PHOSPHORYLATION HOTSPOT IN THE C-TERMINAL DOMAIN OF OCCLUDIN REGULATES THE DYNAMICS OF EPITHELIAL JUNCTIONAL COMPLEXES

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List of materials included

Supplemental figures S1 through S8
Table S1

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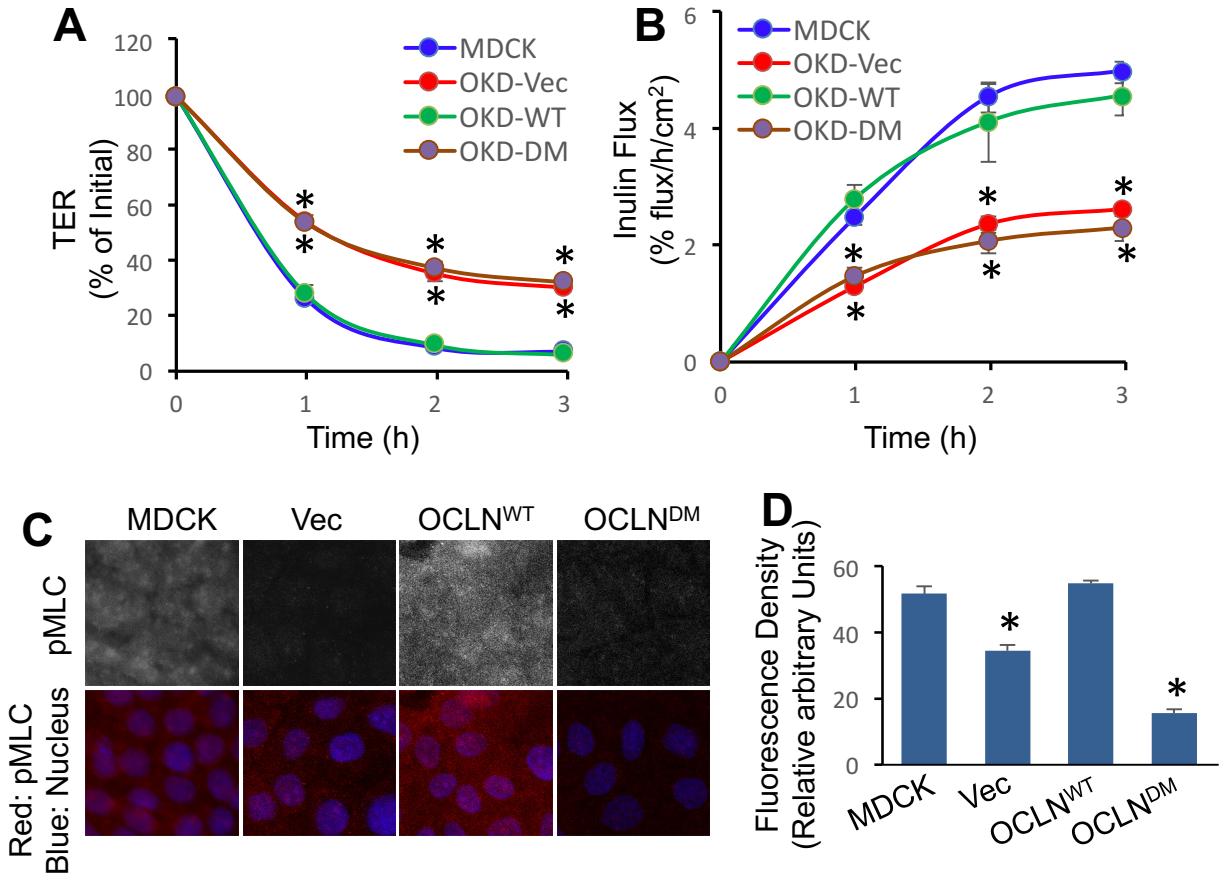

Figure S1: Effect of ORM deletion on actin cytoskeleton. A & B: MDCK (Blue), OLCN^{VEC} (OKD-Vec; Red), OLCN^{WT} (OKD-WT; Green) and OCLN^{DM} (OKD-DM; Brown) cell monolayers in transwell inserts were incubated with latrunculin A (100 nM). At varying times TER (A) and FITC-inulin flux (B) were measured. Values are mean ± SEM (n = 3). Asterisks indicate the Vec and DM values are significantly ($P<0.05$) different from corresponding values for MDCK and WT cell monolayers. **C & D:** MDCK, OLCN^{VEC}, OLCN^{WT} and OCLN^{DM} cell monolayers in transwell inserts were fixed and stained for pMLC (Red) and nucleus (Blue) (C). Fluorescence in 6 different regions of monolayers were measured (D) and values presented as mean ± SEM. This was repeated once with similar results. Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding values for MDCK and WT cell monolayers.

