Supplementary Materials and Methods

Generation of pTOF-DNMAML1

Platinum® *Pfx* DNA polymerase (Invitrogen) was used to amplify GFP-DNMAML1 cDNA by PCR with primers *Bgl*II-*Pac*I-MAML (5'-GGCCAGATCTTTAATTAAGCCGCCAC CATGGCGCTGCCGCGGCACA-3') and *Xho*I-*Pme*I-GFP (5'- GGCCCTCGAGGTTTAAACT TACTTGTACAGCTCGTCCATG-3') using MSCV-Mam(12-74)-EGFP[1] as a template. Following initial incubation at 94°C for 5 min, PCR was carried out for 35 cycles at 94°C for 15 sec for denaturing, 56°C for 30 sec for annealing, 68°C for 3 min for extension, with extended incubation at 68°C for 5 min after the final extension. The PCR product was subcloned into *Pme*I and *Pac*I sites of pBASTR1[2], resulting in generation of pTOF-DNMAML and verified by DNA sequencing.

RT-PCR for *MAML1-3*

RT-PCR was performed with SYBR reagent (PE Applied Biosystems, Foster City, CA) for *MAML1-3* as demonstrated in Supplementary Figure 3, using the following paired primers: *MAML1*, 5'-CACCAGCCACCGAGTAACTT-3' and 5'-CCCACAGTCCGCTTTGTAAT-3' (amplicon, 105 bp); *MAML2*, 5'-TTTCCTTCACCCAACCAAAG-3' and 5'-GGGCCCATGTTA TCATTTTG-3' (amplicon, 101 bp); *MAML3*, 5'-CGTATATCCAGCAGCAGCAA -3' and 5'-TTTCTGGTCTTCGCTCAGGT-3' (amplicon, 116 bp), and GAPDH5'-CAATGACCCCTTCAT TGACC-3' and 5'-GACAAGCTTCCCGTTCTCAG-3' (amplicon, 106 bp). Amplification was done at 94°C for 1 min for denaturing, 56°C for 45 sec for annealing and 72°C for 30 sec for extension.

Supplementary Table 1 ChIP assay primers

The *N3* (GenBank NG_009819) -12 kb, promoter, and the intron 2 regions containing the CSL binding *cis*-elements were amplified by the primers listed below. The *N3* 3' region has no CSL sites and served as an off target negative control. The HES1 promoter region served as a positive control[3] for ICN1 binding as demonstrated in Supplementary Figure 12.

Targets	Forward Primers	Reverse Primers
N3 -12 kb	5'-GGACCCACCCAAGGTGTC-3'	5'-CTCATGGAGGAGAAATGCAGTC-3'
N3 -2.3 kb	5'-GCACACCCAACCTCGTGAA-3'	5'-GACGTGACTGGCCTCAGTTTC-3'
N3 intron2	5'-GCTGGGCGCCGAGGATAG-3'	5'-AGACCTCGTCCCCATCTCCTAGTC-3'
N3 3' end	5'-TGCCAAAAGAGGAAGCATAAGTA-3'	5'-TCAAAATCCCTGTGTAGCTGAAT-3'
Hes1 promoter	5'-CGTGTCTCCTCCTCCCATT-3'	5'-CCGCTGTTATCAGCACCAG-3'

Supplementary Table 2 TaqMan® Gene Expression Assays

IVL, Hs00846307_s1; CK13, Hs00999762_m1, HES5, Hs01387463_g1; N1, Hs01062014_m1; N2, Hs00225747_m1; N3, Hs00166432_m1; N4, Hs00270200_m1

Supplementary Table 3. **Primary antibodies and titers for Western blotting**

IVL, Mouse monoclonal anti-involucrin (CloneSY5)(Sigma-Aldrich, St. Louis, MO),

Cat# I 9018, (1:1000)

Ivl, Rabbit polyclonal anti-involucrin antibody (Covance, Princeton, NJ),

Cat# PRB-140C, (1:1000)

β-actin, Mouse monoclonal anti-β-actin antibody (AC-74)(Sigma-Aldrich),

Cat# A5316, (1:3000)

α–tubulin, Rabbit polyclonal anti-α-tubulin (Cell Signaling, Danvers, MA),

Cat# 2144, (1:1000)

Histone H1, Mouse monoclonal anti-Histone H1 antibody (AE4)(Santa Cruz, Santa Cruz, CA)

(1:250)

GFP, Rabbit polyclonal anti-GFP antibody (Cell Signaling),

Cat# 2555, (1:1000)

FL-N1, Rat monoclonal anti-N1 antibody (5B5)(Cell Signaling),

Cat# 3447, (1:1000)

ICN1, Rabbit monoclonal anti-cleaved N1 antibody (Val1744) (D3B8)(Cell Signaling),

(1:1000)

ICN1 Rabbit monoclonal anti-N1 antibody (EP1238Y)(Epitomics, Burlingame, CA),

Cat# 1935-1, (1:1000) (to detect retrovirally transduced ICN1 in Figure 5*B*)

FL-N3/ICN3, Rat monoclonal anti-N3 antibody (8G5)(Cell Signaling),

Cat#3446, (1:1000)

Mouse N3, Goat polyclonal anti-N3 antibody (M-20)(Santa Cruz Biotechnology),

Cat# sc-7424, (1:200)

MAML1, Rabbit polyclonal anti MAML1 (Cell Signaling),

Cat# 4608, (1:1000)(50 μg of whole cell lysate was loaded per lane in Supplementary Figure 3*B*)

MAML2: Rabbit polyclonal anti MAML1(Bethyl Laboratories, Montgomery, TX)

(1:1000)(50 μg of whole cell lysate was loaded per lane in Supplementary Figure 3*B*)

MAML3: Rabbit polyclonal anti MAML1 (Bethyl Laboratories)

(1:1000)(50 μg of whole cell lysate was loaded per lane in Supplementary Figure 3*B*)

Supplementary Table 4 Antibodies for Immunostaining

N1, Rabbit polyclonal anti-N1 antibody (Abcam, Cambridge, MA), Cat# ab27526,

(1:500), microwaved at pH 3.0

N3, Rabbit polyclonal anti-N3 antibody (Abcam), Cat# ab23426,

(1:1000), microwaved at pH 3.0

Ki67, Rabbit polyclonal anti-Ki-67 antibody (Novocastra, Bannockburn, IL), Cat# NCL-Ki67-P,

(1:500)

Flg, Rabbit polyclonal anti-Filaggrin antibody (Abcam), Cat# ab81468,

(1:500), microwaved at pH 3.0

CK14, Rabbit polyclonal anti-CK14 antibody (Covance), Cat# PRB-155P,

(1:300), microwaved at pH 6.0

Ivl, Mouse monoclonal anti-involucrin (Sigma-Aldrich), Cat# I 9018,

(1:75), microwaved at pH 6.0

CK13, Mouse monoclonal anti-CK13 antibody (Lab Vision, Fremont, CA), Cat# MS-925-s,

(1:100), microwaved at pH 6.0

Note that deparaffinized sections were blocked and subjected to antigen retrieval at the conditions listed above, followed by incubation with primary antibodies overnight at 4°C at the indicated titers, and then with appropriate secondary antibodies for 30 min at room temperature. Nuclei were counterstained by 4',6-diamidino-2-phenylindole (DAPI)(1:10,000; Invitrogen) in immunofluorescence. Note that GFP was not detected after fixation without interfering Cy2 (green) used to stain IVL and CK13 in the tissues expressing GFP, DNMAML1 (GFP fusion protein) or *N3* shRNA transduced by MigRI or pGIPZ in Figures 2*C*, 6*E* and supplementary Figure 4*B*.

