Supplementary Materials

Relevance of abnormal KCNN1 expression and osmotic hypersensitivity in Ewing sarcoma

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Supplementary materials:

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1. **Supplementary Tables:**

Table S1. Primers used in the experiments.

Gene (RefSeq ID)	**Forward-Primer**	**Reverse-Primer**	**Source**
GAPDH (NM_002046)	ACGACCCCTTCATTGACCTCA	TTTGGCTCCACCCTTCAAGTG	[59]
YWHAZ (NM_003406)	CAACACATCCTATCAGACTGGG	AATGTATCAAGTTCAGCAATGGC	[60]
KCNMA1 (NM_002247)	ACGCTCAAGTACCTGTGGAC	AAGACAACCAGGACTCTGCC	De novo
KCNN1 (Exon 6-8) (NM_002248)	GCGCGTCTGCGAGAGGTA	GTACAGCCAGCTCCCATGAT	De novo
KCNN2 (NM_021614)	TCGTGGTCATGGTCATCGAG	CTCCAGTCATCTGCTCCATTGT	De novo
KCNN4 (NM_002250)	GGCCAAGCTTTACATGAACACGCA	AAAGGTGCCCAGTGGCATTAACAG	De novo
TRPC1 (NM_003304)	ACTGTGTAGGCATCTTCTGTGAACA	GGAGAAAATATACCAGAACAAAGCAAA	[61]
TRPV4 (NM_021625.4)	TCCCATTCTTGCTGACCCAC	AGGGCTGTCTGACCTCGATA	De novo
TRPV6 (NM_018646)	GCCCTGAACAAGTTGCTCAAG	TGTCATAGAGGGCTGCTATGTGTAG	[61]
TRPC6 (NM_004621)	CCGCTGCCTTGCTACGGCTACT	TCTTCCGCACCACTGGGATGTT	[62]
TRMP2 (NM_003307)	ACGTGCTCATGGTGGACTTC	AGGGTCATAGAAGAGCTGCC	[63]
TRPM7 (NM_017672)	CAGAAACCAAGCGCTTTCCT	AATTCAACGGCCAACTGACC	[64]
PIEZO1 (NM_001142864)	CAGGCCTATGAGGAGCTGTC	TTGTAGAGCTCCCGCTTCAT	[65]
PIEZO2 (NM_022068)	GCCCAACAAAGCCAGTTGAA	GGGCTGATGGTCCACAAAGA	[65]

CACNA1A (Cav2.1) (NM_001174080)	GTCTGGGGAAGAAGTGTCCG	GCTCCTCCCTTGGCAATCTT	De novo
CACNA1C (Cav1.2) (NM_199460)	TCCTCATCGTAATTGGCAGCA	TTCAGCTGGGTTTACCTCGG	De novo
CACNA1G (Cav3.1) (NM_018896)	ACACTTGGAACCGGCTTGAC	AGCACACGGACTGTCCTGA	[66]
CACNA1H (Cav3.1) (NM_021098)	CTTCTTCTGCCTCGGTCAGA	TGATTACCAGCATGCTCACG	[67]
CACNA1I (Cav3.1) (NM_021096)	TATGTCTGCCACATCCTGCG	ACCCTTAGTCTTCCCATGGTAGT	De novo
STIM1 (NM_003156)	GCTCCTCTGGGGACTCCT	CAATTCGGCAAAACTCTGCT	[68]
STIM2 (NM_020860)	GACGTCAGTATGCAGAACAGGA	TCAAATTCTTTTTCGGCCTTT	[68]
ORAI1 (NM_032790)	ACCTCGGCTCTGCTCTCC	GATCATGAGCGCAAACAGG	[68]
ORAI2 (NM_032831)	TACCTGAGCAGGGCCAAG	TGGCCACCATGGCAAAG	[68]
ORAI3 (NM_152288)	ACGTCTGCCTTGCTCTCG	GAGTGCAAAGAGGTGCACAG	[68]
KCNN1_Exon1	TCTGAGCTGGAGCCACG	CAGCTCTCCGAGTCCGTAGG	De novo
KCNN1_Exon1-4	TCTGAGCTGGAGCCACG	GCTGGGCTCCTGCACTGAC	De novo
KCNN1_Exon3-4	GAGCCCCGCAGGTCAGT	TTGGCCACTACCACCTGGAG	De novo
KCNN1_Exon5-6	ATGCCTCATCAGCCTCTCCA	CCACCATGAACAGCTGGATCT	De novo
KCNN1_Exon9-11	GCCATCCATCAGGCTCAGAA	AGGTCGGTAAGCGTGTTAGC	De novo
KCNN1_Exon12 (3'UTR)	CGCCACCAGACCCCTAAAT	ACTGTACAAGGCTTCCCGGA	De novo
EWSR1-FLI1	CAGCCTCCCACTAGTTACCC	GGTGAGGCCAGAATTCATG	[69]