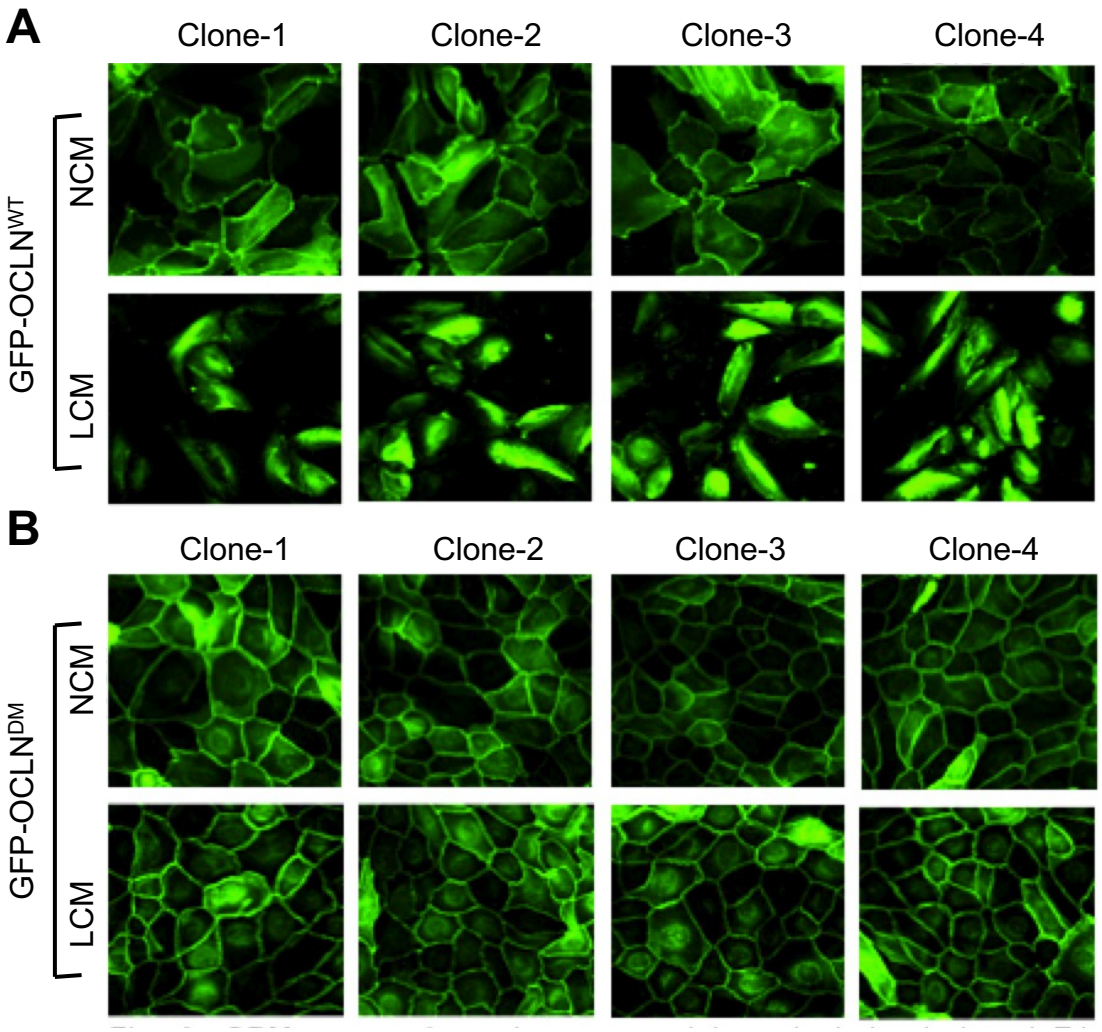

Figure S2: ORM deletion attenuates Ca2+ depletion-mediated disassembly of occludin from the TJ. $OCLN^{WT}$ (A) and $OCLN^{DM}$ (B) cell monolayers from different clones were incubated with low calcium medium (LCM) or normal calcium medium (NCM) for 16 hr. Live-cell images for EGFP fluorescence were captured before and after incubation.