Supplementary Figure Legends

Supplementary Figure 1 Calcium (Ca^{2+}) induces cell-cell contact in a temporal and dose-dependent manner.

EPC2-hTERT cells maintained in medium containing low Ca^{2+} (0.09 mM) were stimulated with Ca^{2+} at indicated concentrations for 72 hrs in (*A*) or at 0.6 mM for indicated time periods in (*B*). Phase contrast images were acquired using a Nikon Eclipse TS100 microscope. Scale bar, 50 μm.

Supplementary Figure 2 Ca^{2+} induces *HES5*, *IVL* and *CK13* in primary and immortalized human esophageal cells.

Cells were stimulated with Ca^{2+} at indicated concentrations for 72 hrs in (*A*), (*C*) and (*D*), or at 0.6 mM for indicated time periods in (*B*) and subjected to real-time RT-PCR to determine *HES5*, *IVL* and *CK13* mRNA levels. (*A*) and (*B*), EPC2-hTERT cells; (*C*), EPC2 cells; (*D*), EPC1-hTERT cells. β-actin served as an internal control. *, $P< 0.001$ vs. 0.09 mM Ca^{2+} in (*A*), (*C*) and (*D*); *, $P< 0.001$ vs. time 0 in (*B*)(n=3).

Supplementary Figure 3 The MAML family is expressed in human esophageal cells.

(*A*) RT-PCR was done for indicated cycles to determine *MAML1*, *MAML2* and *MAML3* mRNA in EPC1-hTERT and EPC2-hTERT cells as described in Supplementary Materials and Methods. *GAPDH* served as an internal control. *, non-specific amplicon. (*B*) Western blotting determined MAML1-3 expression in EPC1-hTERT and EPC2-hTERT cells. β-actin served as a loading control.

Supplementary Figure 4 Notch inhibition impairs squamous differentiation of human esophageal epithelia reconstituted in organotypic 3D culture.

Cells were grown in 3D culture in the presence or absence of 1 μM compound E (GSI) or DNMAML1, and subjected to H&E staining in (*A*), IF for CK14 (red) and CK13 (green) in (*B*), and IHC for FLG in (*C*). Arrows indicate FLG. (*A*), EPC1-hTERT cells; (*B*) and (*C*), EPC2-hTERT cells. Ep., epithelium; Str., stroma; Scale bar, 50 μm.

Supplementary Figure 5 DNMAML1 repression restores Ca^{2+}-induced esophageal squamous differentiation.

EPC2-hTERT expressing DNMAML1 in the Tet-Off system were stimulated with 0.6 mM Ca^{2+} for 72 hrs in the presence or absence doxycycline (DOX). In (*A*), cells were transiently transfected with 8x*CSL-luc* reporter construct prior to Ca^{2+} and DOX treatment. In (*B*)-(*D*), real-time RT-PCR was done to determine *HES5*, *IVL* and *CK13* mRNA levels where β-actin served as an internal control. *, $P< 0.001$ vs. 0.6 mM Ca^{2+} + Dox (+); n=6 in (*A*)-(*D*). In (*E*), Western blotting demonstrates enhanced IVL induction upon DNMAML1 repression in the presence of DOX. DOX (-), 0 μg/ml DOX; DOX (+), 2 μg/ml DOX.

Supplementary Figure 6 Ca^{2+} induces *N1* and *N3* mRNA in primary and immortalized human esophageal cells.

Cells were stimulated with indicated concentrations of Ca^{2+} for 72 hrs in (*A*), (C) and (D) or 0.6 mM Ca^{2+} for indicated time periods in (*B*), and subjected to real-time RT-PCR to determine mRNA for indicated Notch family members. (*A*) and (*B*), EPC2-hTERT cells; (*C*), EPC2 cells; (*D*), EPC1-hTERT cells. β-actin served as an internal control. *, $P< 0.001$ vs. 0.09 mM Ca^{2+}; *ns*, not significant vs. 0.09 mM Ca^{2+}; (n=3) in (*A*)-(*C*). *ns*, not significant vs. time 0 (n=3) in (*D*).

Supplementary Figure 7 Notch regulates *N3* through CSL-dependent transcription.

EPC2-hTERT cells were stimulated with 0.6 mM Ca^{2+} for 72 hrs in the presence or absence of GSI in (*A*), DNMAML1 in (*B*), or 2 μg/ml doxycycline (DOX) to repress *DNMAML1* expression in the Tet-Off system in (*C*) and (*D*). In (*A*), Compound E (GSI) was used at indicated concentrations. Nuclear extracts were prepared in (*A*), (*B*) and (*D*) to determine ICN3 by Western blotting where Histone H1 served as loading controls. In (*C*), *N3* mRNA was determined by real-time RT-PCR where β-actin served as an internal control. *, $P< 0.001$ vs. 0.6 mM Ca^{2+} + Dox (+)(n=3).

Supplementary Figure 8 Ca^{2+} induces ICN3 in primary mouse esophageal keratinocytes.

MEK3N cells, primary wild-type mouse esophageal keratinocytes[4] were stimulated with 0.6 mM Ca^{2+} for 72 hrs in the presence or absence of GSI. Compound E (GSI) was used at indicated concentrations. Whole cell lysates were used in to determine Ivl and ICN3 induction by Western blotting where β-actin served as loading controls.

Supplementary Figure 9 Ca^{2+} may induce *N1* mRNA through a CSL-independent mechanism.

EPC2-hTERT cells were stimulated with 0.6 mM Ca^{2+} for 72 hrs in the presence or absence of GSI or DNMAML1 to determine *N1* mRNA expression by real-time RT-PCR. Compound E (GSI) was used at indicated concentrations. β-actin served as an internal control. *, $P< 0.001$ vs. 0.6 mM Ca^{2+} + 0 μM GSI (n=3). Note that DNMAML1, unlike GSI, failed to suppress Ca^{2+}-stimulated *N1* mRNA induction.

Supplementary Figure 10 ICN1 directly induces squamous differentiation markers through CSL-dependent transcription in esophageal keratinocytes.

EPC2-hTERT cells expressing either DNMAML1 or GFP (control) were transduced with ICN1 to determine (*A*) 8x*CSL-luc* activity, (*B*) *HES5* mRNA, (*C*) *IVL* mRNA, and (*D*) C*K13* mRNA. Luciferase assays and real-time RT-PCR determined the reporter activity and the mRNA levels, respectively. β-actin served as an internal control in (*B*)-(*D*). *, $P< 0.001$ vs. GFP + vector control; #, $P< 0.001$ vs. GFP + ICN1; n=6 in (*A*) and n=3 in (*B*)-(*D*).

Supplementary Figure 11 N1 contributes to Ca^{2+}-stimulated induction of N3, *HES5* and squamous differentiation markers.

EPC2-hTERT cells were stimulated with Ca^{2+} for 72 hrs following *N1* siRNA transfection to determine indicated mRNA species by real-time RT-PCR. β-actin served as an internal control. *, $P< 0.001$ vs. 0.09 mM Ca^{2+} + siRNA control; #, $P< 0.001$ vs. 0.6 mM Ca^{2+} + siRNA control; n=3.

Supplementary Figure 12 N1 binds to *HES1* promoter, but not the *N3* distal (-12 kb) and proximal (-2.3 kb) regions.

