasing CBX4 protein levels and, importantly, that this effect
547 occurs at a post-transcriptional level.

548

549 **SALL1 stabilizes CBX4 avoiding its degradation via the proteasome**

550 Different mechanisms may contribute to increase the levels of a given protein,
551 including changes in subcellular localization, solubility, or alteration in protein
552 stability due to reduced degradation. The results described above led us to test the
553 hypothesis that SALL1 could stabilize CBX4.

554 To this end, we analyzed the half-life of CBX4 by using a time-course
555 experiment with CHX. HEK 293FT cells were transfected with WT SALL1-YFP,
556 SALL1 Δ SUMO-YFP or GFP- β -Gal and treated with 50 μ g/ml of CHX in presence or
557 absence of 10 μ M of the proteasome inhibitor MG132. Cells were collected at
558 different time points (0, 4, 8 and 16 hours after initiation of treatment) and the levels
559 of endogenous CBX4 were analyzed by Western blot.

560 As shown Figure 5A, the levels of CBX4 began to decrease after 4 h of CHX
561 treatment in cells expressing GFP- β -Gal. However, in SALL1 WT or

562 SALL1 Δ SUMO-transfected cells the reduction in CBX4 levels was slower than in
563 control cells. Quantification of six independent experiments is shown in Figure 5B.
564 When cells were co-treated with CHX and MG132 (Figure 5C), proteasome
565 degradation was inhibited and CBX4 levels did not decline at 4 h. Consequently, as
566 shown in the Western blot quantification, no significant differences in the CBX4
567 levels were observed between cells transfected with SALL1, SALL1 Δ SUMO or
568 control (Figure 5D). Overall, these results show that CBX4 protein is more stable in
569 presence of SALL1 or SALL1 Δ SUMO, and that degradation of CBX4 occurs through
570 the ubiquitin proteasome system (UPS). Therefore, we concluded that SALL1
571 stabilizes CBX4 protein slowing down its degradation via the proteasome, and that
572 SUMOylation of SALL1 seems not to be essential for CBX4 stabilization.

573

574 **SALL1 influences CBX4 ubiquitination**

575 Previous reports have shown that CBX4 is ubiquitinated to mediate its
576 degradation through the proteasome (Ning et al., 2017). To investigate a potential
577 relationship between SALL1 expression and CBX4 ubiquitination, we used the bioUb
578 system (Pirone et al., 2017). First, we tested the efficiency of this system to detect the
579 ubiquitinated fraction of CBX4. We transiently transfected HEK 293FT cells with
580 CBX4-HA together with BirA-2A-bioUb or BirA as control. Cells were treated with
581 biotin in presence or absence of the proteasome inhibitor MG132. Protein lysates
582 were processed for bioUb assay (see Materials and Methods) and results were
583 analyzed by Western blot (Figure 6A). Ubiquitinated CBX4 is shown in the elution
584 panel. A band above 100 KDa and a high molecular weight smear, both consistent
585 with ubiquitinated forms of CBX4, are visible. As expected, the levels of
586 ubiquitinated CBX4 increased in presence of the proteasome inhibitor MG132. Anti-

587 Avitag antibodies detecting bioUb also showed an increase in the general
588 ubiquitination levels in presence of MG132, as shown in the elution panel. These
589 results confirmed the modification of CBX4 by ubiquitination and its degradation via
590 UPS.

591 Next, to test whether SALL1 could increase CBX4 stability by impairing its
592 ubiquitination and subsequent proteasomal degradation, we studied CBX4
593 ubiquitination in the inducible HEK 293FT_TripZ-SALL1-2xHA cells. These cells
594 were transiently transfected with *CBX4-YFP* together with *BirA-2A-bioUb* or *BirA* as
595 control. The cells were treated or not with 1 µg/ml of doxycycline to induce SALL1
596 expression, in the presence or absence of 10 µM MG132. Protein lysates were
597 processed for bioUb assay, and the results were analyzed by Western blot (Figure
598 6B). A statistically significant reduction of CBX4 ubiquitination was observed in
599 presence of high levels of SALL1 (Figure 6B, C, in the elution panel compare lane 4
600 with lane 2). However, in the presence of MG132, no significant differences were
601 appreciated between induced and not induced cells (Figure 6C, in the elution panel
602 compare lanes 6 and 8).

603 To further analyze the ubiquitination of endogenous CBX4, we transiently
604 expressed SALL1-2xHA or pcDNA3 as a control in HEK 293FT cells. After lysis,
605 total ubiquitinated material was isolated from the cells by pulldown using TUBES
606 (see Materials and Methods), and analyzed by Western blot (Figure 6D, E). In
607 presence of SALL1, the levels of ubiquitinated CBX4 were reduced when compared
608 with cells transfected with the control plasmid (elution panel, compare lanes 1 and 2).
609 No significant differences were appreciated when cells were treated with MG132
610 (elution panel, lane 4 *versus* lane 3). Quantification of ubiquitinated CBX4 in relation
611 to the CBX4 input is shown in Figure 6E. Taken together, these results indicated that

612 SALL1 is able to stabilize CBX4 protein by reducing its ubiquitination and
613 subsequent degradation via the UPS.

614

615 **SALL1 modulates the number and size of CBX4-containing Pc bodies, as well as**
616 **the expression of CBX4 target genes**

617 Although SALL1 does not colocalize with CBX4 in Pc bodies, the finding that
618 SALL1 modulates CBX4 protein levels prompted us to investigate a potential effect
619 of SALL1 expression on CBX4-containing Pc bodies. We transiently transfected
620 SALL1-YFP or its mutant SALL1 Δ SUMO-YFP in U2OS cells. GFP- β -Gal was
621 transfected as control. Transfected cells were stained with a specific CBX4 primary
622 antibody and the number and area of CBX4-containing Pc bodies were examined in
623 more than 100 cells per condition (Figure S6). Using confocal microscopy and image
624 analysis with Fiji software (Figure 7A, B), we observed that Pc bodies were
625 significantly larger and more abundant in cells expressing SALL1 or SALL1 Δ SUMO
626 than in cells expressing β -Gal. No significant differences in the number of bodies
627 were observed between cells expressing SALL1 and SALL1 Δ SUMO. However, the
628 area of the Pc bodies was significantly smaller in SALL1 Δ SUMO compared to
629 SALL1 transfected cells. These results revealed that SALL1 SUMOylation status
630 does not influence the increase in the number of Pc bodies, but it may influence their
631 size.