Table S2. *KCNN1* expression in EwS does not correlate with clinical parameters of disease.

*Contingency coefficient

KCNN1^{low} vs *KCNN1*^{high}		
correlation	r*	*P*
Primary tumor volume <200ml / >=200ml	0.02	0.89
Primary metastasis (no / yes)	0.02	.77
Event of relapse /secondary malignancy (no / yes)	0.03	.78
Time until relapse /secondary malignancy	0.22	.04
Histological response (good / poor)	0.08	.41
Overall survival (dead/alive)	0.00	.99
Gender (male / female)	0.03	.68
Age at diagnosis (< 15 years / >= 15 years)	0.00	1.0
Tumor location (axial vs non-axial)	0.02	.78

2. Supplementary Figures:

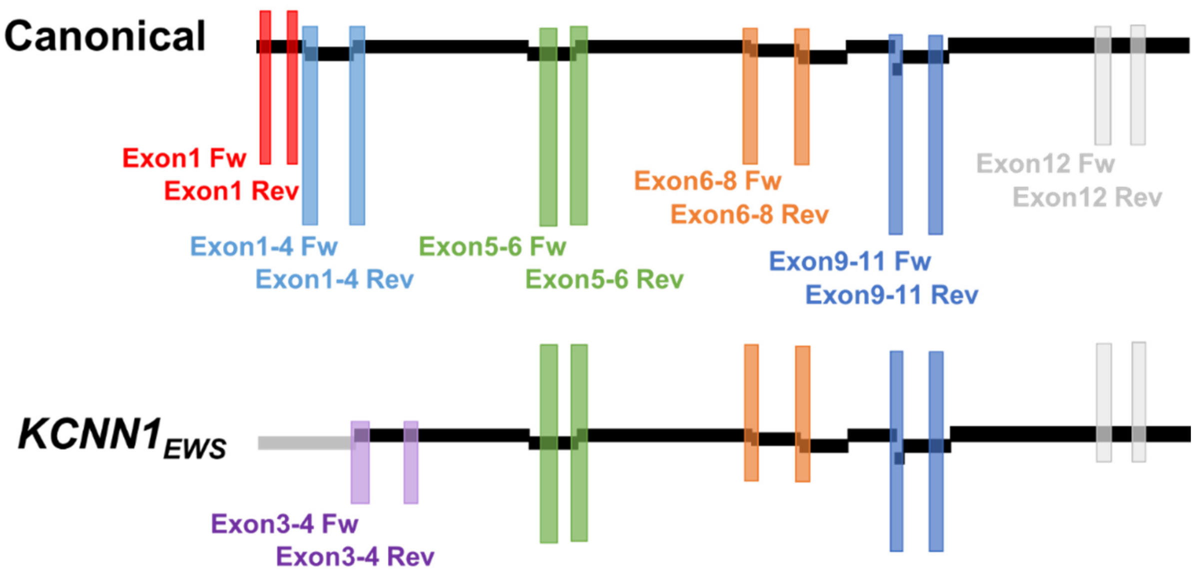

Figure S1. Primer binding sites for exon-detecting *KCNN1* primers.