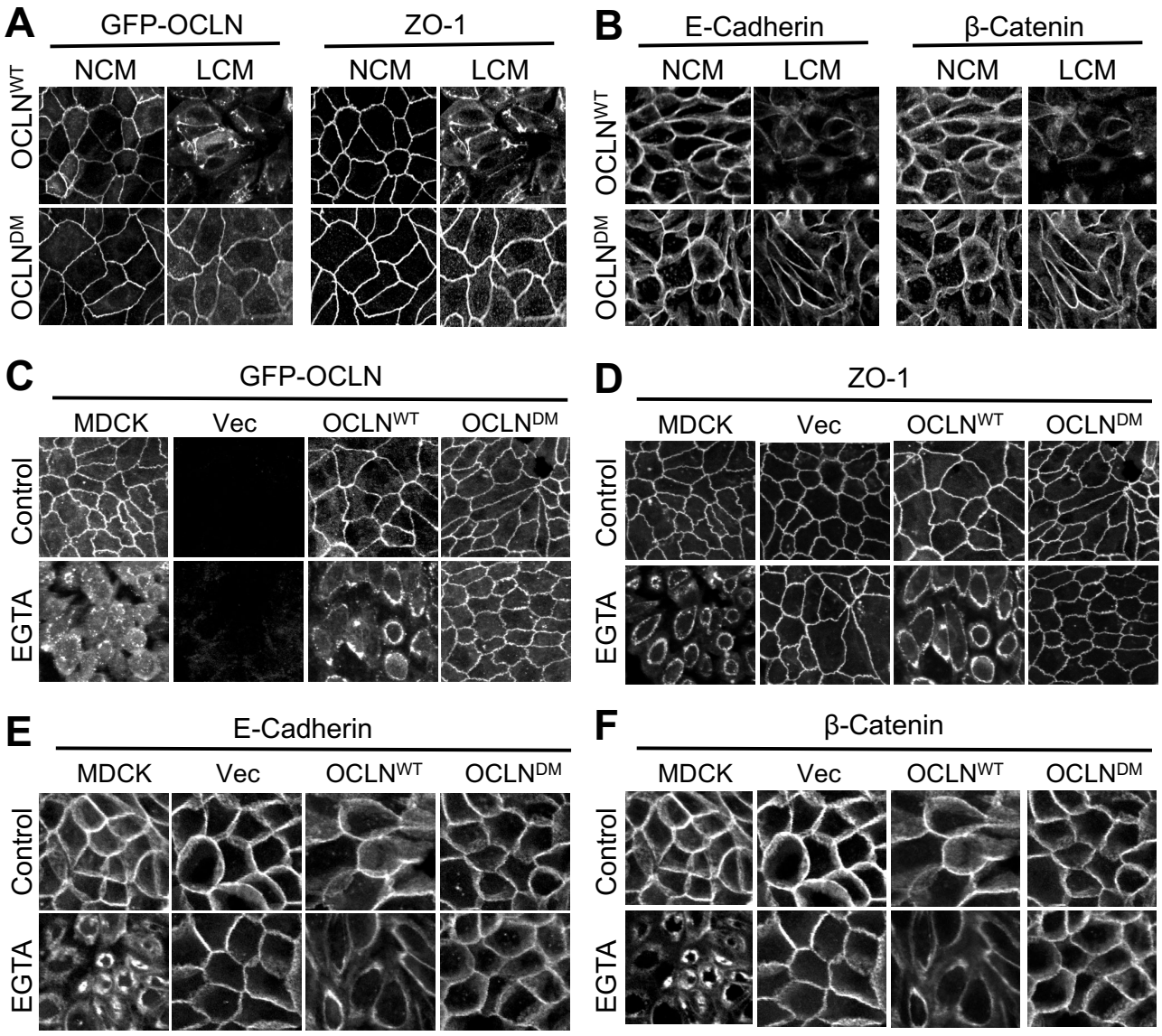

Figure S3: Deletion of ORM attenuates Ca^{2+} depletion-induced redistribution of TJ and AJ proteins. A & B: $OCLN^{WT}$ and $OCLN^{DM}$ cell monolayers were incubated with low Ca^{2+} medium (LCM) or normal Ca^{2+} medium (NCM) for 16 hr. Fixed cell monolayers were stained for EGFP-occludin/ZO-1 (A) and E-cadherin/β-Catenin (B). **C-F:** MDCK, Vec, $OCLN^{WT}$ and $OCLN^{DM}$ cell monolayers were incubated with or without 4 mM EGTA. Cell monolayers fixed at 0, 5 and 30 min of EGTA treatment were stained for EGFP-occludin (C), ZO-1 (D), E-cadherin (E) and β-catenin (F).