632 Finally, since SALL1 increases CBX4 protein levels, as well as the size and
633 number of Pc bodies, and increased formation of Pc bodies may lead to stronger
634 transcriptional repression of several PRC1 target genes (Gonzalez et al., 2014;
635 Soshnikova, 2014; Cheutin and Cavalli, 2018), we hypothesized that SALL1

636 overexpression could lead to a stronger transcriptional repression of CBX4 targets,
637 including HOX genes.

638 To test this possibility, HEK 293FT cells were transiently transfected with
639 SALL1-YFP, SALL1 Δ SUMO-YFP or GFP- β -Gal as control, and the expression
640 levels of several direct CBX4 target genes (*HOXA11*, *HOXB4*, *HOXB7*, *HOXB13*,
641 *HOXC6*, *HOXC10*, *HOXC12*, *HOXD13* and *GATA4*) were analyzed by RT-qPCR.
642 Significant differences in the expression of *HOXB4*, *HOXB13*, *HOXC6*, *HOXC10* and
643 *GATA4* were observed between wild-type SALL1 and β -Gal expressing control cells
644 (Figure 7C). However, no significant differences were observed between
645 SALL1 Δ SUMO-transfected cells and control cells.

646 Taken together, these results indicate that high SALL1 levels modulate the
647 transcriptional repression capacity of CBX4 on some of its target genes. Interestingly,
648 SUMOylation of SALL1 seemed to be necessary for this transcriptional effect.

649

650

651 **DISCUSSION**

652

653 In this work, we have confirmed that SALL1 and CBX4 proteins interact with
654 each other. Although both proteins can be SUMOylated and contain validated (CBX4
655 (Merrill et al., 2010)) or predicted (SALL1) SIM motifs, our results suggest that the
656 SALL1/CBX4 interaction does not depend on the SUMOylation status of SALL1, nor
657 the mutation of its putative SIMs. We note the possible contribution of the
658 endogenous SALL1 to the interaction, as dimers with the endogenous WT SALL1
659 and exogenous mutants could be formed, bridging the interaction of mutant SALL1
660 with CBX4.

661 Neither SALL1 WT nor the SALL1 Δ SUMO or SALL1 Δ SIM mutant forms
662 showed colocalization with CBX4 in Pc bodies, a subset of nuclear bodies that have
663 been defined as centers of chromatin regulation for transcriptional repression of target
664 genes (Entrevan et al., 2016). This observation indicates that the SALL1-CBX4
665 interaction does not occur in this specific cellular compartment. Despite this, we
666 demonstrate that SALL1, as well as its SUMOylation-deficient mutant form, increase
667 the number and size of CBX4-containing Pc bodies. We speculate that a dynamic and
668 transitory interaction with SALL1 in the nucleoplasm may indirectly influence Pc
669 body formation by altering CBX4 levels. In fact, we demonstrated that SALL1
670 stabilizes and increases CBX4 protein levels in a post-translational manner, reducing
671 its ubiquitination with subsequent reduction of its degradation via the proteasome.

672 Different hypothetical scenarios could explain the SALL1-mediated
673 stabilization of CBX4. As a transcriptional repressor, SALL1 could inhibit the
674 transcription of ubiquitin E3 ligase(s) involved in CBX4 modification or could
675 facilitate the binding and/or the recognition of CBX4 by DUBs (Ning et al., 2017).

676 Interestingly, SALL1 was found to interact with members of the UPS, which might
677 disrupt CBX4 homeostasis (Bozal-Basterra et al., 2018). Importantly, we show that
678 high SALL1 levels increase CBX4-mediated transcriptional repression of some of its
679 target genes. Although SUMOylation of SALL1 does not seem to affect its ability to
680 regulate CBX4 protein levels, it seems to be important for SALL1 to modulate CBX4
681 transcriptional repression activity: only when SALL1 is SUMOylated, the recruitment
682 of CBX4 on the chromatin results in a functional effect. In a speculative scenario, one
683 possible explanation of these results could be the involvement of a third component.
684 For instance, SUMOylation of SALL1 could facilitate the simultaneous interaction
685 with other members of the PRC1, such as RING1 or PHC1. Interestingly, those
686 factors were also found as possible SALL1 interactors in the proximity proteomics
687 analysis that hinted initially to a possible SALL1/CBX4 interaction (Bozal-Basterra et
688 al., 2018). Otherwise, SUMOylation of SALL1 could facilitate the interaction of
689 CBX4 with co-factors required for gene repression (Cheng et al., 2014).

690 These highly speculative hypotheses can be summarized into the model shown
691 in Figure 8. SALL1 (in its unmodified or SUMOylated form) would interact with
692 CBX4. This interaction would result in less ubiquitination of CBX4 with its
693 consequent stabilization (Figure 8). Thus, CBX4 would be recruited on chromatin,
694 where it would act as a transcriptional repressor of its target genes. In its SUMOylated
695 form, SALL1 could interact, not only with CBX4, but also with repression cofactors
696 or other components of PRC1, which could be recruited on chromatin along with
697 CBX4 (Figure 8, left). The recruitment of transcriptional cofactor(s), or various
698 components of PRC1, would result in the activation of the multiprotein complex with
699 consequent repression of the target genes.

700 In an alternative hypothesis, SUMOylated SALL1 could enhance CBX4
701 repression capacity by facilitating its SUMOylation. The SUMOylation of CBX4 is
702 known to be necessary for its repression activity on the chromatin (Kang et al., 2010).
703 We observed that, in the presence of high levels of SALL1, the SUMOylation of
704 CBX4 increased (data not shown). However, this was probably the result of
705 increasing the total levels of the protein. In addition, SALL1 was demonstrated to
706 interact with UBC9 and SUMO1 in a yeast two-hybrid system (Netzer et al., 2002).
707 Interestingly, some members of the SUMOylation pathway were also found in the
708 proximity proteomics analysis of SALL1 (Bozal-Basterra et al., 2018). In this
709 alternative hypothetical scenario, once SUMOylated SALL1 promotes CBX4
710 stabilization impairing its ubiquitination, it would be able also to promote CBX4
711 SUMOylation by recruiting an E3 SUMO ligase or other components of the
712 SUMOylation machinery (Figure 8, right). In this regard, the K224 residue involved
713 in CBX4 SUMOylation, and the adjacent K209 and K247 residues were predicted as
714 putative ubiquitination sites by UbPred (<http://www.ubpred.org/>). This raises the
715 interesting possibility that modification of CBX4 by ubiquitin and SUMO would be
716 mutually exclusive events. Whether this is the case, and whether SALL1 is involved
717 in this regulation, would require further investigation.

