The secreted protein DEL-1 activates a β3 integrin–FAK–ERK1/2–RUNX2 pathway and promotes osteogenic differentiation and bone regeneration

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The integrin-binding secreted protein developmental endothelial locus-1 (DEL-1) is involved in the regulation of both the initiation and resolution of inflammation in different diseases, including periodontitis, an oral disorder characterized by inflammatory bone loss. Here, using a mouse model of bone regeneration and *in vitro* cell-based mechanistic studies, we investigated whether and how DEL-1 can promote alveolar bone regeneration during resolution of experimental periodontitis. Compared with WT mice, mice lacking DEL-1 or expressing a DEL-1 variant with an Asp-to-Glu substitution in the RGD motif ("RGE point mutant"), which does not interact with RGD-de-

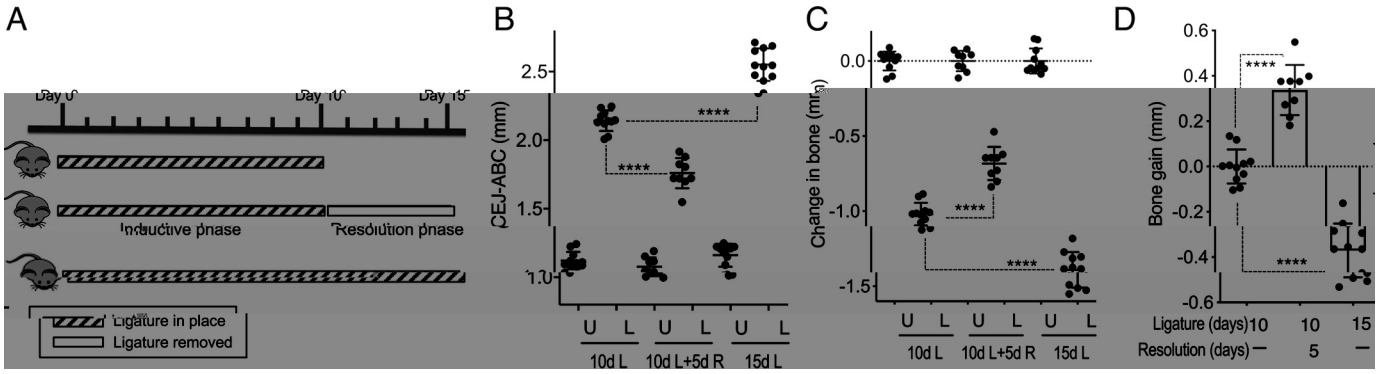

Figure 1. Bone gain during periodontitis resolution. *A*, outline of the model used. *B*, measurement of bone heights (distance from CEJ to ABC) in groups of WT C57BL/6 mice (8–10 weeks old) after 10 or 15 days of ligature (10d L or 15d L) or after 10 days ligature followed by 5 days without ligatures to enable resolution (10d L + 5d R). *C*, data from *B* were transformed to show bone loss in ligated (*L*) sites *versus* unligated (*U*) contralateral sites. *D*, data from *C* were transformed to show bone gain (or loss; negative values) relative to the 10d L group (baseline). Data are means ± S.D. (*error bars*) ($n = 9$–11 mice/group; pooled from two independent experiments). ****, $p < 0.0001$ (one-way ANOVA and Dunnett's post-test).