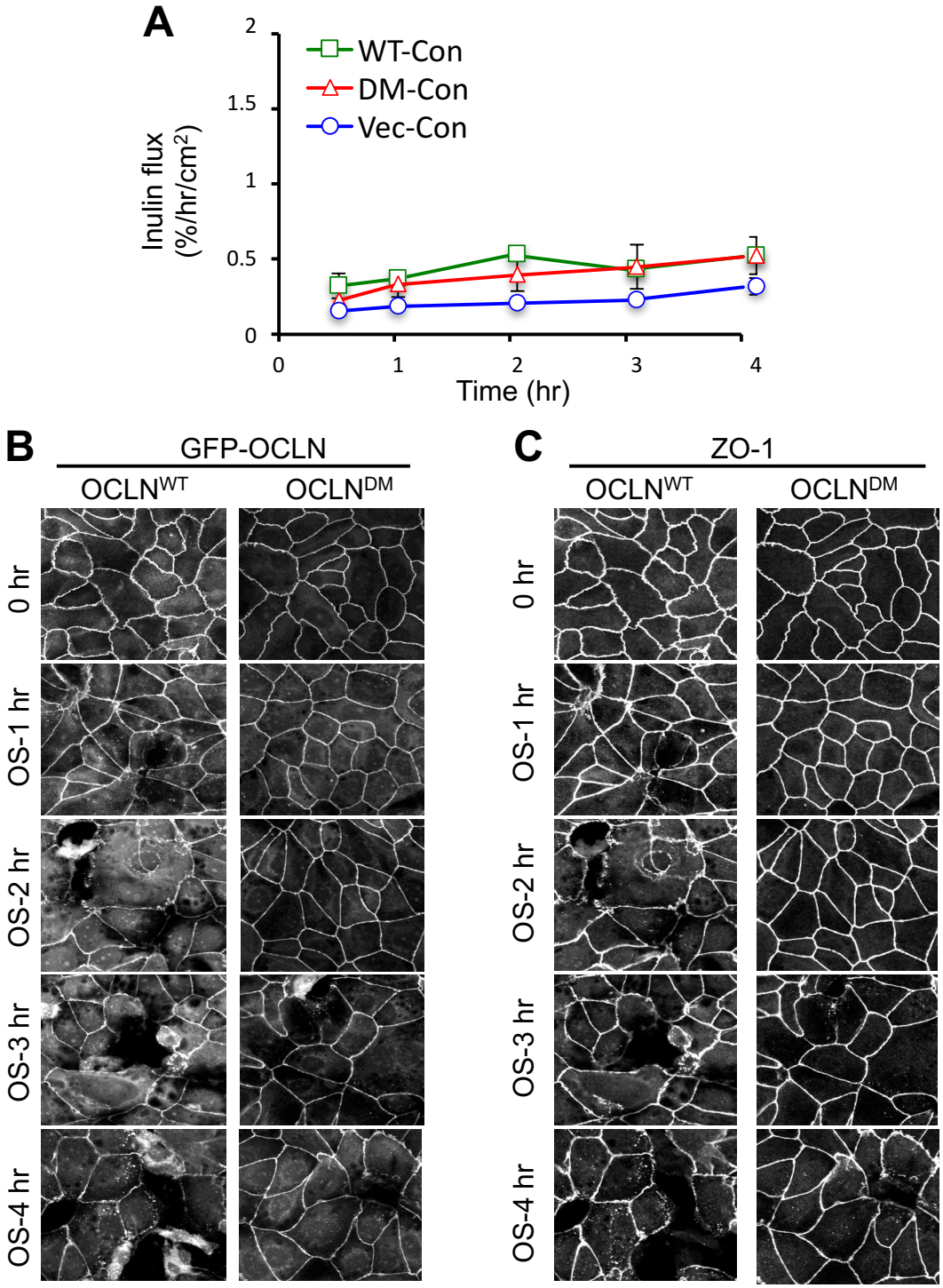

Fig S4: Deletion of ORM attenuates osmotic stress-induced redistribution of TJ proteins. $OCLN^{WT}$, $OCLN^{DM}$ and Vec cell monolayers were grown in transwell inserts and inulin flux (A) was measured for 4 hr. These data represent the control values for data in Fig. 4A. **B & C:** Following osmotic stress for varying times, cell monolayers were fixed and stained for EGFP-occludin (B) and ZO-1 (C); these images represent the individual images corresponding to those in Fig. 4B.