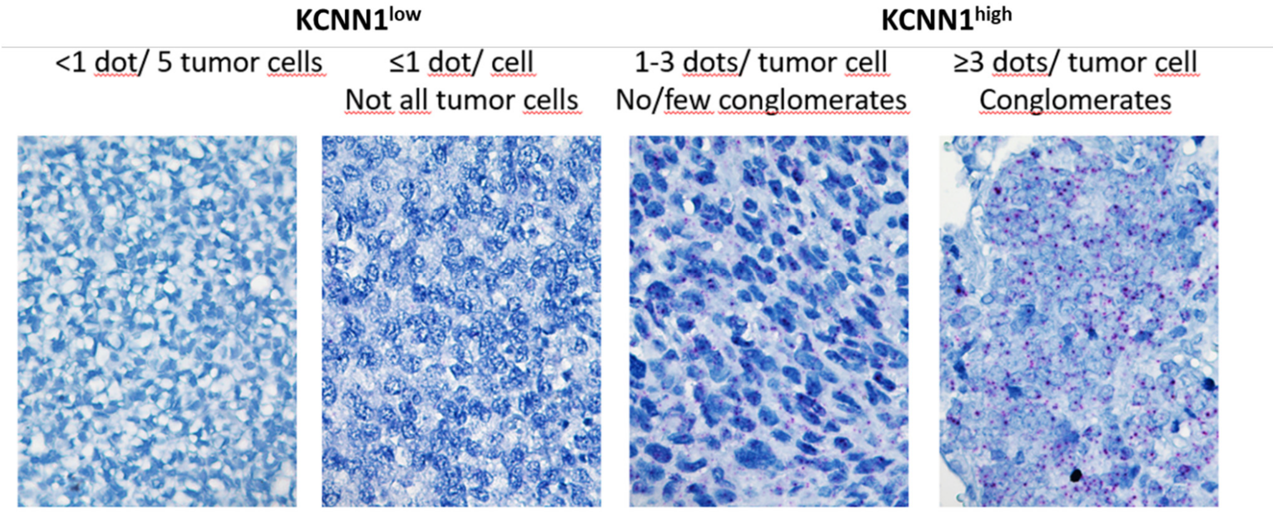

Figure S2. Scoring algorithm of RNAish. Representative images show criteria for classification into KCNN1low and KCNN1high groups, respectively, based on the number of RNAish signal (purple dots).

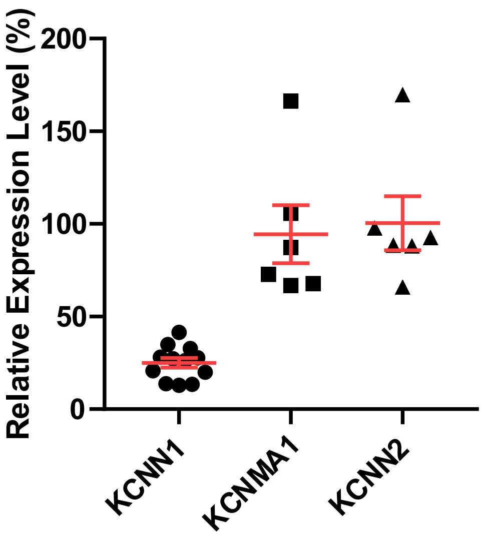

Figure S3. si*KCNN1* transfection interferes selectively with the expression of *KCNN1*. Scatter plot depicts relative gene expression of target genes compared to siScrambled transfection 24 following transfection (N=3, n_{KCNN1}=12, n_{KCNMA1}=6, n_{KCNN2}=6).