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Results

Model to study bone regeneration during periodontitis resolution

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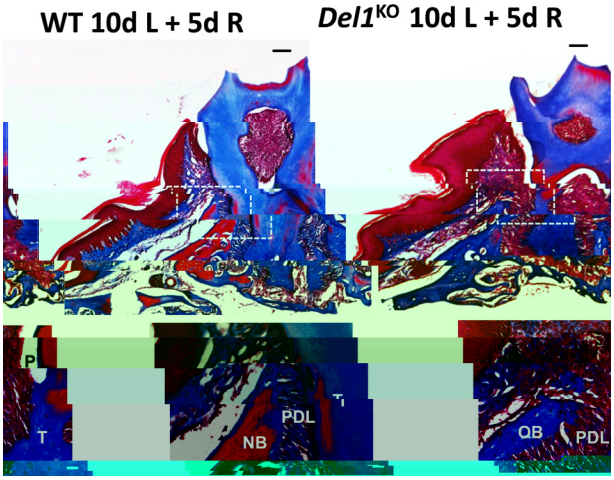

Figure 2. Del1^{KO} mice fail to generate new bone. *Del1*^{KO} mice and WT littermates were subjected to LIP for 10 days followed by 5 days without ligatures to enable resolution. Coronal sections were stained with modified Masson's trichrome, which stains old bone *blue* and new bone *red*. Shown are representative images (*scale bars*, 100 μm) and insets (*scale bars*, 25 μm) from resolution sites. *NB*, new bone; *OB*, old bone; *PDL*, periodontal ligament; *P*, pocket; *T*, tooth.

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DEL-1 promotes bone gain in vivo during resolution of periodontitis

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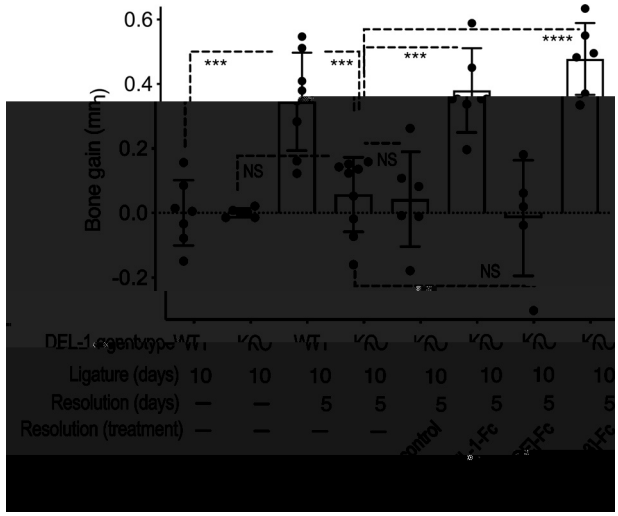

Figure 3. DEL-1 promotes bone gain during resolution. *Del1*^{KO} mice and WT littermates (8–10 weeks old) were subjected to LIP for 10 days followed (or not) by 5 days of resolution, with or without local injection with DEL-1-Fc (1 μg) or equal molar amounts of Fc control or mutants. Treatments were performed daily (days 10–14) in *Del1*^{KO} mice. Bone heights were measured, and CEJ-ABC data were transformed to indicate bone gain as outlined in Fig. 1. Data are means ± S.D. (*error bars*) (n = 5–9 mice/group). ***, $p < 0.001$; ****, $p < 0.0001$; *NS*, not significant (one-way ANOVA and Dunnett's post-test for comparing the various treatments with untreated KO; two-tailed unpaired Student's t test for comparing WT mice with resolution *versus* WT mice without resolution, as well as KO mice with resolution *versus* KO mice without resolution).