EPC2-hTERT cells were stimulated with Ca^{2+} for 48 hrs and subjected to ChIP assays for *HES1* promoter (*A*) and *N3* (*B*) with indicated antibodies. *, $P< 0.001$ vs. 0.09 mM Ca^{2+}; n=3.

Supplementary Figure 13 N3 is required for ICN1-induced esophageal squamous differentiation.

EPC2-hTERT cells stably expressing two independent shRNA sequences directed against *N3* (N3-A and N3-B) or scrambled non-silencing control shRNA were transduced with ICN1 in (*A*)-(*D*) or stimulated with 0.6 mM Ca^{2+} for 72 hrs in (*E*), and subjected to real-time RT-PCR in (*A*), (*C*)-(*E*) or Western blotting in (*B*) to determine indicated molecules. In (*A*)-(*D*), cells were harvested 72 hrs after retrovirus infection. β-actin served as an internal or loading control. *, $P< 0.001$ vs. ICN1 + scramble; #, $P< 0.001$ vs. ICN1 + scramble; n=3 in (*A*), (C) and (*D*). *, $P< 0.001$ vs. 0.09 mM Ca^{2+} + scramble; #, $P< 0.001$ vs. 0.6 mM Ca^{2+} + scramble; n=3 in (*E*).

Supplementary Figure 14 Model for regulation of esophageal squamous differentiation by a crosstalk between N1 and N3.

In esophageal keratinocytes, cell-cell contact allows cell surface ligands (e.g. JAG1, JAG2 and Dll1) to bind N1. N1 activation results in ICN1 generation and nuclear translocation. ICN1 activates N3 transcription directly in a CSL-dependent fashion. N3 is activated subsequently. N3 is required and cooperates with N1 during esophageal squamous differentiation. ICN1 and ICN3 cooperatively induce other CSL-dependent downstream target molecules HES5, IVL and CK13. GSI and DNMAML1 inhibit Notch signaling. Ligands responsible for Notch activation and the roles of HES5 in esophageal squamous differentiation remain to be elucidated. Red arrows indicate CSL-dependent transcription.

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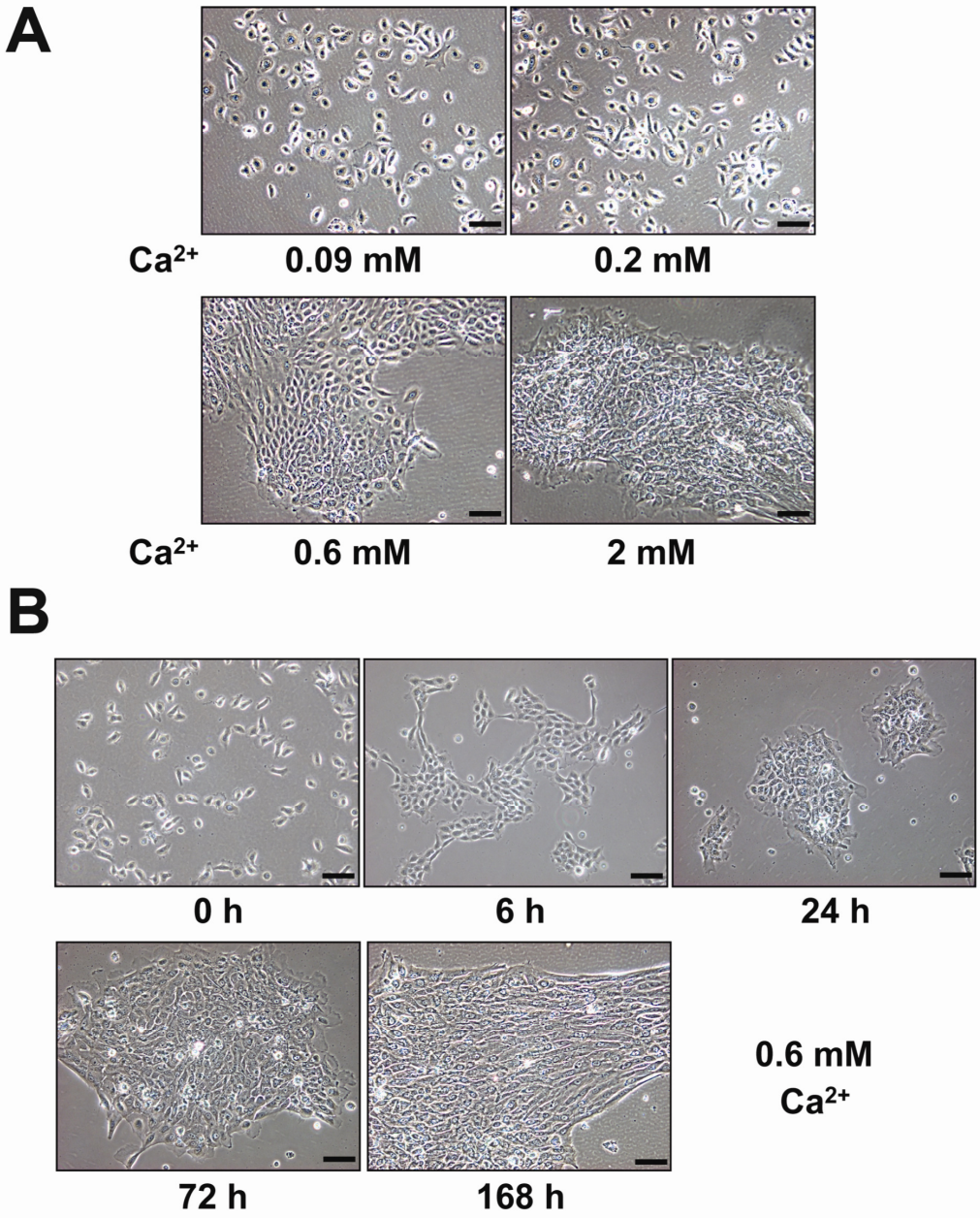
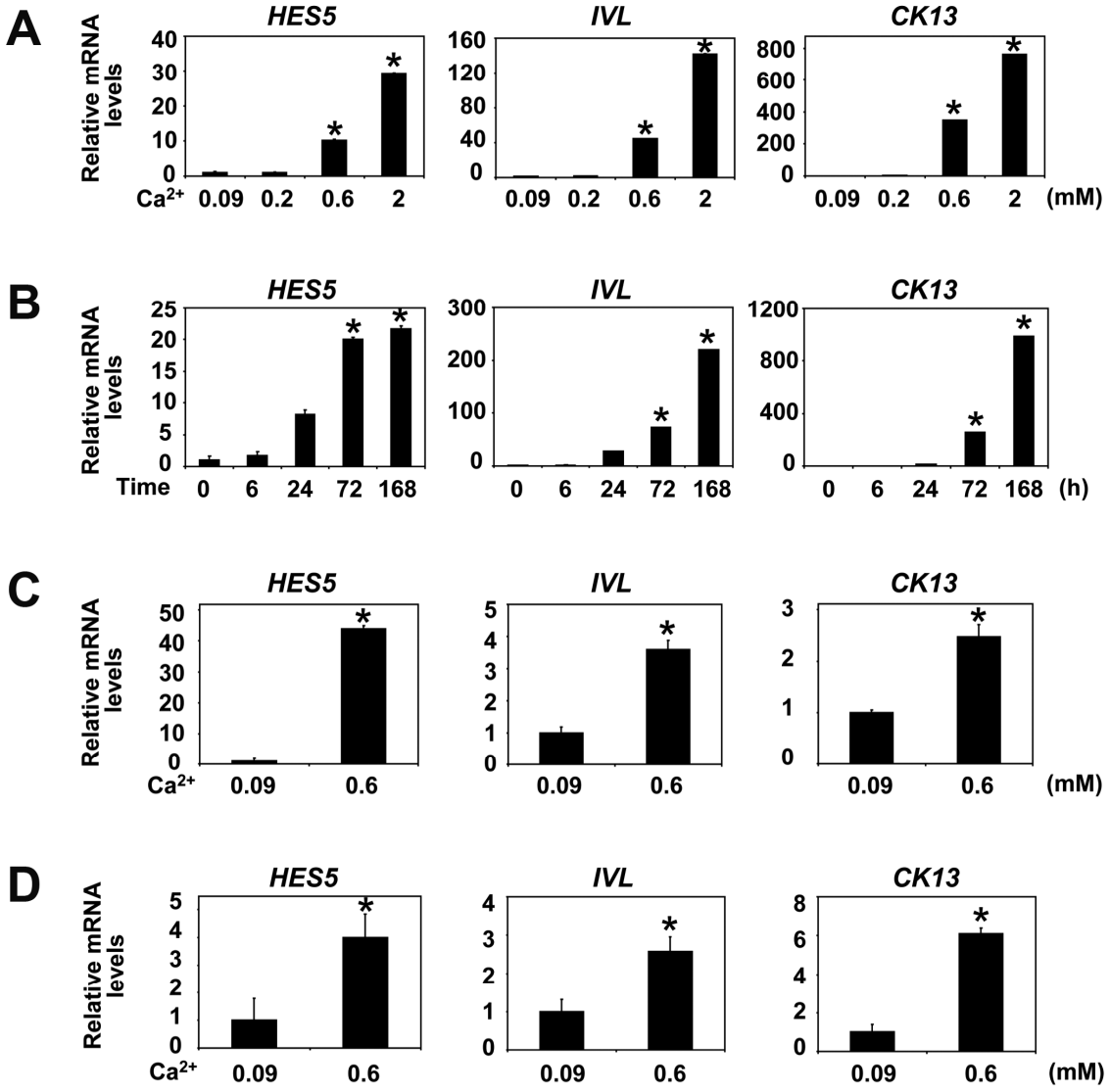


