Supplemental Materials

Molecular Biology of the Cell

Nanes et al.

Supplemental Figures

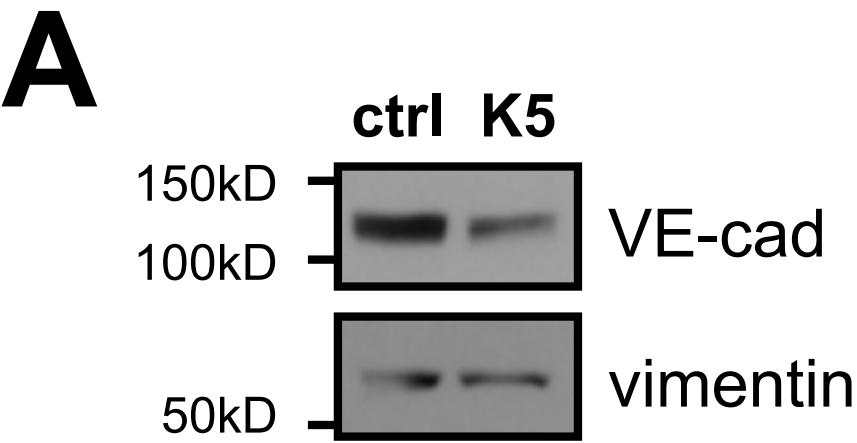
Figure S1. K5-induced down-regulation of VE-cadherin is rapid and selective. **(**A) K5–FLAG was expressed in primary dermal microvascular endothelial cells by adenoviral transduction. After 24 hours, cells were harvested, and the lysates analyzed by Western blot. (B) K5–FLAG was expressed in primary human keratinocyte cultures by adenoviral transduction. After 48 hours, cells were harvested, and the lysates analyzed by Western blot.

Figure S2. K5-induced down-regulation of VE-cadherin requires ubiquitin ligase activity. (A) K5–FLAG was expressed in primary dermal microvascular endothelial cells by adenoviral transduction. After 6 hours, cells were treated with 50 μM MG-132 to disrupt the ubiquitin–proteasome system, or with vehicle as a control. After 24 hours, cells were fixed and processed for immunofluorescence. (B) CHO cell lines stably expressing VE-cadherin were transfected with wild-type K5–GFP or a K5 mutant lacking ligase activity (RING mutant; Means et al., 2007). After 24 hours, cells were fixed and processed for immunofluorescence. Bars: 20 μm.

Figure S3. K5 targets two membrane-proximal lysine residues on VE-cadherin. K5–FLAG was expressed in CHO cell lines stable expressing wild-type or mutant (K626R K633R) VE-cadherin by adenoviral transduction. After 30 hours, cells were fixed and processed for immunofluorescence.

Figure S4. MARCH proteins are expressed in endothelial cells and exogenous expression disrupts endothelial junctions. (A). RT-PCR analysis of MARCH expression in primary dermal microvascular endothelial cells. (B) MARCH2-GFP expression in MEC cells leads to decreased expression of VE-cad (top, red) but not PECAM-1 (middle, red) or TfR (bottom, red). (C) Expression of a MARCH2-GFP mutant lacking ligase activity (RING domain mutant) fails to promote VE-cad downregulation in primary dermal microvascular endothelial cells cells. (D) MARCH4-GFP expression in primary dermal microvascular endothelial cells cells leads to decreased expression of VE-cad (top, red) and PECAM-1 (middle, red), but not TfR (bottom, red). Bars: 20 μm. Asterisks indicate cells expressing MARCH proteins.