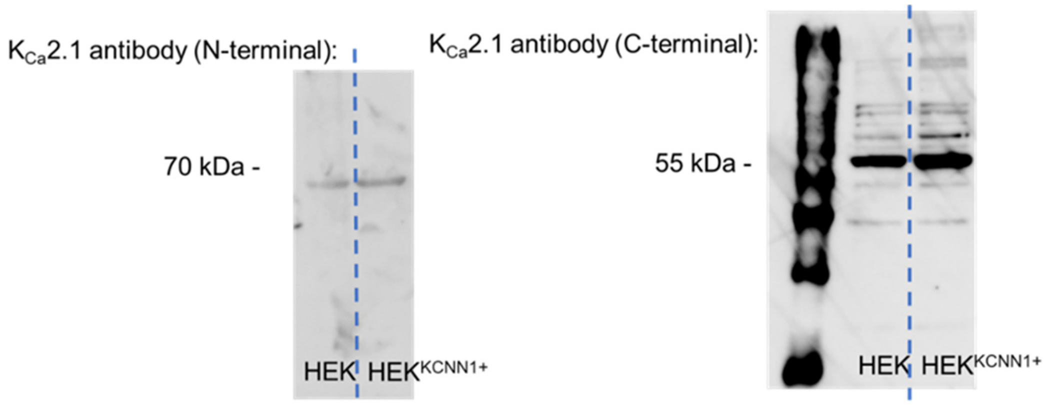

Figure S4. anti-*KCa2.1* antibodies do not specifically recognize the *KCa2.1 channel*.

Representative Western Blot chemiluminescent images from N=3 blots depict anti-*KCa2.1* signals against wild-type HEK293 cells (HEK) or HEK293 cells transfected with KCNN1 (HEK $^{KCNN1+}$), divided by blue dashed lines. The two types of available antibodies target peptide sequences from either the N- or the C-terminus of the protein.

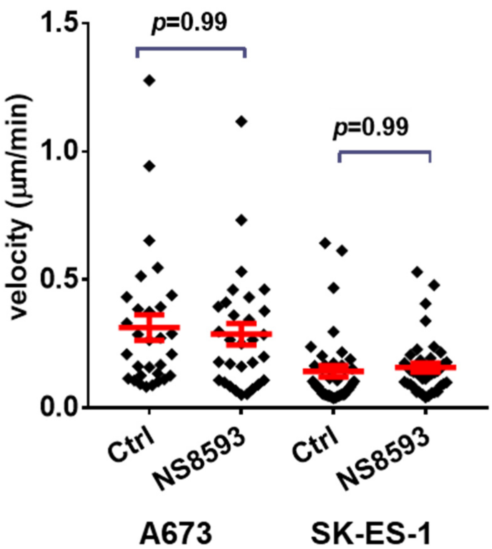

Figure S5. Membrane-permeable *KCa2.1* inhibitor NS8593 does not affect EwS cell migration.

Mean cell velocities from individual cells in the presence of 1 µM NS8593 or control (0.1% DMSO). A673: N=3, n=30, SK-ES-1: N=4, n=40

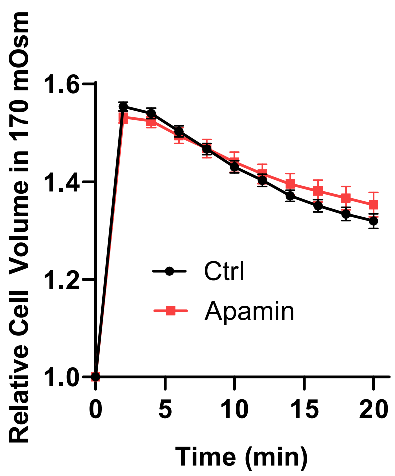

Figure S6. Apamin does not affect TC-71 cell regulatory volume decrease.

RVD performance of TC-71 cells treated with *siKCNN1* (red, N=4, n=32) or with scrambled siRNA (black, N=4, n=38), indicated by changes in their relative cell volume in a hypoos-motic solution (170 mOsm). The volume is normalized to their initial volume in an isosmotic solution

3. **Supplementary Data:**

KCNN1 transcripts derived from EWS cell lines using capillary sequencing. Forward and reverse sequencing primers are highlighted in yellow.

I. **NM_002248.5** (Homo sapiens potassium calcium-activated channel subfamily N member 1 (KCNN1), transcript variant 1, mRNA) **– 1809 bp**