Supplementary Figure 1



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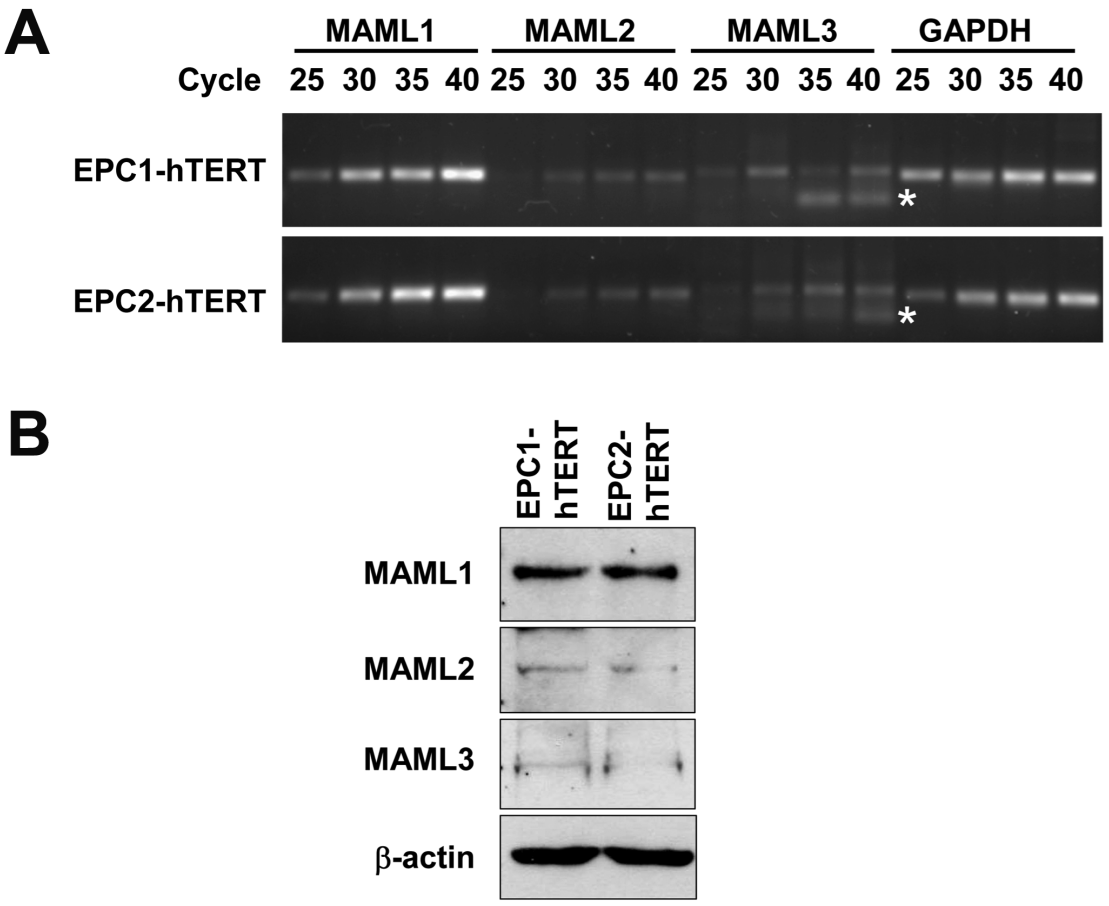