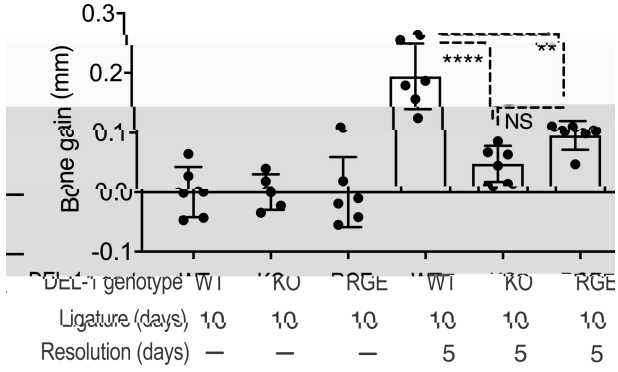

Figure 4. The ability of DEL-1 to promote bone gain during resolution depends on the RGD motif. WT, *Del1*^{KO} mice, and *Del1*$^{RGE/RGE}$ mice (8–10 weeks old) were subjected to LIP for 10 days, followed (or not) by 5 days of resolution. Bone regeneration on day 15 was calculated relative to the bone height at day 10, which was taken as the baseline (CEJ–ABC data were transformed to indicate bone gain as outlined in Fig. 1). Data are means ± S.D. (*error bars*) (n = 6 mice/group). **, $p < 0.01$; ****, $p < 0.0001$; *NS*, not significant (one-way ANOVA and Tukey's post-test).

DEL-1 promotes Runx2 expression and osteogenic differentiation of MC3T3-E1 cells in a manner dependent on β3 integrin, FAK, and ERK1/2

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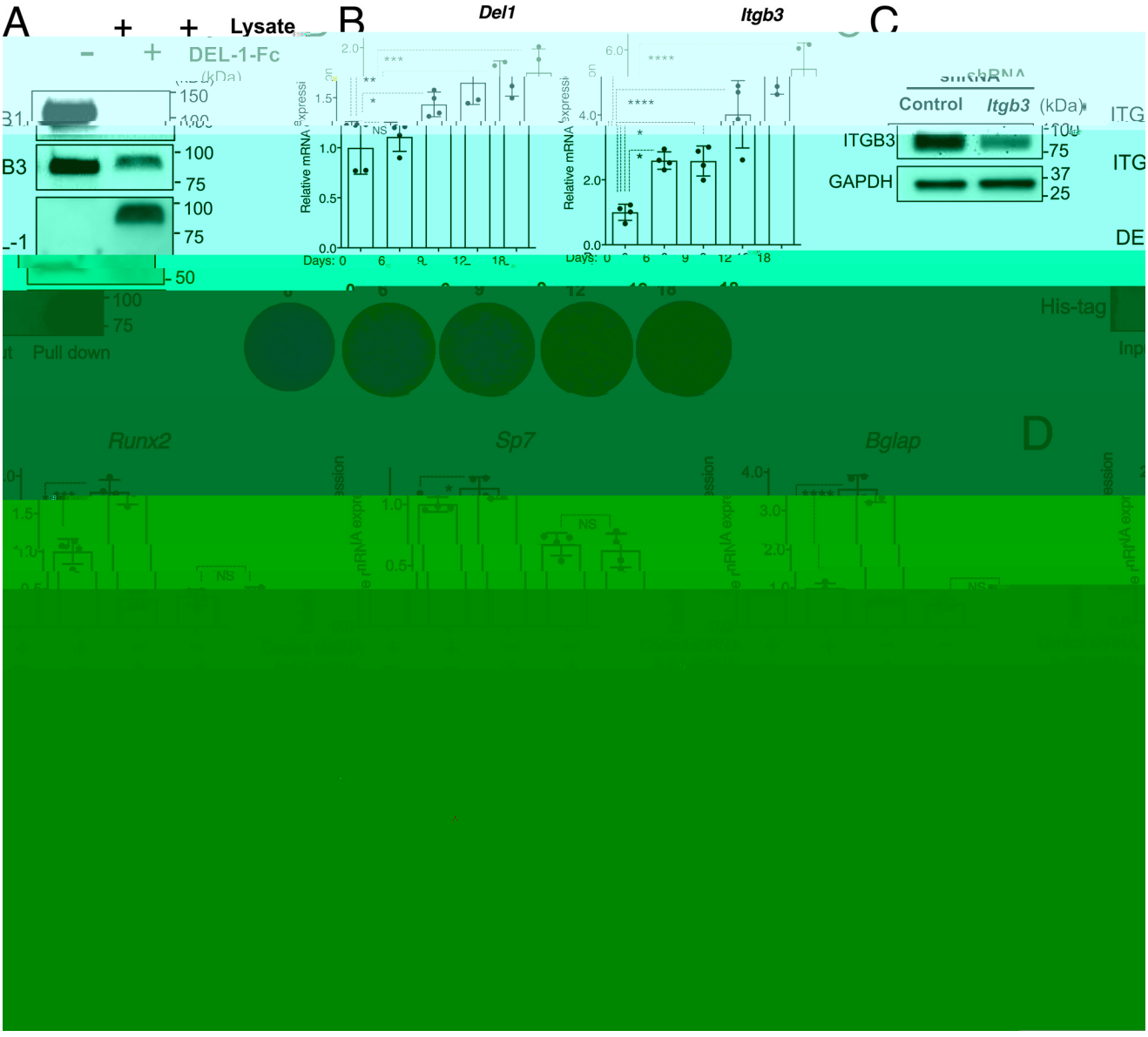