Figure S1


A
ctrl
K5
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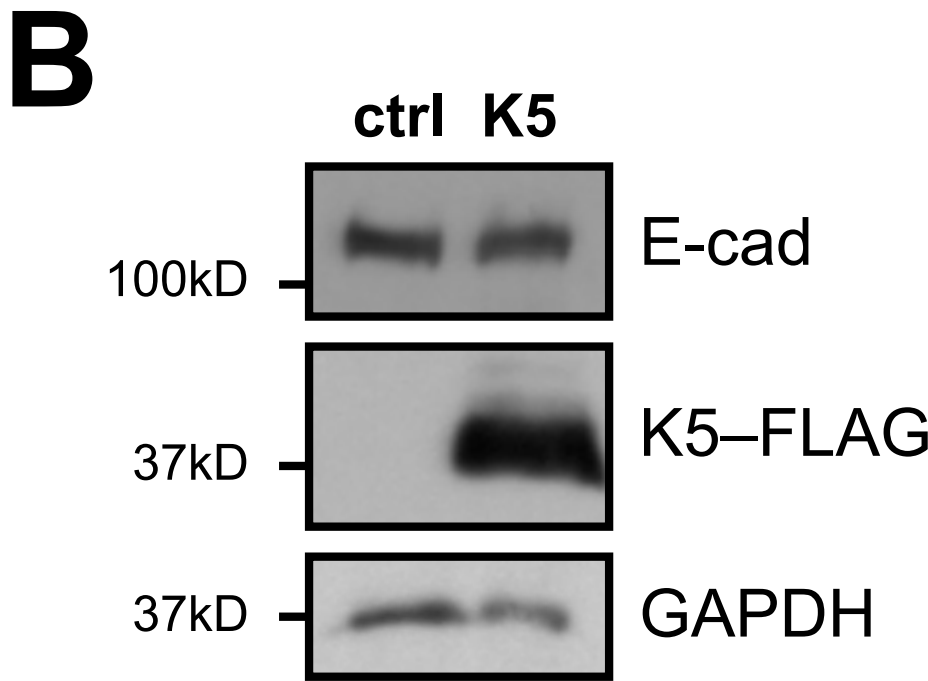
100kD
VE-cad
50kD
vimentin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ctrl
K5
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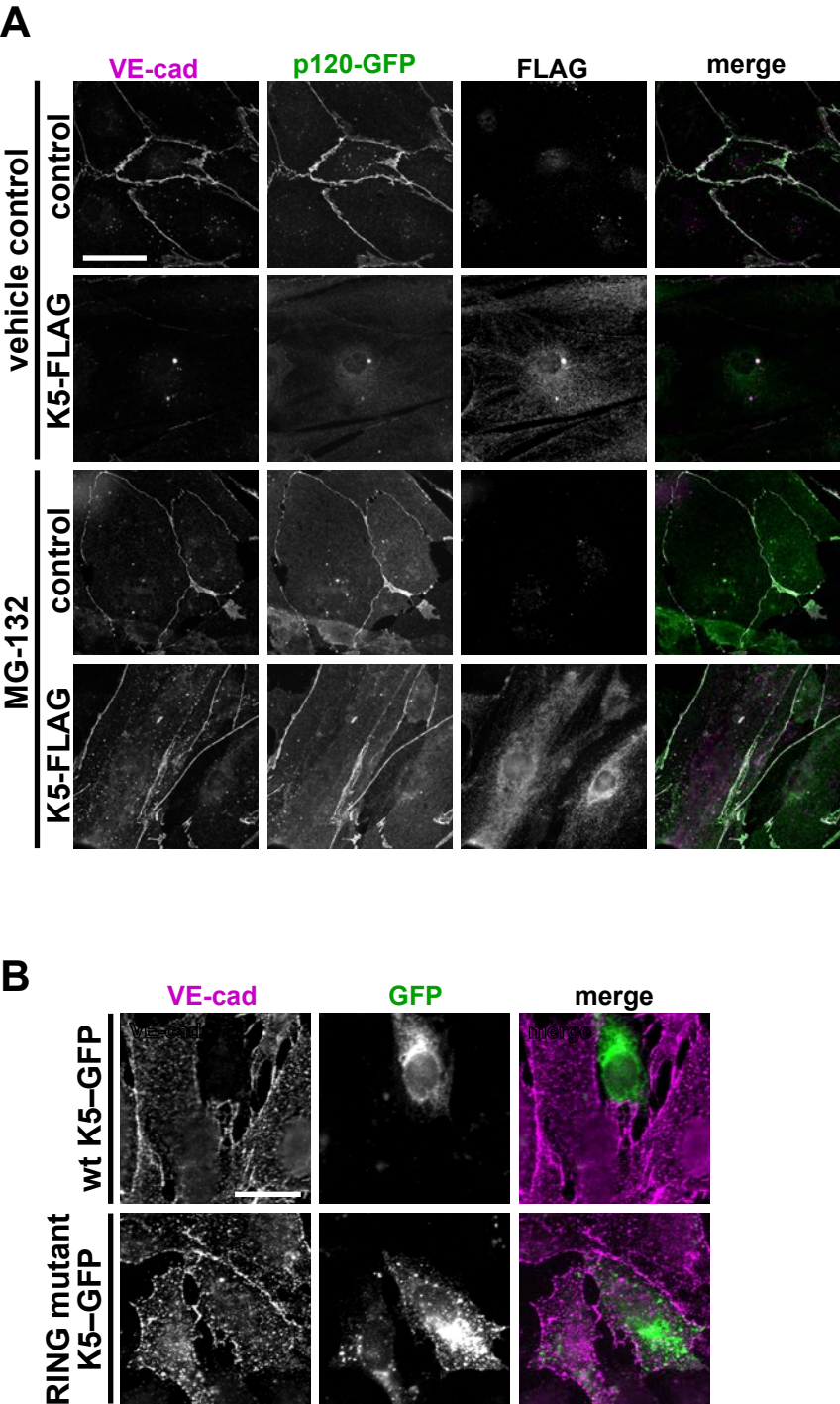
E-cad
37kD
K5–FLAG
37kD
GAPDH