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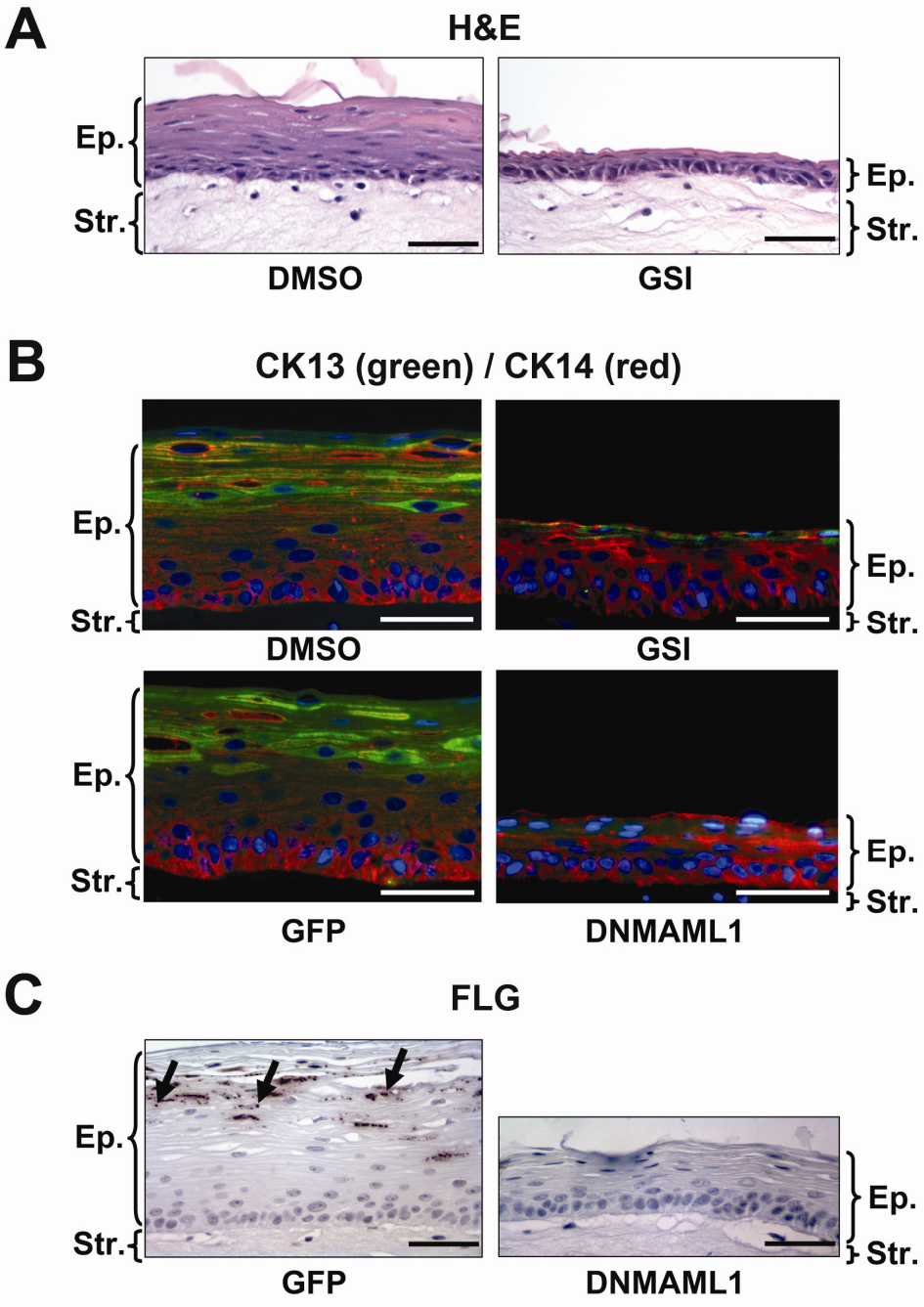

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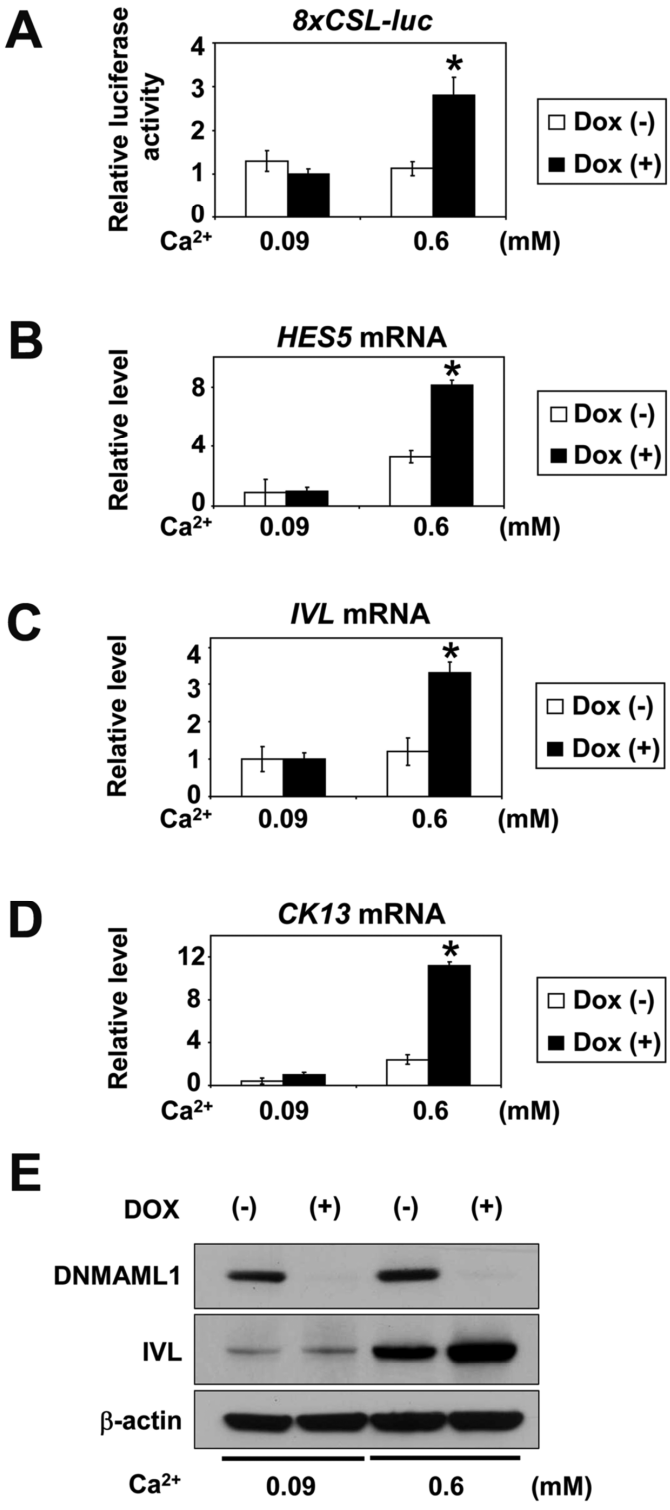

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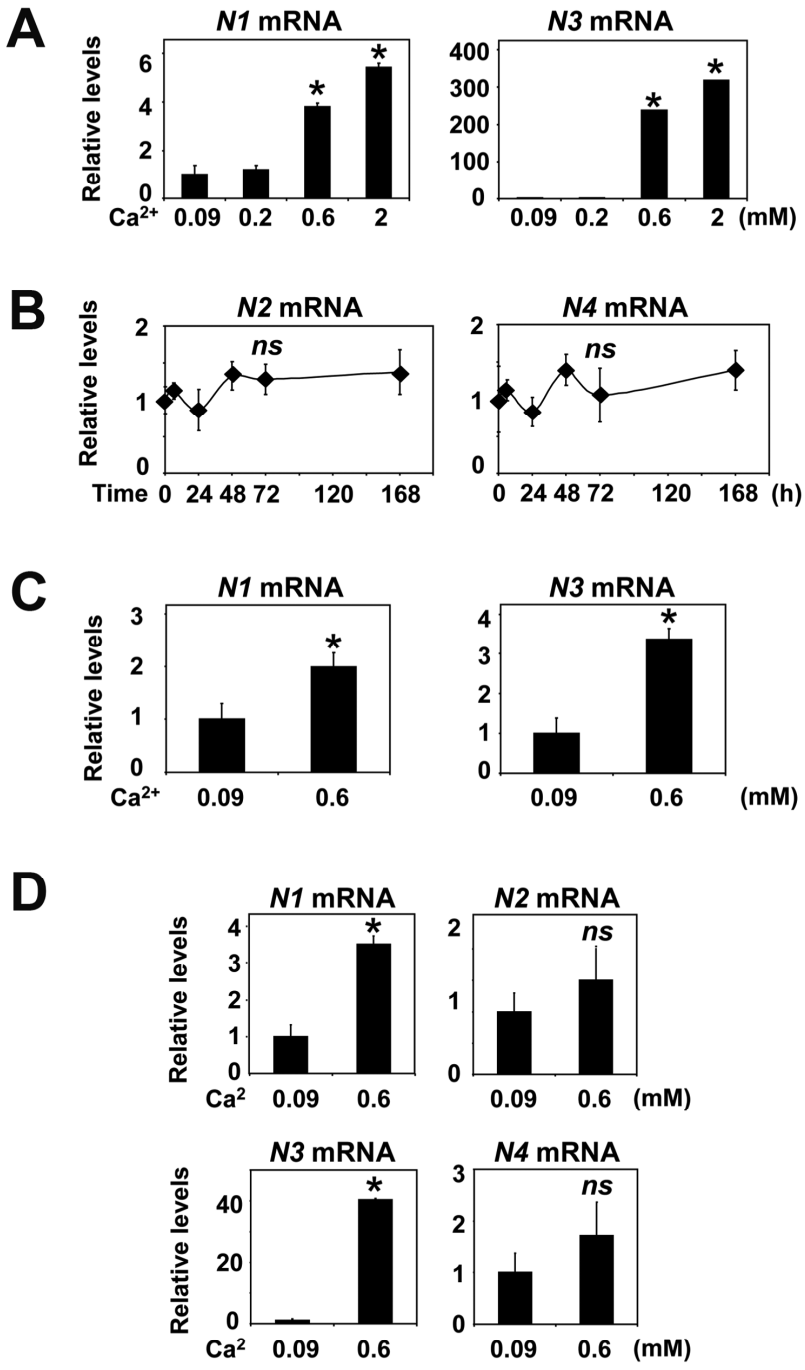