718 Additional experiments are necessary to further test the non-mutually exclusive
719 hypotheses for SALL1-mediated regulation of CBX4. Our results suggest that SALL1
720 plays an important role in the control of the expression of key developmental genes
721 through the post-transcriptional regulation of CBX4. Where and when this regulation
722 takes place *in vivo* during development deserves further investigation.

723

724

725 **CONFLICT OF INTEREST**

726 The authors declare that the research was conducted in the absence of any
727 commercial or financial relationships that could be construed as a potential conflict of
728 interest.

729 **AUTHORS CONTRIBUTIONS**

730 I.G., L.P., J.D.S. and R.B. designed experiments and analyzed data. I.G.,
731 L.P., J.A.R., J.D.S. and R.B. wrote the manuscript. I.G., L.P., V.M., E.L., C.P.,
732 V.L., E.G.-L., M.G. and O.B.-G. developed experimental protocols, performed
733 experiments, and analyzed data. A.C.O.V., A.M.A., J.A.R. and M.S.R. provided
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735

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746

747 **DATA AVAILABILITY**

748 The data that support the findings of this study are available from the
749 corresponding author upon reasonable request.

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993 **FIGURE LEGENDS**

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995 **Figure 1. SALL1 and CBX4 do not colocalize in nuclear bodies. (A-F)** Confocal
996 images of U2OS cells showing expression of SALL1-YFP, SALL1 Δ SUMO-YFP or
997 SALL1 Δ SIM-YFP (green), and endogenous CBX4 (magenta in **A-C**) or endogenous
998 SUMO2/3 (magenta in **D-F**). Nuclei were stained with DAPI. Black and white
999 pictures show single green or magenta channels. Green arrowheads indicate SALL1
1000 bodies, magenta arrowheads indicate Pc bodies (in **A-C**), or SUMO bodies (in **D-F**)
1001 and white arrowheads indicate colocalization of SALL1 and SUMO2/3 (in **D-F**).
1002 Pictures were taken with a Leica DM IRE2 confocal microscope using a 63X
1003 objective. Scale bars indicate 5 micrometers.

1004

1005 **Figure 2. SALL1 interacts with CBX4 in a SUMOylation-independent manner.**

1006 **(A)** Validation of the interaction between human SALL1 and human CBX4 or
1007 *Drosophila melanogaster* Pc proteins using BioID-based biotin pulldown in
1008 transfected HEK 293FT cells. In the Input panel, the relative expression of the HA-
1009 tagged SALL1 proteins (the full-length protein or a TBS-related truncation mutant) is
1010 shown. One asterisk indicates SALL1-HA, while two asterisks indicate SALL1⁸²⁶-
1011 HA. Negative controls (single expression of each individual protein) are shown in
1012 lanes 4-7. Anti-GAPDH was used as loading control. As shown in the Elution panel,
1013 CBX4-BirA* interact preferentially with full-length SALL1-HA (lane 1). Anti-biotin
1014 blot shows the efficiency of the different pulldowns. **(B)** Validation of the interaction
1015 between SALL1 and CBX4 using GFP-Trap. The Input panel shows the expression of
1016 epitope-tagged SALL1 and CBX4 proteins in transfected HEK 293FT cells. YFP
1017 alone and HA empty vector were used as controls. Lanes 1 and 2 of the Elution panel,

1018 show that CBX4 interacts with SALL1 full length and the truncated form. **(C)**
1019 SUMO-related SALL1 mutants interact with CBX4. Western blot analysis of proteins
1020 extracted from HEK 293FT cells transfected with the indicated plasmids. Pulldowns
1021 were performed using GFP-Trap. As shown in the Elution panel, (lanes 4, 5, and 6),
1022 interaction between CBX4 and WT SALL1 or SALL1 mutants was readily detected
1023 in all blot images. **(D)** Graph showing that CBX4 levels increase when co-expressed
1024 with WT SALL1-YFP, SALL1 Δ SUMO-YFP or SALL1 Δ SIM-YFP. The intensity of
1025 CBX4 bands in blots was quantified using ImageJ, normalized to β -Actin and
1026 reported as fold change relative to the YFP alone control. The mean plus SEM of 3
1027 independent experiments is plotted. P-values were calculated using Mann Whitney
1028 test. (*) P-value < 0.05. **(A, B, C)** Antibodies used are indicated to the left. Molecular
1029 weight markers are indicated to the right in KDa.

1030

1031 **Figure 3. SALL1 and CBX4 interact in the nucleoplasm. (A-D)** Confocal pictures
1032 of a proximity ligation assay (PLA) showing *in situ* interaction of SALL1 and CBX4
1033 in the nucleus of U2OS cells, visualized as magenta spots. Cells were transfected with
1034 SALL1-HA or with the empty pcDNA3 vector as negative control. Antibodies used in
1035 the assay are indicated in magenta. Panel **A** shows SALL1 and CBX4 interaction,
1036 while panels **B-D** are negative controls. **(E)** Quantification of PLA signals per cell as
1037 in panels **A-D**. Bars represent mean plus SEM of 3 independent experiments. P-values
1038 were calculated using One-way ANOVA test. (***) P-value < 0.001.

1039

1040 **Figure 4. SALL1 influences the levels of CBX4. (A)** Western blot showing protein
1041 levels of CBX4-HA when co-expressed with SALL1-YFP or YFP alone in HEK
1042 293FT cells. Actin expression was used as loading control. **(B)** Western blot showing

1043 expression levels of endogenous CBX4 protein in parental HEK 293FT cells (lane 1)
1044 or in HEK 293FT cells stably expressing GFS-SALL1 (lane 2). **(C)** Confocal
1045 microscopy images showing inducible expression SALL1-2xHA in HEK
1046 293FT_TripZ-SALL1-2xHA cells. Cells were treated with different concentrations of
1047 doxycycline (Dox) to induce SALL1 expression as indicated. SALL1-2xHA was
1048 detected using anti-SALL1 primary antibody (green). Cell nuclei were stained with
1049 DAPI (blue). **(D)** Western blot analysis showing expression levels of endogenous
1050 CBX4 in HEK 293FT_TripZ-SALL1-2xHA cells treated with increasing
1051 concentrations of Dox. **(E)** Quantification of the expression levels of endogenous
1052 CBX4 in HEK 293FT_TripZ-SALL1-2xHA cells treated with increasing
1053 concentrations of Dox. Three independent experiments as the one shown in panel D
1054 were performed. The intensity of CBX4 bands was quantified using ImageJ, and the
1055 values were normalized to the levels of Actin. P-value was calculated using One-way
1056 ANOVA test. (*) P-value < 0.05. **(F)** RT-qPCR analysis of *SALL1* and *CBX4* mRNA
1057 expression in HEK 293FT_TripZ-SALL1-2xHA cells treated with increasing
1058 concentrations of Dox. SALL1 and CBX4 expression were normalized using *GAPDH*
1059 expression and shown as fold change relative to untreated control. **(A, B, D)**
1060 Molecular weight markers are shown to the right in KDa. Antibodies were used as
1061 indicated to the left. **(E, F)** The mean plus SEM of at least three independent
1062 experiments is shown.