Figure 5. DEL-1 promotes osteogenic differentiation and mineralization in a β3 integrin–dependent manner. *A*, His-tagged DEL-1-Fc bound to cobalt-agarose beads was incubated with cell membrane protein lysates from MC3T3-E1 cells, and pulled-down proteins were analyzed by immunoblotting using antibodies to β1 and β3 integrins. Input (10%) represents lysates directly subjected to immunoblotting. DEL-1-Fc was 82 kDa, and endogenous DEL-1 was 52 kDa. *B*, expression of *Del1* and β3 integrin (*Itgb3*) in MC3T3-E1 cells cultured in osteogenic medium at the indicated time points determined by qPCR. Shown in the *bottom panel* is mineralization nodule formation in MC3T3-E1 cultures in osteogenic medium at the same time points. *C–E*, control or β3 integrin shRNA-transfected MC3T3-E1 cells were cultured in osteogenic medium with DEL-1-Fc (2 μg/ml) or an equal molar concentration of Fc control. *C*, knockdown of β3 integrin (ITGB3) confirmed by immunoblotting. *D*, analysis of expression of *Runx2* (day 6), *Sp7* (day 9), *Bglap* (day 12), typical early, middle, and late osteogenic markers, respectively, using qPCR. Data were normalized to *Gapdh* mRNA and expressed relative to Fc-treated and control shRNA-transfected cells, set as 1. *E*, representative images of mineralized nodule formation, detected by Alizarin Red S staining, after 12 days; the *right side* shows quantified results. Data are means ± S.D. (*error bars*) ($n = 4$ (B and D) or $n = 6$ (*E*) cell cultures/group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; *NS*, not significant (*B*, one-way ANOVA and Dunnett's post-test; *D* and *E*, two-tailed unpaired Student's *t* test).