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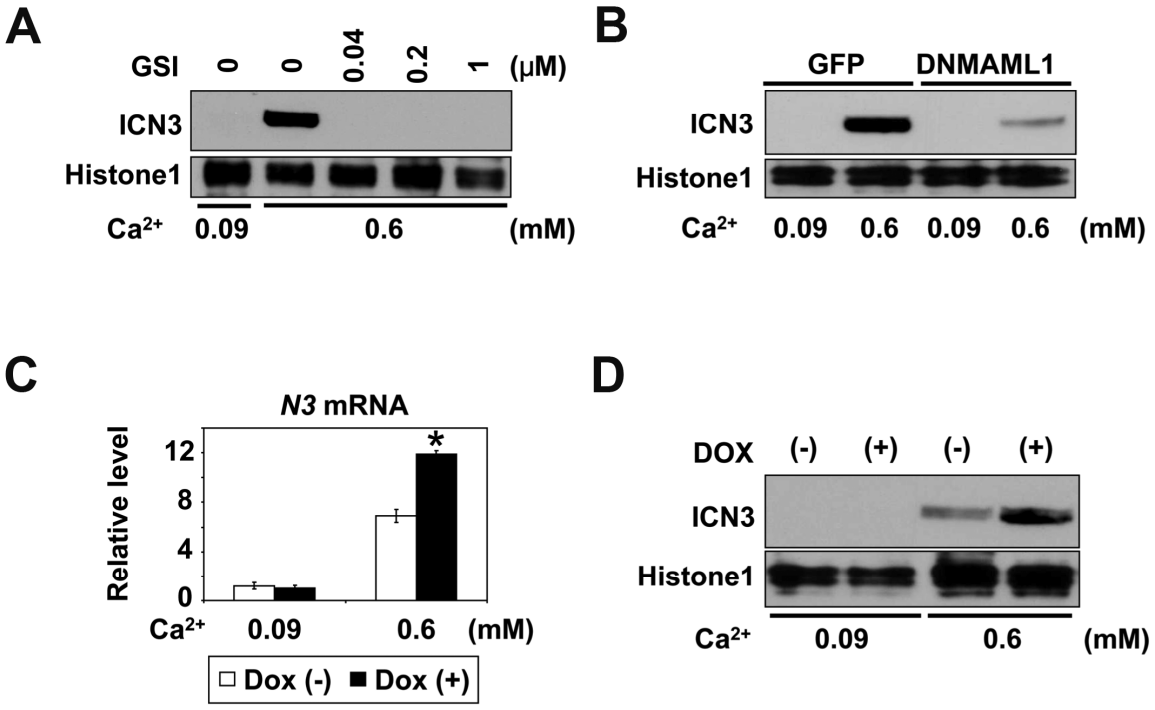

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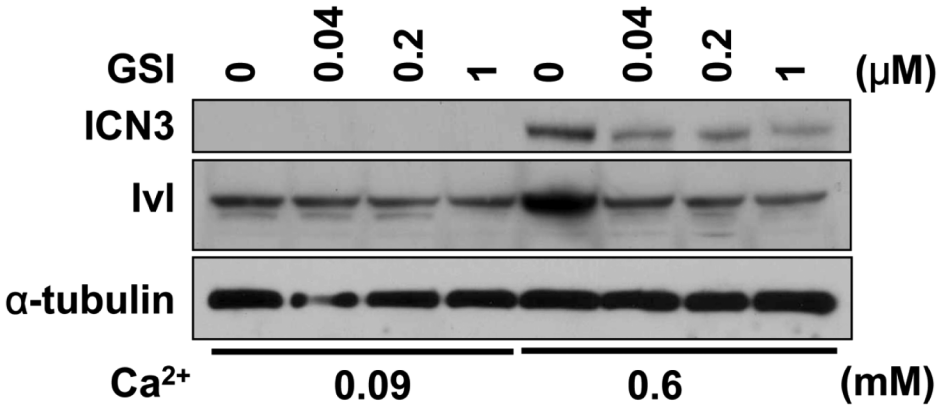

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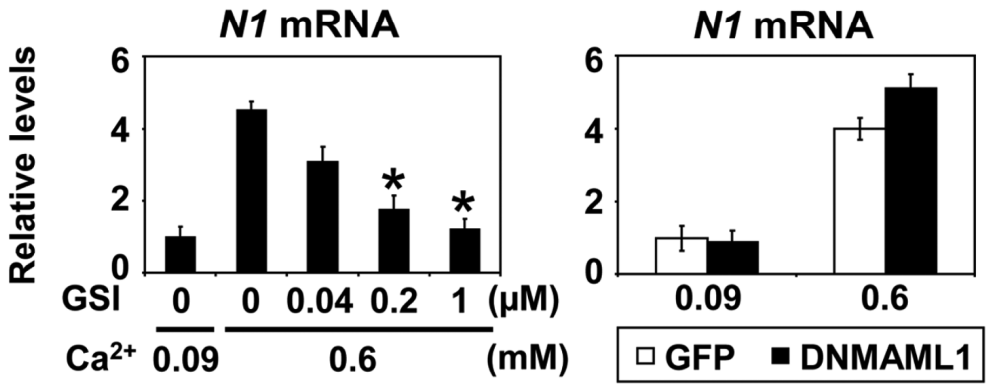