1063

1064 **Figure 5. SALL1 stabilizes CBX4 protein.** **(A, C)** Western blot analysis of
1065 cycloheximide (CHX) chase experiments performed in HEK 293FT cells transfected
1066 with *SALL1-YFP*, *SALL1ΔSUMO-YFP* or *GFP-β-Gal*. Cells were treated with 50
1067 μg/ml of CHX in the absence **(A)** or presence **(C)** of 10 μM of the proteasome

1068 inhibitor MG132. Cells were collected at different time points (0, 4, 8 and 16 hours
1069 after initiation of treatment) and endogenous CBX4 levels were analyzed by Western
1070 blot. Vinculin was used as loading control. Molecular weight markers are shown to
1071 the right in KDa. Antibodies were used as indicated to the left. **(B, D)** CBX4 levels
1072 were quantified after CHX treatment alone **(B)** or in combination with MG132 **(D)**,
1073 normalized to Vinculin, and data from six different independent experiments were
1074 pooled together. Graphs show mean plus SEM. P-values were calculated using One-
1075 way ANOVA test. (*) P-value < 0.05; (**) P-value < 0.01.

1076

1077 **Figure 6. CBX4 ubiquitination is reduced in presence of SALL1.** **(A)** Western blot
1078 analysis of HEK 293FT cells transfected with *CBX4-HA* together with *CMV-BirA-2A-*
1079 *bioUb* or *BirA* as a negative control. Cells were treated with 50 μ M of biotin in the
1080 presence or absence of 10 μ M MG132. Protein lysates were subjected to pulldown
1081 with streptavidin beads and the results were analyzed by Western blot. Two asterisks
1082 indicate monoubiquitinated CBX4-HA protein and the vertical line indicates the
1083 polyubiquitination smear. **(B)** Western blot analysis of HEK 293FT_TripZ-SALL1-
1084 2xHA cells transiently transfected with *CBX4-YFP* together with *BirA-2A-bioUb* or
1085 *BirA* as control. The cells were treated or not with 1 μ g/ml of doxycycline (Dox), in
1086 presence or absence of 10 μ M of MG132. Protein lysates were incubated with
1087 streptavidin beads to isolate bioUb conjugated proteins and results were analyzed by
1088 Western blot. β -Actin was used as loading control. **(C)** The levels of ubiquitinated
1089 CBX4-YFP in Dox induced and not induced cells, in presence (right panel) or
1090 absence (left panel) of MG132, were quantified and normalized to the CBX4 levels in
1091 the input. **(D)** Western blot analysis of endogenous CBX4 in HEK 293FT cells
1092 transfected with *CMV-SALL1-2xHA* (lanes 2 and 4) or with pcDNA3 control plasmid

1093 (lanes 1 and 3), in presence (lanes 3 and 4) or absence (lanes 1 and 2) of 10 μ M
1094 MG132. **(E)** Quantification of ubiquitinated CBX4 in the elution panel normalized to
1095 the CBX4 levels in the input, in cells expressing or not SALL1-HA, in presence (right
1096 panel) or absence (left panel) of MG132. **(A, B, D)** Molecular weight markers are
1097 shown to the right in KDa. Antibodies were used as indicated to the left. **(C, E)**
1098 Graphs represent mean plus SEM. P-values were calculated on n= 4 using Mann
1099 Whitney test. (*) P-value < 0.05.

1100

1101 **Figure 7. SALL1 expression increases the number and size of CBX4-containing**
1102 **Pc bodies and enhances downregulation of CBX4 targets.** **(A, B)** Graphs represent
1103 the number of CBX4-containing Pc bodies **(A)** and their mean area in pixels
1104 quantified using Fiji software **(B)** in U2OS cells expressing SALL1-YFP,
1105 SALL1 Δ SUMO-YFP or GFP- β -Gal as a negative control. **(C)** Graph showing the
1106 mRNA expression levels of several CBX4 target genes in HEK 293FT cells
1107 expressing SALL1-YFP, SALL1 Δ SUMO-YFP or GFP- β -Gal as control. Data shown
1108 correspond to the mean plus SEM of at least 5 independent RT-qPCR experiments.
1109 Gene expression data were normalized to *GAPDH* and are shown as relative fold
1110 change over β -Gal expressing cells (magenta line). P-values were calculated using
1111 One-way ANOVA test. (*) P-value < 0.05; (**) P-value < 0.01.

1112

1113 **Figure 8. SALL1 influences regulation of CBX4 target genes.** Hypothetical model
1114 showing speculative scenarios whereby SALL1 could influence CBX4-mediated
1115 regulation of target genes. Binding to SALL1 (SUMOylated or non-SUMOylated)
1116 could stabilize CBX4 by interfering with its ubiquitination and its consequent
1117 degradation by the proteasome. CBX4 stabilization entails an increment of its protein

1118 levels and its accumulation in Pc bodies. Binding to SUMOylated SALL1 increases
1119 CBX4-mediated transcriptional repression of its target genes. At least two non-
1120 exclusive hypothetical mechanisms might underlie this effect. Under one hypothetical
1121 scenario (left side), it could be due to the concurrent recruitment of other essential
1122 cofactors. In another hypothetical scenario (right side), SUMOylated SALL1 could
1123 increase CBX4 transcriptional repression by facilitating its SUMOylation through
1124 recruitment of SUMOylation machinery components. Discontinuous arrows indicate
1125 speculative events that have not been proven experimentally.

1126

1127

1128 **SUPPLEMENTARY FIGURES**

1129

1130 **Supplementary Figure S1. SALL1 localizes to nuclear bodies.** Endogenous SALL1
1131 **(A)** and transiently expressed SALL1-YFP **(B)** localize to nuclear bodies in U2OS
1132 cells. In contrast, YFP alone, used as a control, shows a homogenous distribution in
1133 the nucleus and cytoplasm **(C)**. Pictures were taken with an AxioD Fluorescent
1134 microscope using 100X objective. Scale bars indicate 5 micrometers.