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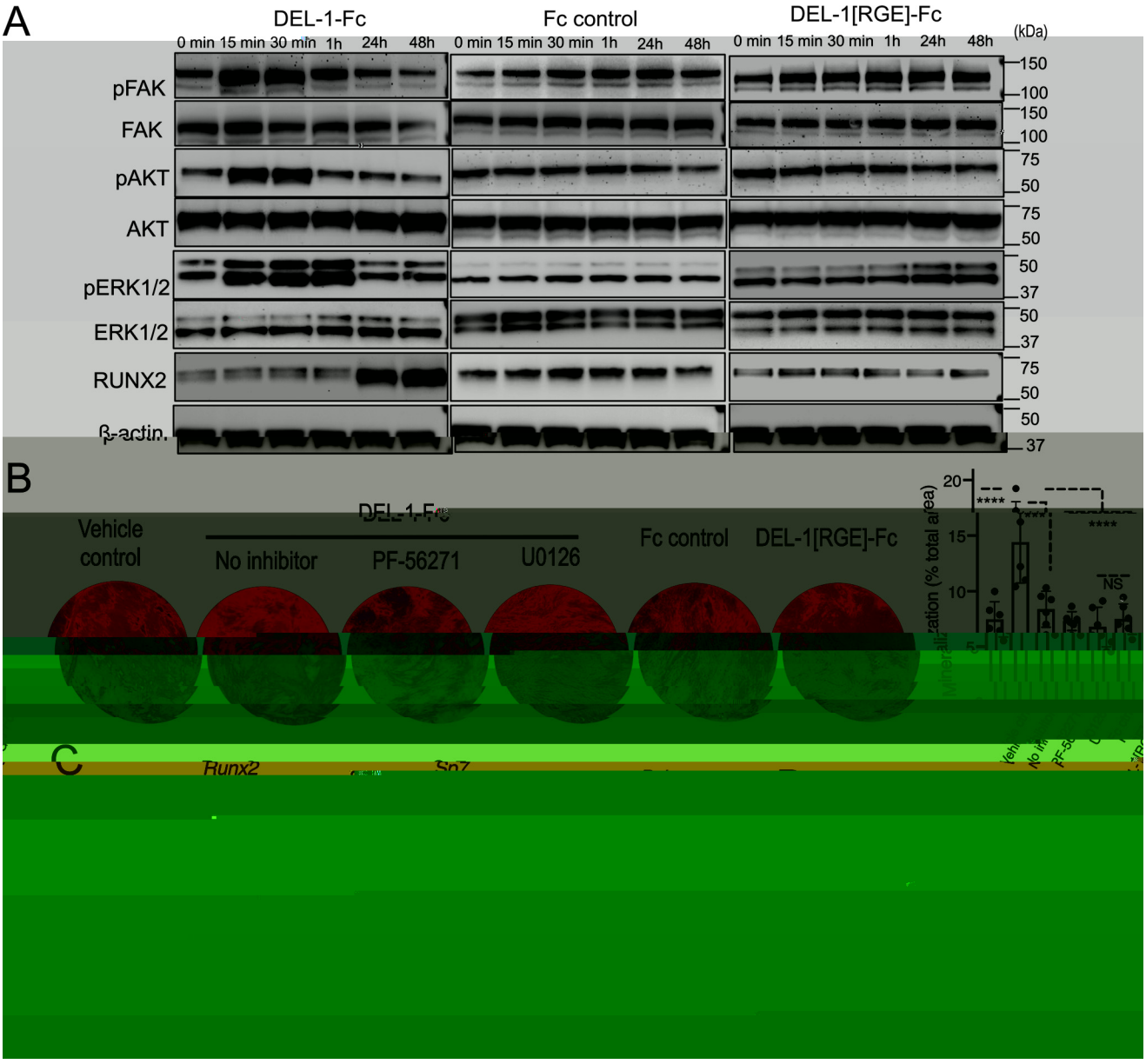

Figure 6. DEL-1 promotes osteogenic differentiation and mineralization in a manner dependent on FAK and ERK1/2. *A*, MC3T3-E1 osteoblastic progenitor cells were incubated in growth medium with 1 μg/ml DEL-1-Fc or DEL-1[RGE]-Fc or equal molar concentration of Fc control for the indicated times. Immunoblot analysis was performed with specific antibodies against phosphorylated and total FAK, AKT, and ERK1/2 as well as against *Runx2* and β-actin (loading control). *B* and *C*, MC3T3-E1 cells were cultured in osteogenic medium in the presence or DEL-1-Fc (1 μg/ml) or equal molar amounts of Fc control, DEL-1[RGE]-Fc, or DEL-1[E1–3]-Fc. In some DEL-1-Fc–treated groups, the cells were pretreated with PF-562271 (1 μM) or U0126 (10 μM); these inhibitors were added 1 h prior to DEL-1-Fc. Medium was changed every 3 days and was supplemented, as appropriate, with fresh DEL-1-Fc (or mutants/controls thereof) in the presence or absence of fresh signaling inhibitors. Shown are representative images of mineralized nodule formation, detected by Alizarin Red S staining, at day 15 of differentiation (*B*, left) and the mineralization area in each culture quantified and expressed as a percentage of the total area (*B*, *right*). *C*, analysis of MC3T3-E1 cells (treated as in *B*) for the expression of *Runx2* (at day 6), *Sp7* (at day 9), and *Bglap* (at day 12), typical early, middle, and late osteogenic markers, respectively, using qPCR. Data were normalized to *Gapdh* mRNA and expressed relative to medium-only–treated control, set as 1. *D*, Western blot analysis of *Runx2* protein expression at 48 h in MC3T3-E1 cells, incubated in growth medium treated with DEL-1-Fc, in the presence or absence of the indicated concentrations of U0126 or PF-562271, which were added 1 h earlier than DEL-1-Fc. Numerical data are means ± S.D. (*error bars*) ($n = 6$ cultures/group). **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; *NS*, nonsignificant (one-way ANOVA and Tuckey's post-test).