CGAGGAGACCCCTGCACCCTAGGGTCAGTGCAGGAGCCCAGCCGCTGAGCCATGCCGGGCCCCG
GGCGGCCTGCAGCGAGCCCAACCCCTGCACCCAGGTAGTCATGAACAGCCACAGCTACAATGGC
AGCGTGGGGCGGCCGCTGGGCAGCGGGCCGGGCGCCCTGGGACGAGACCCTCCGGACCCTGAG
GCCGGCCACCCCCCACAACCCCCGCACAGCCCGGGCCTCCAGGTGGTAGTGGCCAAGAGTGAGC
CAGCCCGGCCCTCACCCGGCAGCCCCCGGGGGCAGCCCCAGGACCAGGACGATGACGAGGATG
ATGAGGAAGATGAGGCCGGCAGGCAGAGAGCCTCGGGGAAACCCTCAAATGTGGGCCACCGCC
TGGGCCACCGGCGGGCGCTCTTCGAGAAGCGGAAGCGCCTCAGCGACTATGCCCTCATTTTCGGC
ATGTTTGGCATCGTCGTCATGGTGACGGAGACCGAGCTGTCCTGGGGGGTGTACACCAAGGAGTC
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CCTCTACCATGCCCGGGAGATCCAGCTGTTCATGGTGGACAACGGGGCTGATGACTGGCGCATCG
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GGCCGACGTGGACGTGCTGCTGTCCATCCCCATGTTCCTGCGCCTCTACCTGCTGGGCCGGGTGA
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CACCTTCAACACGCGCTTCGTCATGAAGACACTCATGACCATCTGCCCCGGCACCGTGCTGCTGG
TCTTCAGCATCTCCTCCTGGATCATCGCAGCCTGGACCGTGCGCGTCTGCGAGAGGTACCACGAC
AAGCAGGAAGTGACCAGCAACTTCCTGGGGGCCATGTGGCTGATTTCCATCACCTTCCTCTCCAT

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GAGTGTGAAGATCGAGCAAGGGAAGCTGAACGACCAGGCTAACACGCTTACCGACCTAGCCAA
GACCCAGACCGTCATGTACGACCTTGTATCGGAGCTGCACGCTCAGCACGAGGAGCTGGAGGCC
CGCCTGGCCACCCTGGAAAGCCGCTTGGATGCGCTGGGTGCCTCTCTACAGGCCCTGCCTGGCCT
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GGCCCTGCCCGCCACCAGACCCCTAAATCTTGGCCATCGTGTGGCCGCCACCTCCGGGAAGCCTT
GTACAGT

II. **NM_001386974.1** Homo sapiens potassium calcium-activated channel subfamily N member 1 (KCNN1), transcript variant 2, mRNA- **1833 bp**

CCCTCTCCCCTCCAGCCTTGTCCGCCCGCTCGGGCCGAGCCCCGCAGGTCAGTGCAGGAGCCCAG
CCGCTGAGCCATGCCGGGCCCCGGGCGGCCTGCAGCGAGCCCAACCCCTGCACCCAGGTAGTCA
TGAACAGCCACAGCTACAATGGCAGCGTGGGGCGGCCGCTGGGCAGCGGGCCGGGCGCCCTGG
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AACCCTCAAATGTGGGCCACCGCCTGGGCCACCGGCGGGCGCTCTTCGAGAAGCGGAAGCGCCT
CAGCGACTATGCCCTCATTTTCGGCATGTTTGGCATCGTCGTCATGGTGACGGAGACCGAGCTGT
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CTCCCAGGCCCGGCCCCGGCCCCCAAGACCAGGCAGCCCGGAGCTCCCCCTGCCGGTGGACGCC
CGTGGCCCCCTCGGACTGCGGGTGACGGCCCTGCCCGCCACCAGACCCCTAAATCTTGGCCATCG
TGTGGCCGCCACCTCCGGGAAGCCTTGTACAGT

I. **NM_002248.5** ΔEx3-4– **1230 bp**

CGAGGAGACCCCTGCACCCTAGGCTGTTCATGGTGGACAACGGGGCTGATGACTGGCGCATCGC
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GACCCAGACCGTCATGTACGACCTTGTATCGGAGCTGCACGCTCAGCACGAGGAGCTGGAGGCC
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GGCCCTGCCCGCCACCAGACCCCTAAATCTTGGCCATCGTGTGGCCGCCACCTCCGGGAAGCCTT
GTACAGT