1135

1136 **Supplementary Figure S2. Characterization of CBX4 and SALL1 nuclear**
1137 **bodies. (A-D)** Endogenous CBX4 (green) does not colocalize with SUM2/3, SUMO1,
1138 nor PML bodies or with SC35 (magenta) in U2OS cells. **(E-I)** SALL1-YFP (green)
1139 partially colocalizes with endogenous SUMO1 (magenta) in U2OS cells **(E)**. Similar
1140 results were obtained for the SALL1 Δ SUMO and SALL1 Δ SIM mutants **(F, G)**.
1141 SALL1 does not colocalize with PML **(H)** nor with SC35 **(I)**. Green and magenta
1142 channels are shown independently in black and white. Nuclei were stained with DAPI

1143 (blue). White arrowheads indicate colocalization, green arrowheads indicate domains
1144 where mainly CBX4 (**A-D**) or SALL1 (**E-I**) proteins are present, magenta arrowheads
1145 indicate domains where mainly SUMO2 (**A**), SUMO1 (**B, E-G**), PML (**C, H**) or SC35
1146 (**D, I**) are present. Pictures were taken using a Leica DM IRE2 confocal microscope
1147 with a 63X objective, except for pictures in C that were taken using an AxioD
1148 Fluorescent microscope and objective 40X. Scale bars indicate 5 micrometers.

1149

1150 **Supplementary Figure S3. SALL1 SUMOylation sites and SIMs are conserved**

1151 **throughout evolution. (A)** SALL1 schematic representation. Ovals represent the zinc
1152 fingers (ZF) distributed along the protein. Blue rectangle represents the poly-
1153 glutamine (PQ) domain. In magenta, SUMO consensus sites mutated in
1154 SALL1 Δ SUMO and, in blue, predicted SIMs mutated in SALL1 Δ SIM. **(B)** SALL1
1155 fused to HA tag was SUMOylated in the presence (black circles) of bioSUMO3,
1156 transiently transfected in HEK 293FT cells. Asterisks indicate the modified SALL1
1157 (SUMO-SALL1) that is shifted if compared with the size of non-modified SALL1
1158 (arrowhead). Anti-tubulin staining was used as a loading control. Molecular weight
1159 markers are shown to the right in KDa. SALL1 Δ SUMO fused to HA tag is not
1160 SUMOylated in presence of bioSUMO3. In the input the expression of WT and
1161 SUMO mutant of SALL1 are shown. **(C)** In magenta, SUMO consensus sites in
1162 SALL1 that were mutated in SALL1 Δ SUMO and, in blue, the predicted SIMs of
1163 SALL1, mutated in SALL1 Δ SIM mutant. **(D)** Evolutionary conservation of the
1164 SUMOylation and SIM sites in SALL1 homologues in the indicated species.
1165 Asterisks indicate identical residues, colons and semicolons indicate conservative and
1166 semi-conservative changes, respectively.

1167

1168 **Supplementary Figure S4. CBX4 and SALL1 localize to the nucleoplasm.**

1169 Endogenous CBX4 (**A**) and endogenous SALL1 (**B**) shown in green localize to
1170 nuclear bodies in U2OS cells (**A'**, **B'**). Increasing the intensity reveals the localization
1171 of both proteins in the nucleoplasm (**A''**, **B''**). Single green channels are shown in
1172 black and white. Pictures were taken using a Leica DM IRE2 confocal microscope
1173 with a 63X objective.

1174

1175 **Supplementary Figure S5. SALL1 SUMOylation is independent of CBX4. *In***

1176 *vitro* SUMOylation of SALL1 with SUMO1 or SUMO2/3 in the presence of growing
1177 quantities of CBX4 (in μ l). Wheat germ was added as negative control. The vertical
1178 bar indicates the SUMOylated forms of SALL1, the empty arrowhead indicates the
1179 unmodified SALL1. Molecular weight markers are shown to the right in KDa.