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Endogenous DEL-1 promotes osteogenic differentiation of primary calvarial osteoblastic cells through its RGD motif

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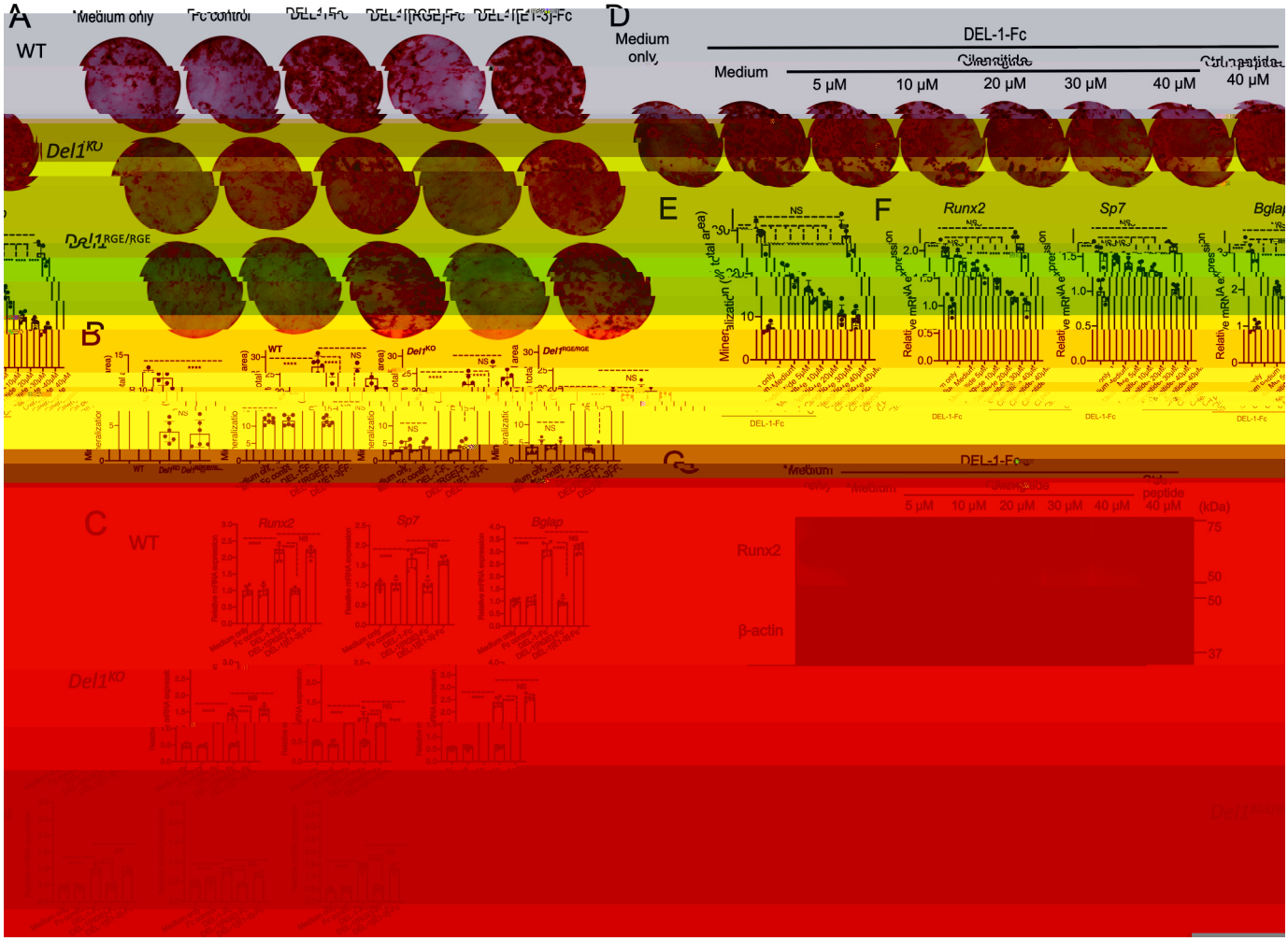