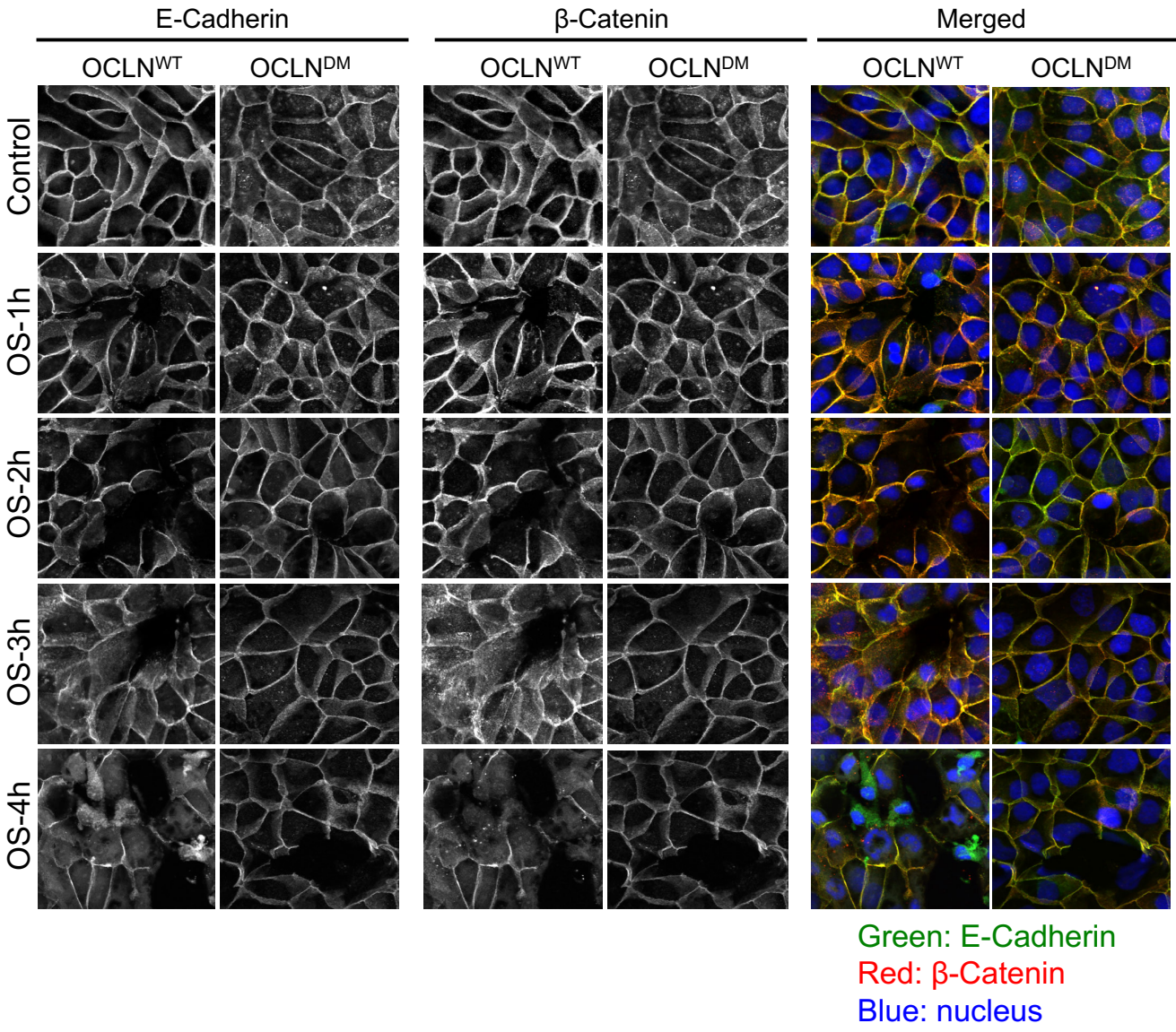

Fig S5: Deletion of ORM attenuates osmotic stress-induced redistribution of AJ proteins. $OCLN^{WT}$, $OCLN^{DM}$ and Vec cell monolayers on transwell inserts were exposed to osmotic stress for varying times. Fixed cell monolayers were co-stained for E-cadherin and β-catenin.