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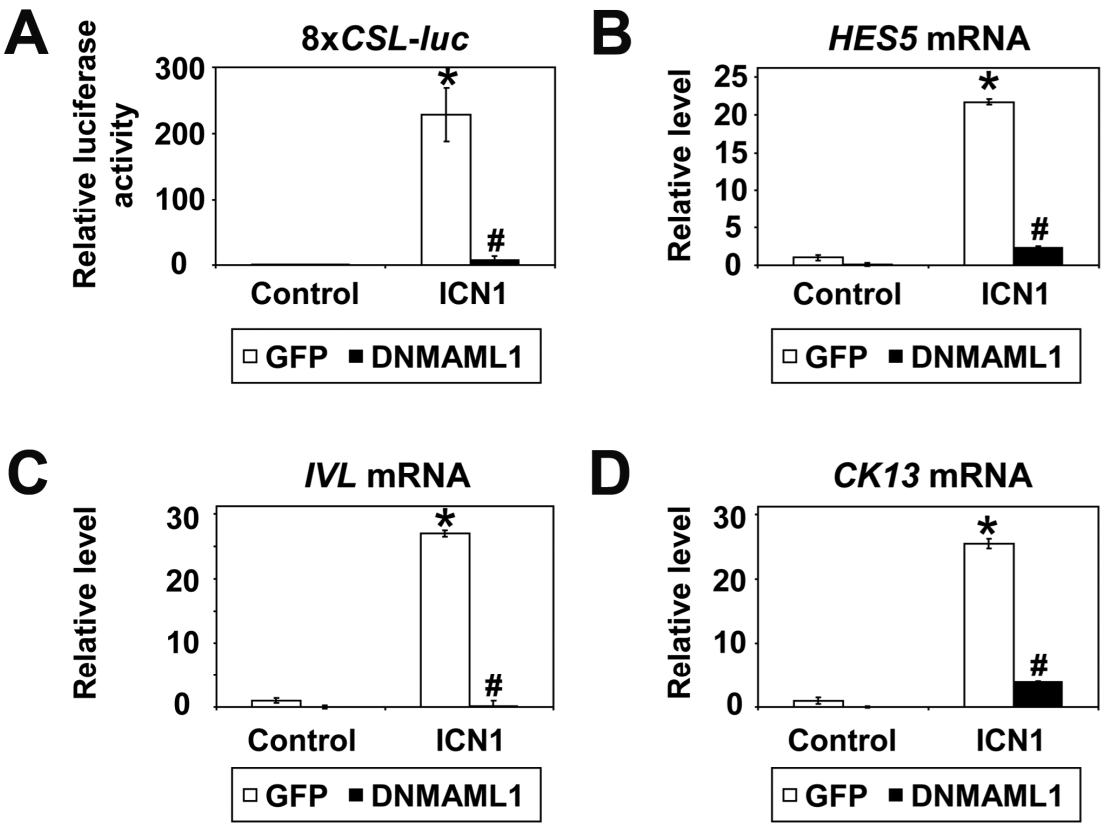

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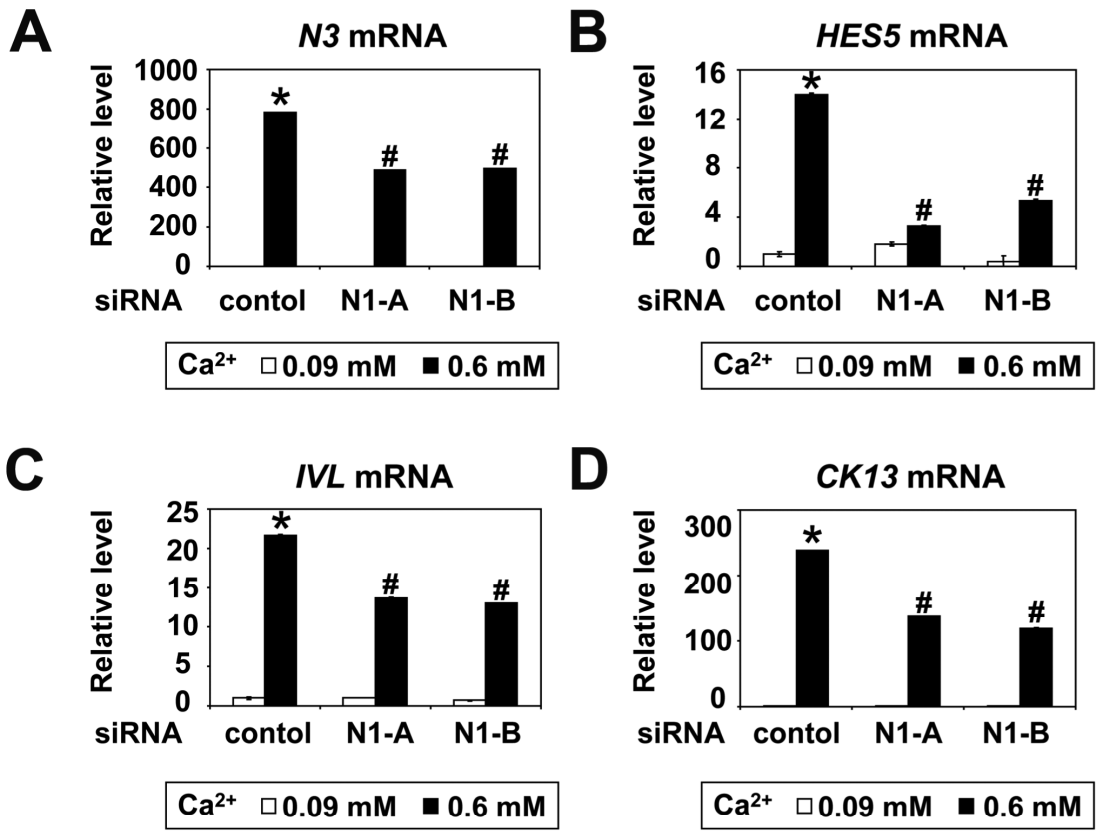

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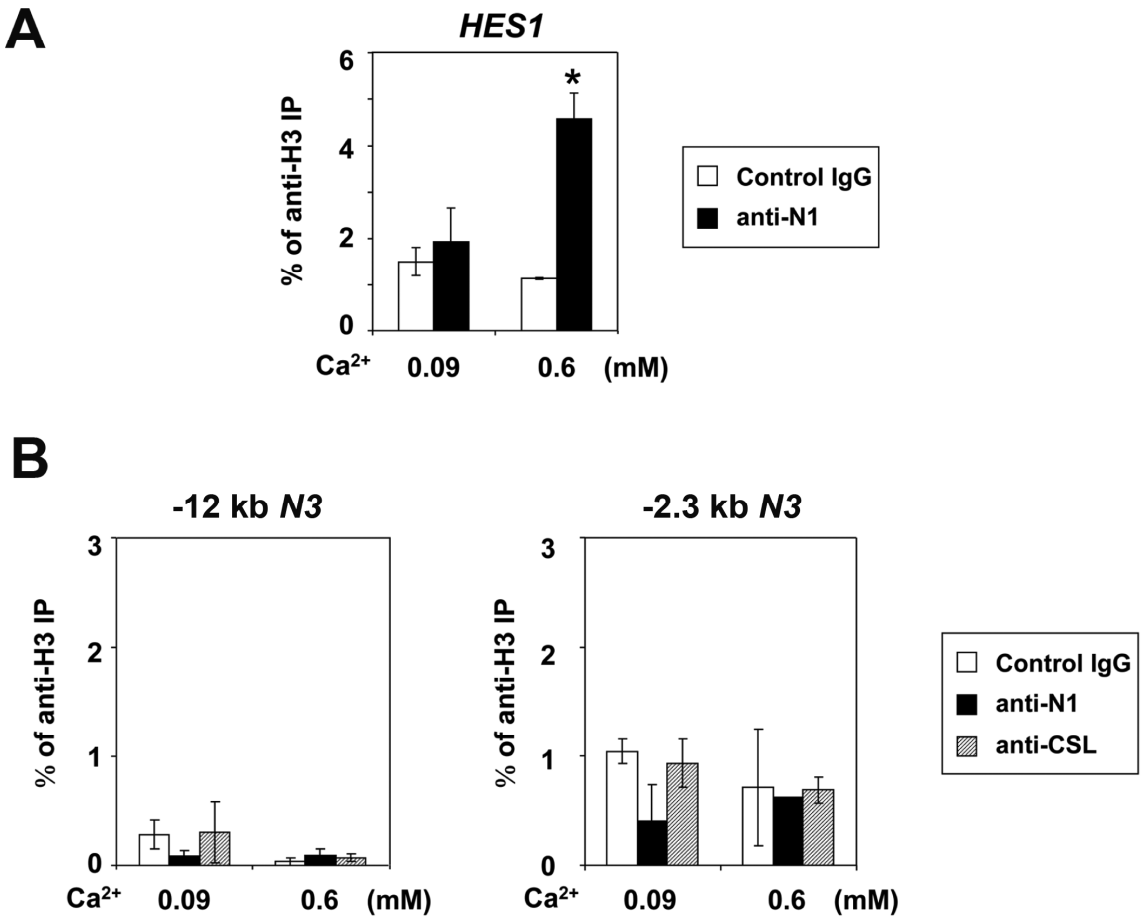