Figure 7. Endogenous DEL-1 induces osteogenic differentiation in primary calvarial osteoblast progenitors through its RGD motif. Primary osteoblastic progenitor cells were isolated from the calvariae of 3-day-old WT, *Del1*^{KO}, or *Del1*$^{RGE/RGE}$ mice. The cells were cultured in osteogenic medium in the presence (or not) of DEL-1-Fc (1 μg/ml) or equal molar amounts of Fc control, DEL-1[RGE]-Fc, or DEL-1[E1–3]-Fc and were compared for mineralized nodule formation (*A* and *B*) and osteogenic gene expression (*C*). Shown are representative images of mineralized nodule formation, detected by Alizarin Red S staining, on day 15 of differentiation (*A*) and mineralization area in each culture quantified and expressed as a percentage of the total area (*B*). *C*, primary calvarial osteoblast progenitors from the same strains of mice, treated similarly as above, were assayed by qPCR for expression of *Runx2* (at day 6), *Sp7* (at day 9), and *Bglap* (at day 12), typical early, middle, and late osteogenic markers, respectively. Data were normalized to *Gapdh* mRNA and expressed relative to the medium-only–treated groups of the WT cells, set as 1. *D–G*, primary osteoblastic progenitor cells, isolated from the calvariae of 3-day-old WT mice, were cultured in osteogenic medium in the presence (or not) of DEL-1-Fc (1 μg/ml) with or without cilengitide (5, 10, 20, 30, or 40 μM) or 40 μM RGD control peptide and assayed for mineralization nodule formation (*D* and *E*) and osteogenic gene expression (*F*). Shown are representative images of mineralized nodule formation, detected by Alizarin Red S staining, on day 15 of differentiation (*D*), and the mineralization area in each culture was quantified and expressed as a percentage of the total area (*E*). *F*, primary calvarial osteoblast progenitors from WT mice were treated as above and assayed by qPCR for expression of *Runx2* (at day 6), *Sp7* (at day 9), and *Bglap* (at day 12), respectively. Data were normalized to *Gapdh* mRNA and expressed relative to the control peptide-only–treated group, set as 1. *G*, Western blot analysis of *Runx2* protein expression at 48 h in WT primary osteoblastic progenitor cells incubated in growth medium in the presence (or not) of DEL-1-Fc (1 μg/ml) with cilengitide (5, 10, 20, 30, and 40 μM) or control peptide (40 μM), which were added 1 h earlier than DEL-1-Fc. β-Actin served as loading control. Data are means ± S.D. (*error bars*) ($n = 6$ cultures/group). **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; *NS*, nonsignificant (one-way ANOVA and Tukey's post-test).

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Resolution of ligature-induced periodontitis and intervention experiments

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Receptor knockdown by specific shRNA

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Statistical analysis

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Data availability

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Author contributions .- . ., . ., . .
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