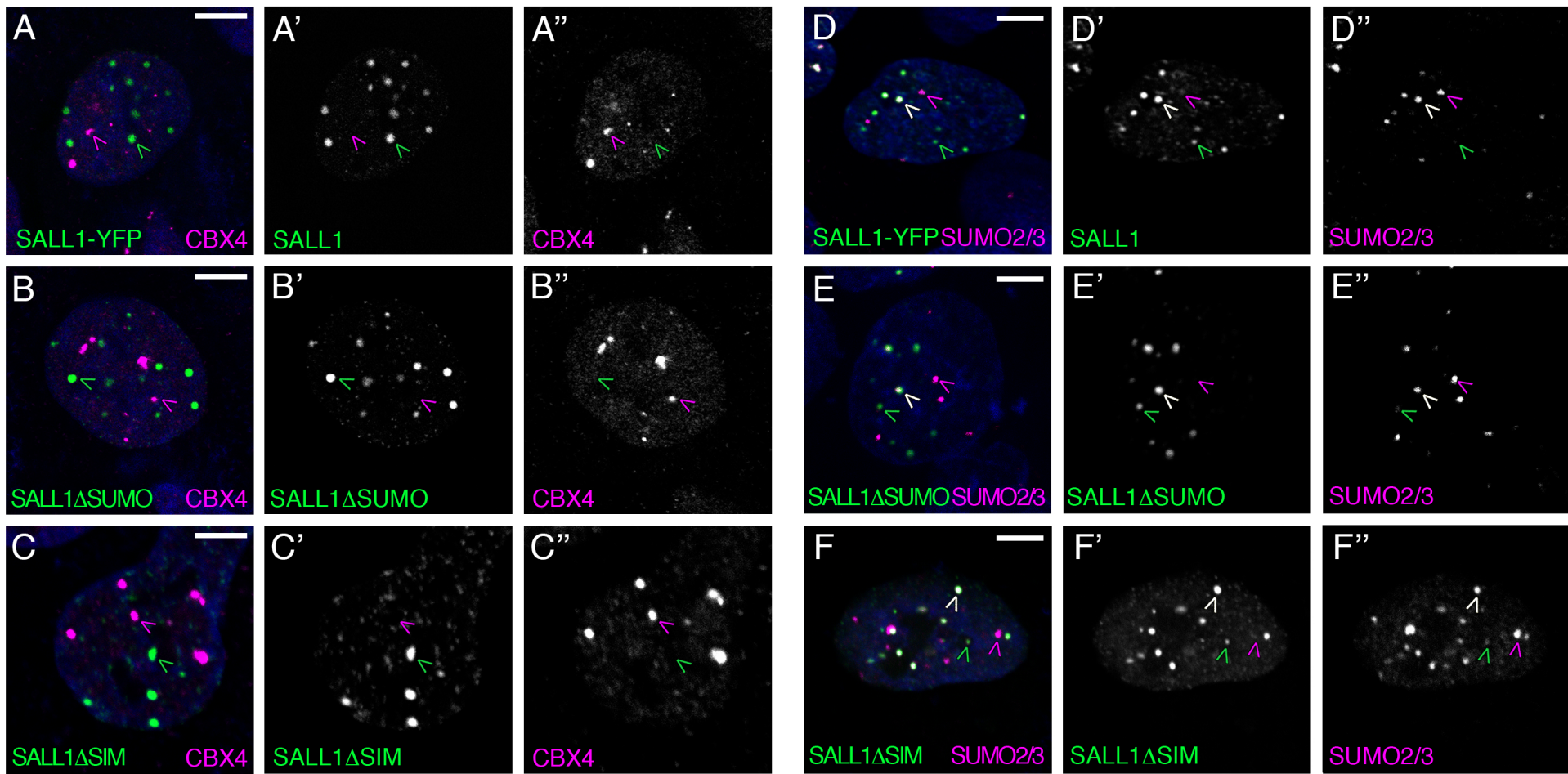
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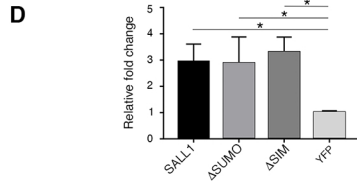
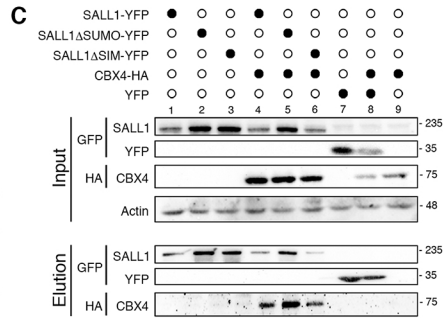
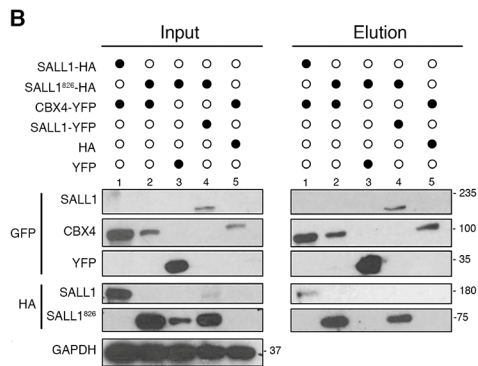
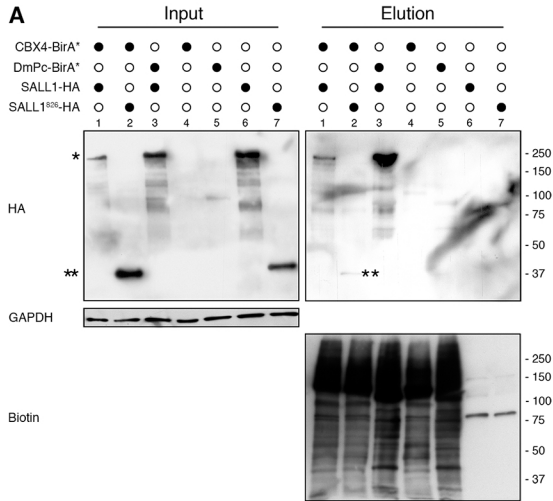
1181 **Supplementary Figure S6. Variation of Polycomb bodies upon SALL1**

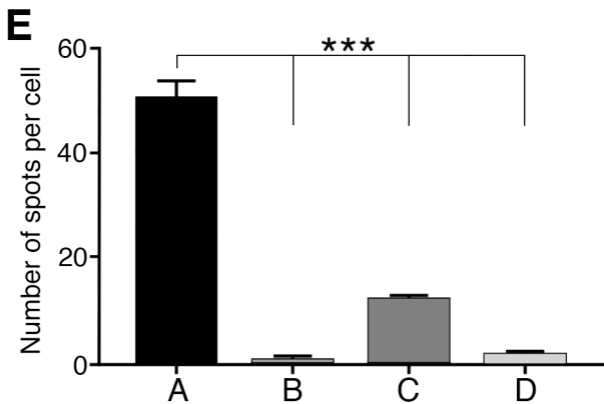
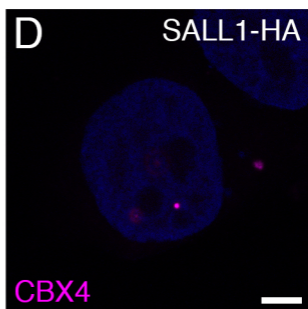
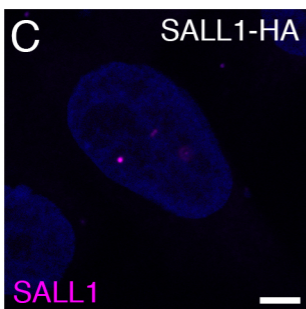
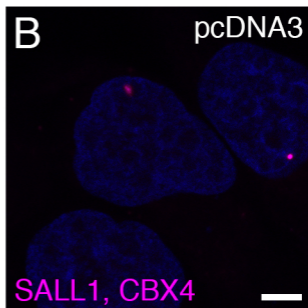
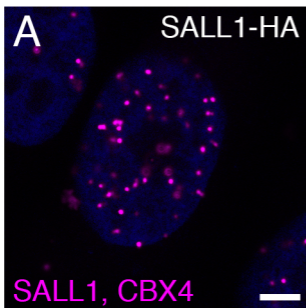
1182 **expression.** Representative composition of independent U2OS cells transfected with
1183 equal amounts of SALL1-YFP, SALL1 Δ SUMO-YFP or GFP- β -Gal plasmids,
1184 stained for endogenous CBX4. Nuclei were labelled with DAPI (not shown). Pictures
1185 were taken using a Leica DM IRE2 confocal microscope with a 63X objective, using
1186 the same settings for all the conditions.