Figure S2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VE-cad
p120-GFP
FLAG
merge
vehicle control
control
K5-FLAG
MG-132
control
K5-FLAG
B
VE-cad
GFP
merge
wt K5–GFP
RING mutant K5–GFP

Figure S3

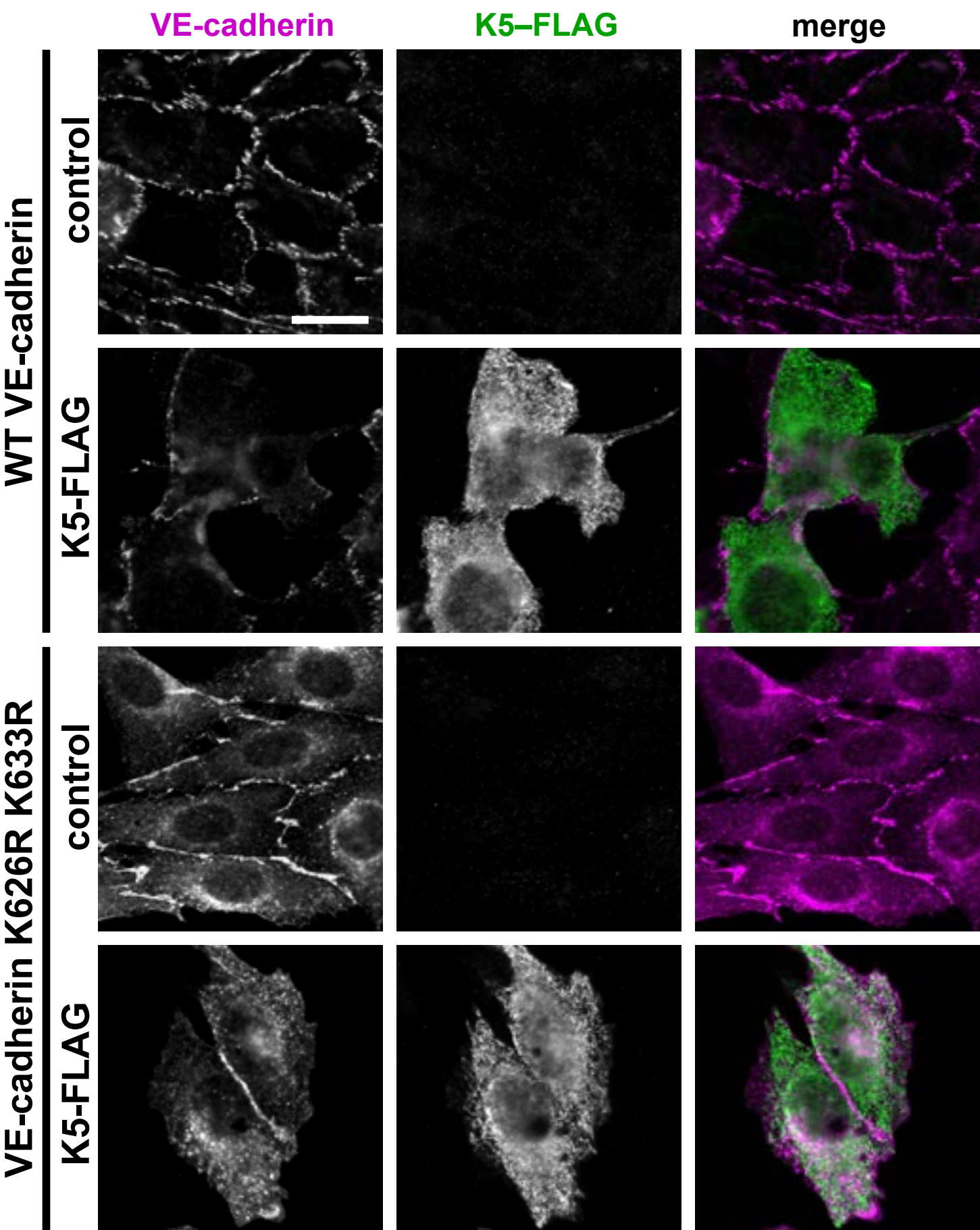
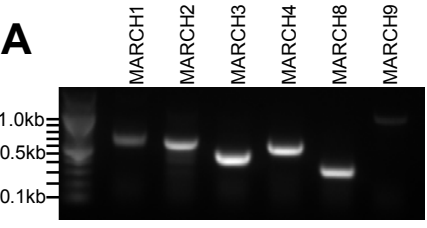
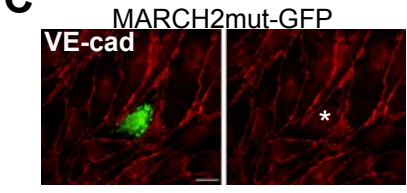