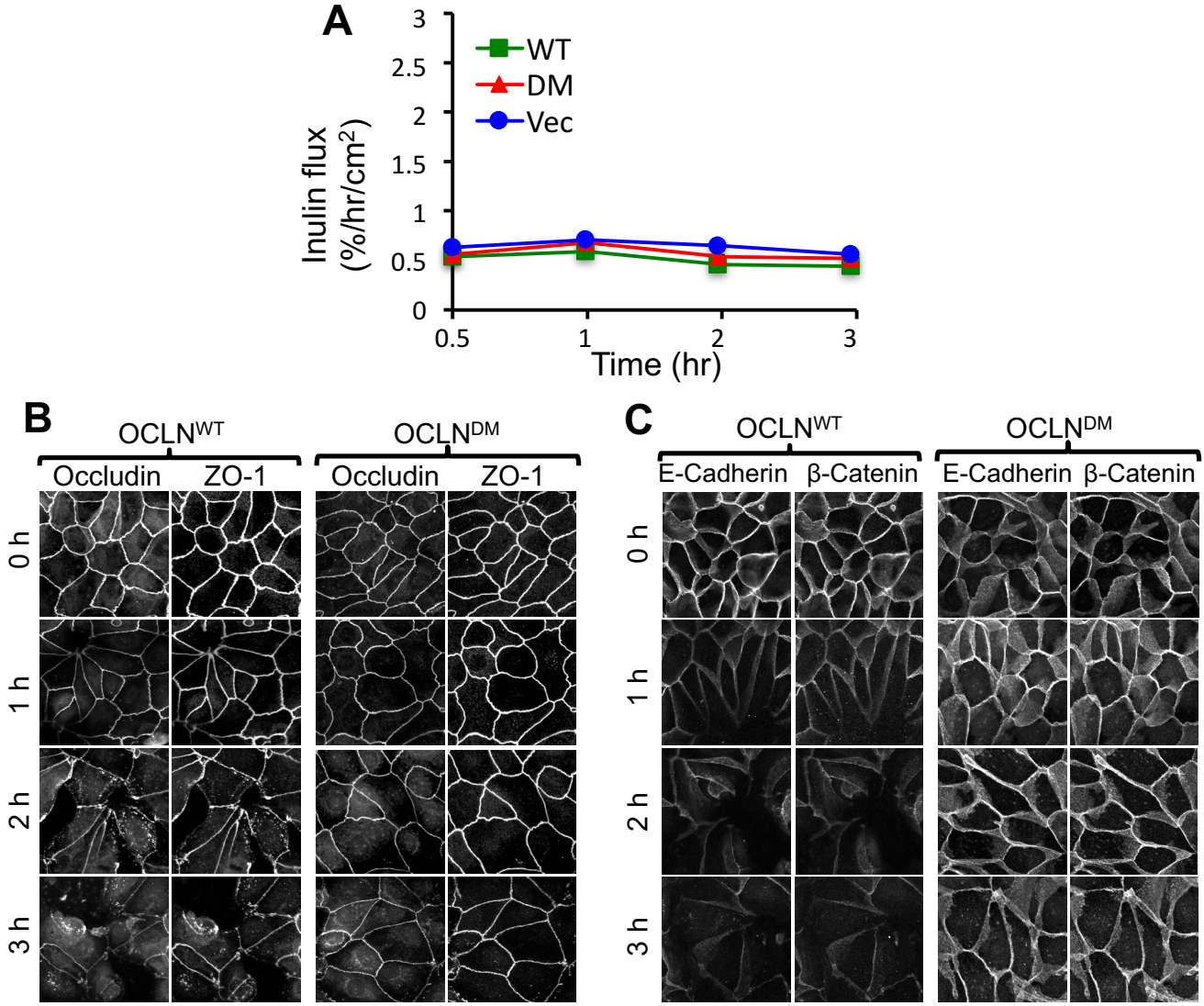

Fig S6: Deletion of ORM attenuates hydrogen peroxide-induced disruption of AJC.
A: $OCLN^{WT}$, $OCLN^{DM}$ and Vec cell monolayers were grown in transwell inserts were exposed to 100 µM hydrogen peroxide and inulin flux was measured at various time points; data represent the control values corresponding to data in Fig. 4D. **B & C:** Cell monolayers were treated with hydrogen peroxide for varying times. Fixed cell monolayers were stained for EGFP-occludin/ZO-1 (B) or E-cadherin/β-catenin (C).