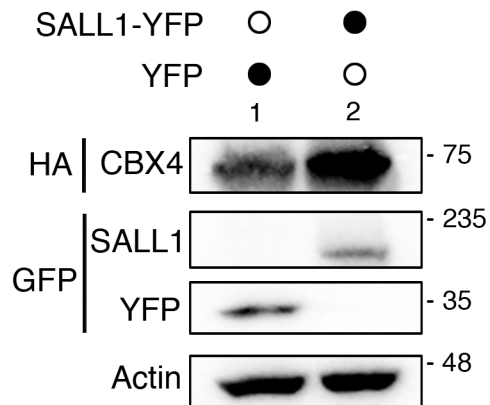
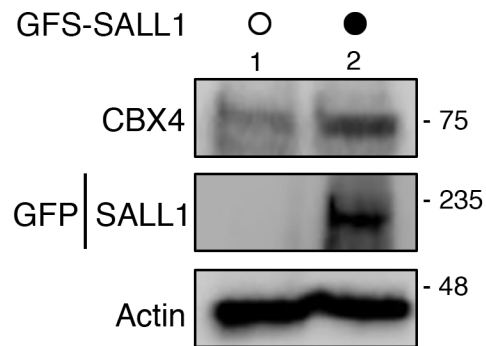
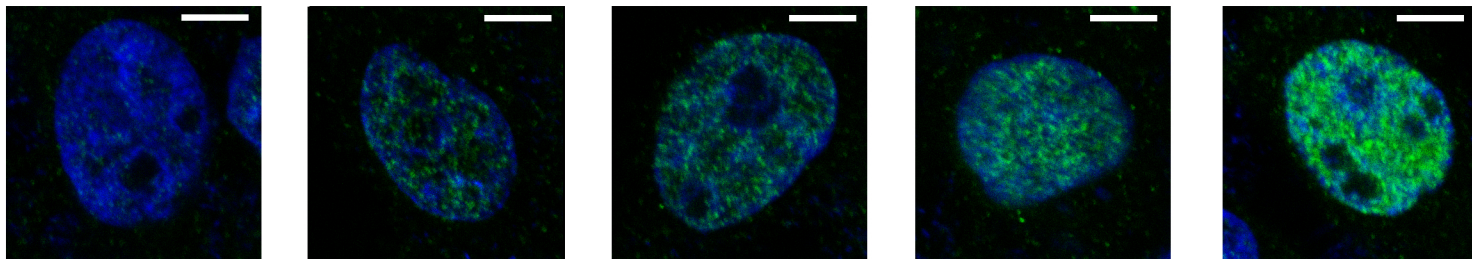
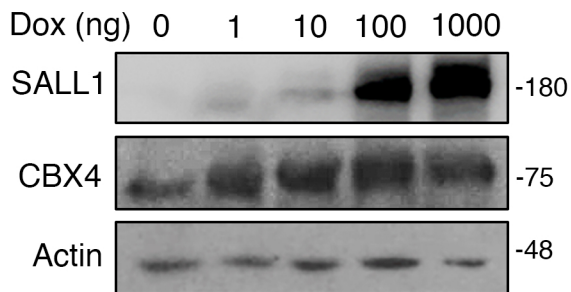
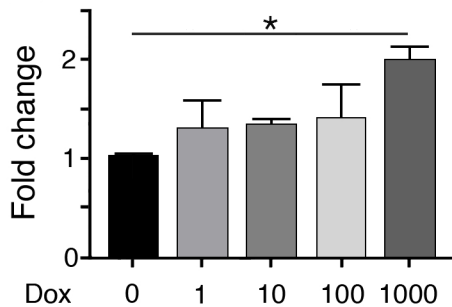
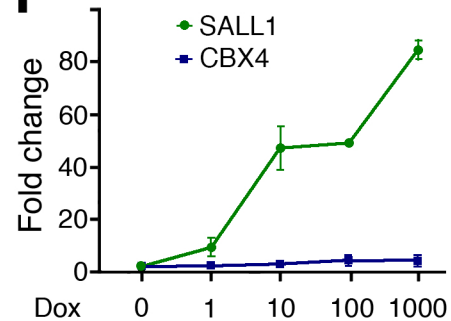
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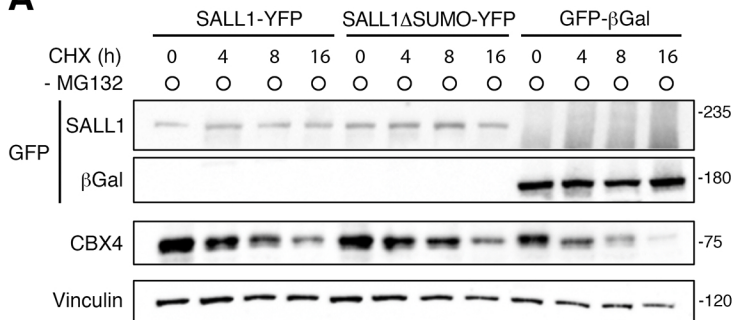
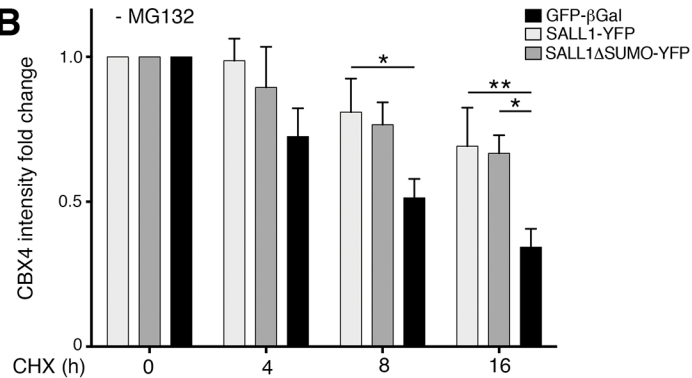
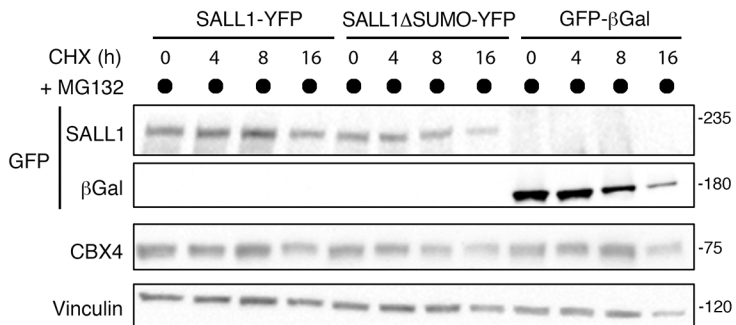
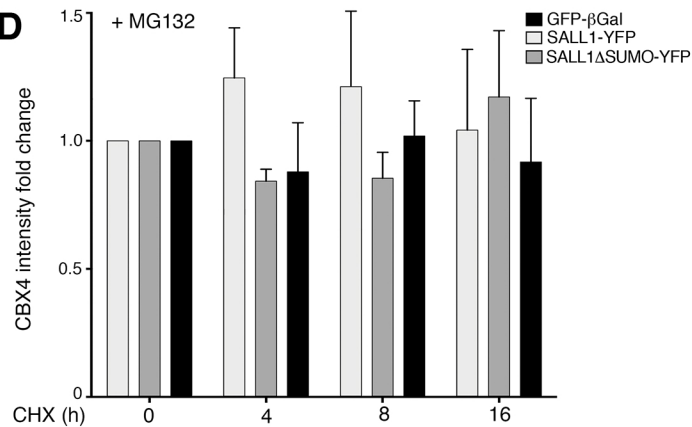
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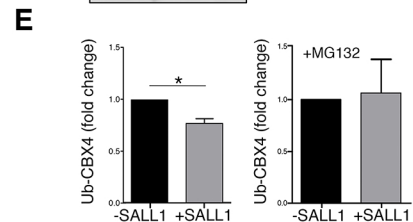
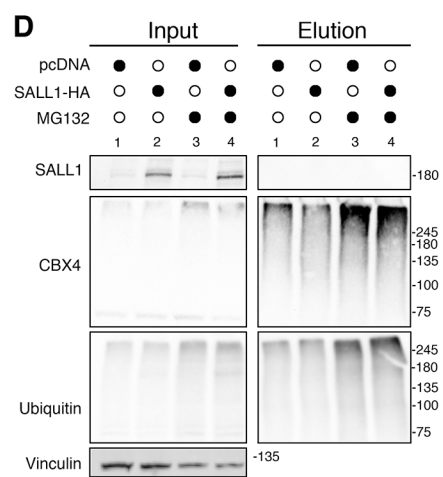
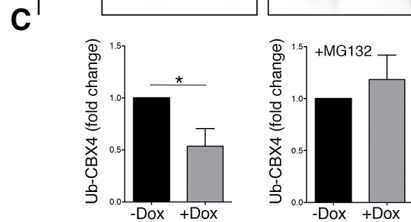
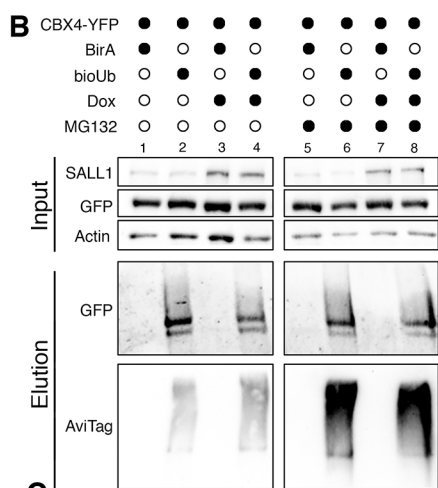
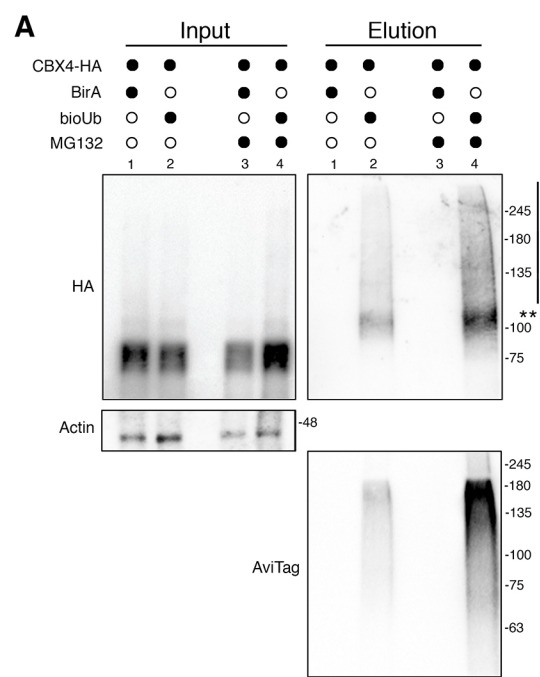