VE-cadherin
K5–FLAG
merge
WT VE-cadherin
control
K5-FLAG
VE-cadherin K626R K633R
control
K5-FLAG

Figure S4


A
MARCH1
MARCH2
MARCH3
MARCH4
MARCH8
MARCH9
1.0kb
0.5kb
0.1kb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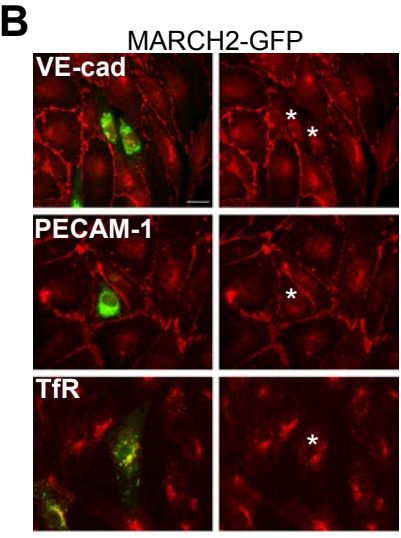
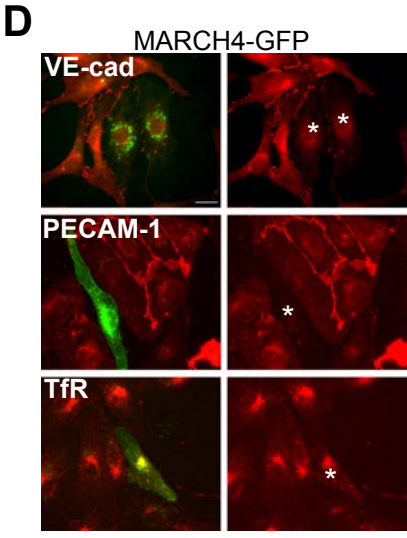
MARCH2mut-GFP
VE-cad