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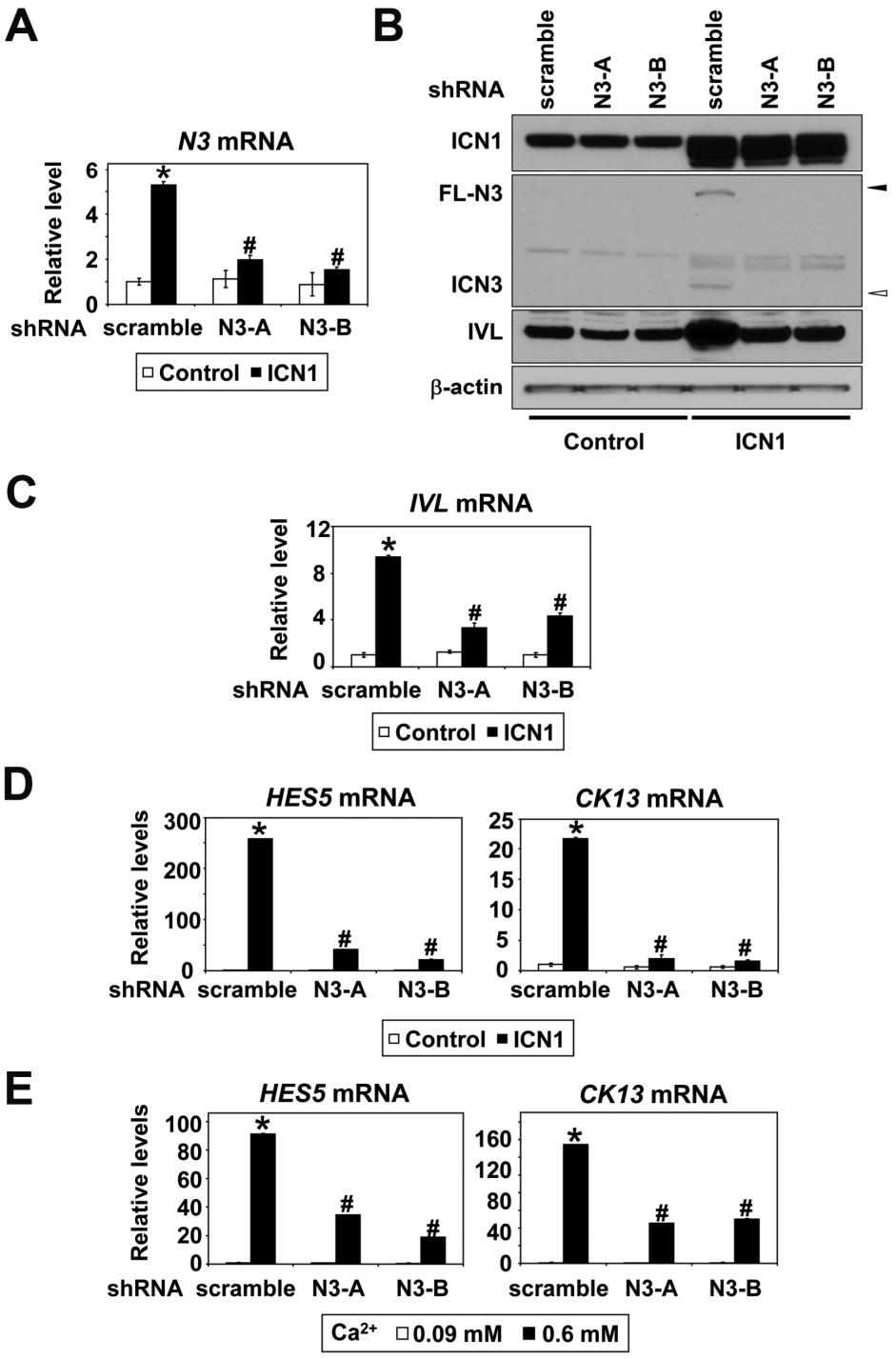

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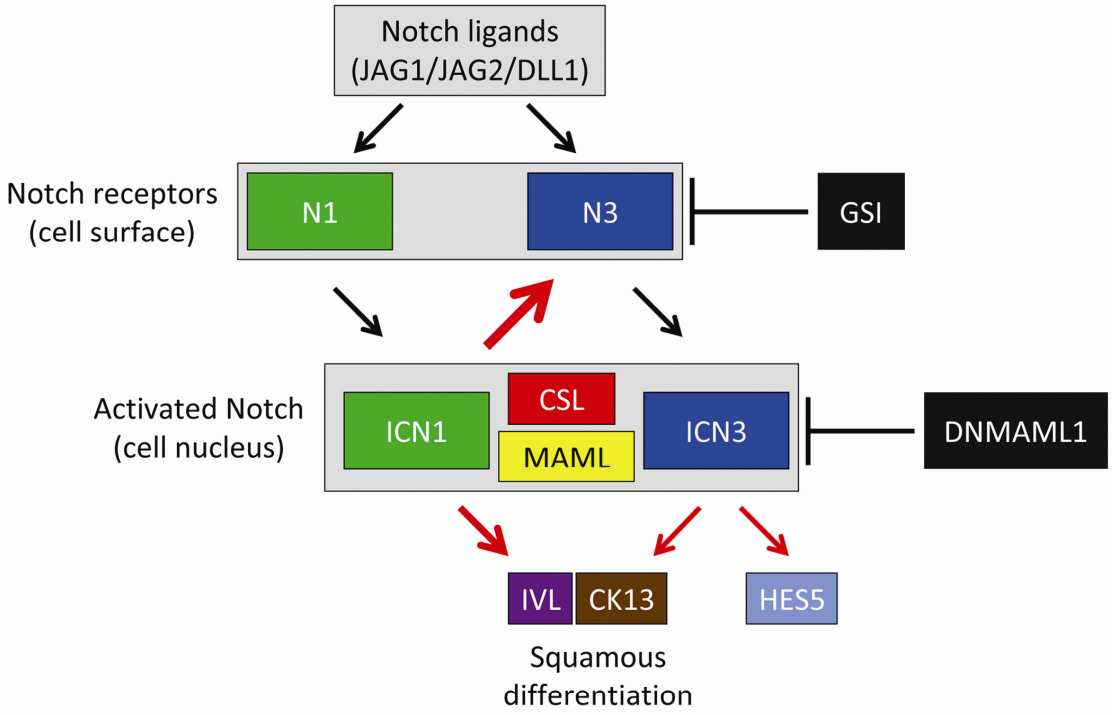

Supplementary Figure 14