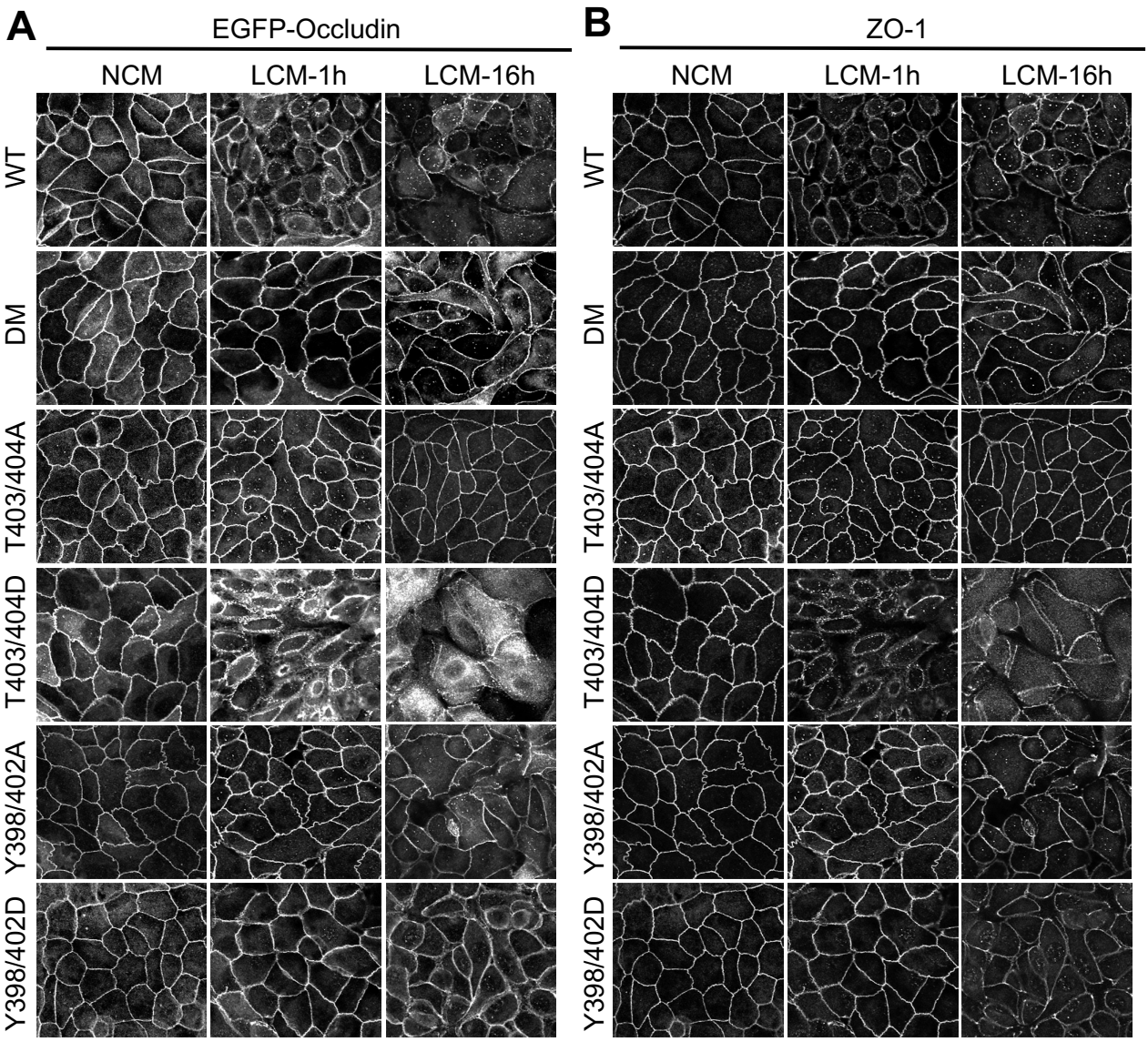

Figure S7: Mutation of phosphorylation sites in ORM alters Ca^{2+} depletion-induced redistribution of TJ proteins. $OCLN^{WT}$, $OCLN^{DM}$, $OCLN^{T403/404A}$, $OCLN^{T403/404D}$, $OCLN^{Y398/402A}$ and $OCLN^{Y398/402D}$ cell monolayers were incubated with NCM or LCM for varying times. Fixed cell monolayers were stained for EGFP-Occludin (A) and ZO-1 (B) by immunofluorescence method.