B
MARCH2-GFP
VE-cad
PECAM-1
TfR


D
MARCH4-GFP
VE-cad
PECAM-1
TfR

Supplemental Tables

Table S1. Primers

VE-cadherin K626R, K633R mutagenesis primers	
forward	5'-G CGG CTC CGG AGG CAG GCC CGC GCG CAC GGC AGG AGC GTG CCG G-3'
reverse	5'-C CGG CAC GCT CCT GCC GTG CGC GCG GGC CTG CCT CCG GAG CCG C-3'
VE-cadherin DEE646–648AAA mutagenesis primers	
forward	5'-CTG GTC ACC TAC GCA GCA GCA GGC GGC GGC GAG ATG-3'
reverse	5'-CAT CTC GCC GCC GCC TGC TGC TGC GTA GGT GAC CAG-3'
VE-cadherin GGG649–651AAA mutagenesis primers	
forward	5'-TAC GAC GAG GAG GCA GCA GCA GAG ATG GAC ACC-3'
reverse	5'-GGT GTC CAT CTC TGC TGC TGC CTC CTC GTC GTA-3'

Underlined codons indicate sites of mutation.

Table S2. Primary antibodies

Target	Antibody	Application
β-catenin	rabbit polyclonal (NeoMarkers, cat. #RB-090-P0)	IF
	rabbit polyclonal (Sigma Aldrich, cat. #C-2206)	WB
c-myc	Rabbit polyclonal (Bethyl Laboratories, cat #A190-105A)	WB
E-cadherin	mouse IgG_{2a} (BD Biosciences, cat. #610182)	WB
FLAG	chicken polyclonal (Bethyl Laboratories, cat. #A190-100A)	IF, WB
GAPDH	rabbit polyclonal (Santa Cruz Biotechnology, cat. #sc-25778)	WB
GFP	rabbit polyclonal (Synaptic Systems Cat. #132002)	WB
N-cadherin	mouse IgG_1 (BD Biosciences, cat. #610920)	WB
p120	rabbit polyclonal (Santa Cruz Biotechnology, cat. #sc-1101)	IF, IP, WB
	rabbit IgG (Abcam, cat. #ab92514)	IHC
PECAM	mouse monoclonal, clone JC70A (DAKO, Cat.# IR61061-2	IF
transferrin rec.	mouse IgG_1, clone H68.4 (Life Technologies, cat #136800)	IF
ubiquitin	mouse IgG_1 (Santa Cruz Biotechnology, cat. #sc-8017)	WB
VE-cadherin	mouse IgG_{2A}, clone BV6 (provided by E. Dejana, FIRC Institute of Molecular Oncology, Milan, Italy; Corada et al., 2001)	IF
	mouse IgG_1 (BD Biosciences, cat. #610252)	IF
	rabbit polyclonal (Novus Biologicals, cat. #18940002)	IP, WB
	goat polyclonal (Santa Cruz Biotechnology, cat. #sc-6458)	IHC
vimentin	mouse IgG (Sigma Aldrich, cat. #V6630)	WB

IF, immunofluorescence; WB, Western blot; IP, immunoprecipitation; IHC, immunohistochemistry