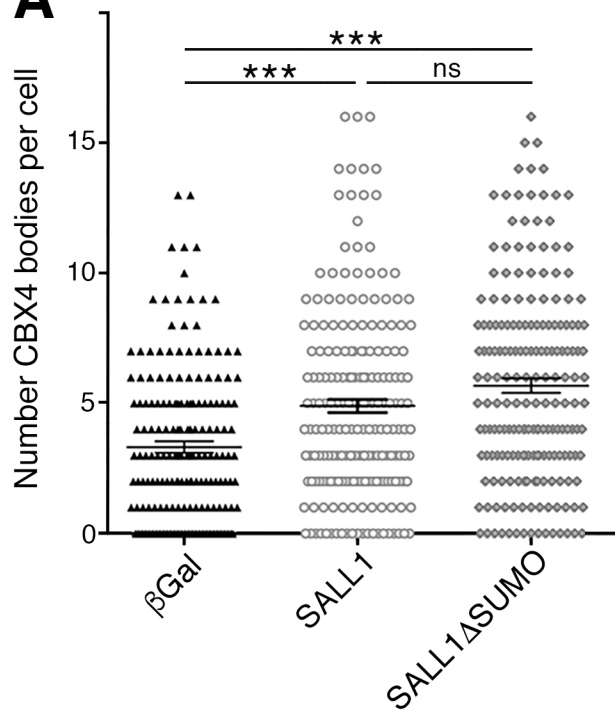
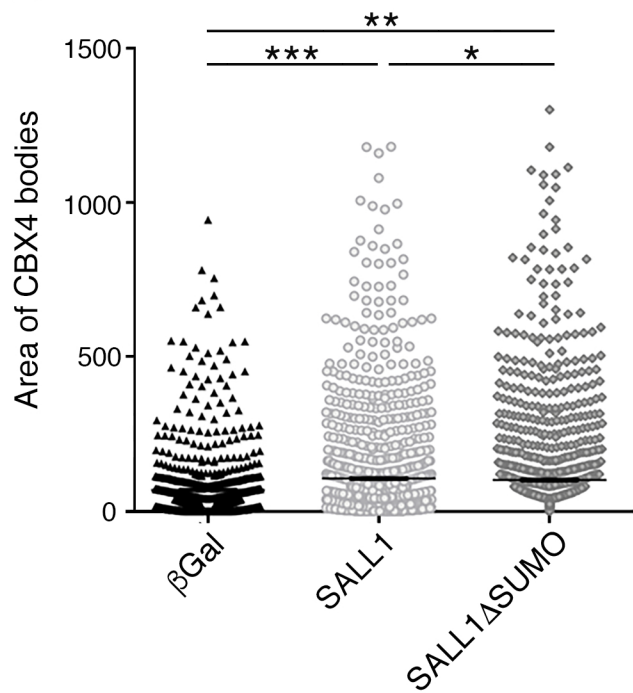




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