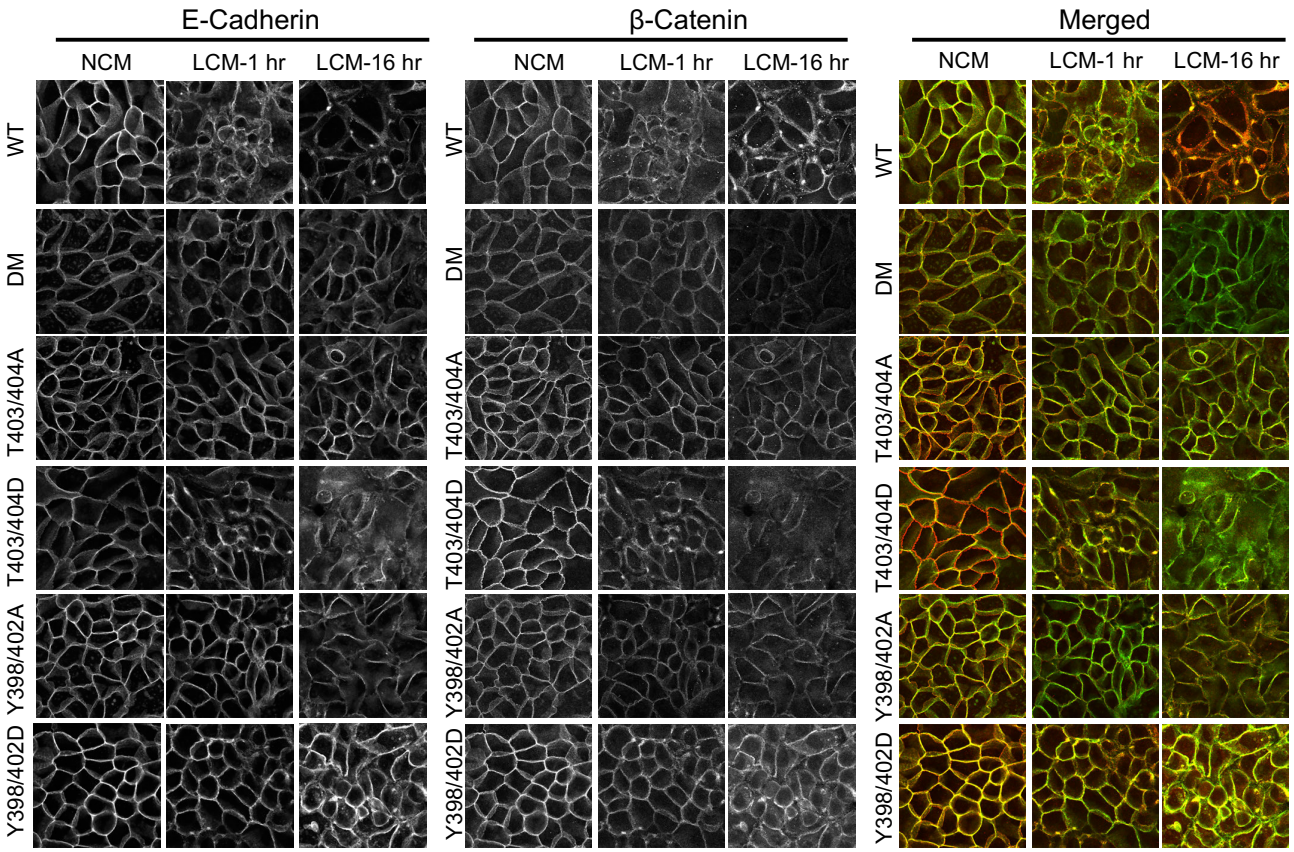

Figure S8: Mutation of phosphorylation sites in ORM alters Ca^{2+} depletion-induced redistribution of AJ proteins. $OCLN^{WT}$, $OCLN^{DM}$, $OCLN^{T403/404A}$, $OCLN^{T403/404D}$, $OCLN^{Y398/402A}$ and $OCLN^{Y398/402D}$ cell monolayers were incubated with NCM or LCM for varying times. Fixed cell monolayers were co-stained for E-Cadherin (green) and β-Catenin (red) by immunofluorescence method.

Table S1: Primers for shRNA constructs.

Top strand 1:
5'-GATCCCGTATGTCAGACCTTATAACGTTGATATCCGCGTTATAAGGTCTGA
CATATTTTTTCCAAA-3'

Bottom strand 1:
5'-AGCTTTTGGAAAAAATATGTCAGACCTTATAACGCGGA TATCAACGTTATA
AGGTCTGACATACGG-3'

Top strand 2:
5'-GATCCCGTATGCTACCACCC ATTAAGTTGATATCCGCTTAATGGGTGGTAGC
ATATTTTTTCCAAA-3'

Bottom strand 2:
5'- AGCTTTTGGAAAAAATATGCTACCACCCATTAAGCGGATATCAACTTAATGGG
TGGT AGCATACGG-3'

Table S2: Details of antibodies used in this article.

Antibody	Vendor	Cat#	Clone #	Source	Dilution For Immuno-fluorescence	Dilution for Western
Anti-β-Catenin	ThermoFisher Sci.	6734 (Lot 2759753)		Rabbit polyclonal	1-300	
Anti-E-cadherin	BD Biosciences	610182 (Lot 49277)	36	Mouse monoclonal	1-500	
Anti-Claudin 2	ThermoFisher Sci.	325600 (Lot QF215342)	12H12	Mouse monoclonal	1-100	1-1000
Anti-Occludin	ThermoFisher Sci.	331500 (Lot RG230928)	OC3F10	Mouse monoclonal	1-100	1-1000
Anti-ZO-1	ThermoFisher Sci.	617300 (Lot 1575558A)		Rabbit polyclonal	1-100	1-1000
Anti-EGFP	ThermoFisher Sci.	632381 (Lot A5033481)	JL8	Mouse monoclonal	1-100	
Anti-β-Tubulin	ThermoFisher Sci.	FCMAB321F	AA2	Mouse monoclonal	1-100	
Anti-pMLC	ThermoFisher Sci.	72013 (Lot 3)		Mouse monoclonal	1-100	
HRP-conjugated anti-mouse IgG	ThermoFisher Sci.	AA4416		Goat polyclonal		1-1000
HRP-conjugated anti-rabbit IgG	Upstate Biotech	12-348		Goat polyclonal		1-1000
Cy3-conjugated anti-rabbit IgG	Sigma-Aldrich	C2306 (Lot SLBQ0938V)		Goat polyclonal	1-100	
AlexaFluor488-conjugated anti-mouse IgG	Life technology	A11029 (Lot 1789729)		Goat polyclonal	1-100	

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