4. Supplementary Method:

Western blot

Protein was extracted from subconfluent HEK293 cells (HEK) and HEK293 cells transfected with pcDNA3-*KCNN1* using radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher, Waltham, USA) and 1% Complete Mini protease inhibitor (Roche, Mannheim, Germany). Total protein concentration was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Western blots of $K_{Ca}2.1$ were performed as described previously (Bulk, E. et al., Oncotarget 8, 112268–112282 (2017)). 15 μg of denatured total cellular protein were applied to each lane of protein gels for electrophoresis at 80 mV. After overnight transfer to PVDF membranes at 4 ºC, we detected total amount of protein on the membrane following manufacturer's instructions. Next, we blocked the PVDF membrane with PBS containing 5% skim milk for 1 h, then incubated the blots overnight at 4ºC with primary antibodies against either the N- (HPA065938, 1:200, Atlas Antibodies, Stockholm, Sweden) or the C-terminus (abx029367, 1:200, Abbexa, Cambridge, UK) of the $K_{Ca}2.1$ protein, recognizing the immunogenic peptide sequences EDDEEDEAGRQRASGKPSNVGHR or KIEQGKLNDQANTLTDLAKTQTVMYDLVSELHAQHEELEARLATLESRLD, respectively. After washing three times with PBS, we applied HRP-conjugated donkey anti-rabbit secondary antibody (1:10000, Donkey Anti-rabbit IgG H&L, #ab6701, Abcam, Cambridge, UK). Chemiluminescence was detected using a Chemidoc MP detection system (Bio-Rad, Hercules, USA), and band intensities were evaluated with the ImageLab software (Bio-Rad, Hercules, USA).

5. References:

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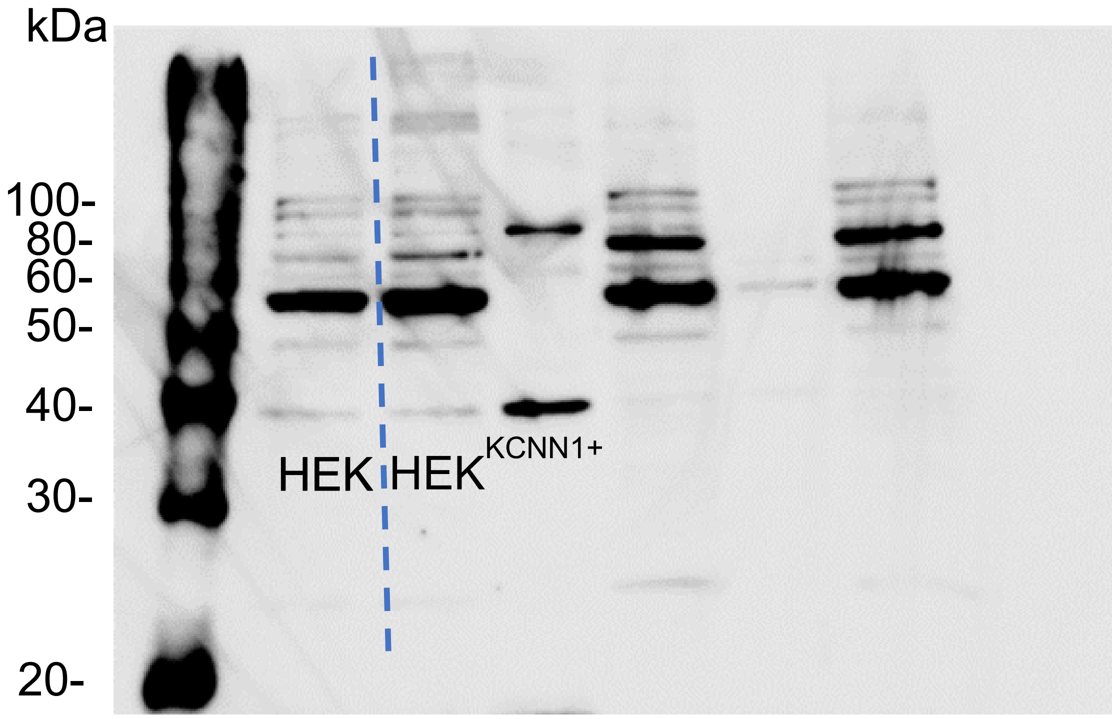
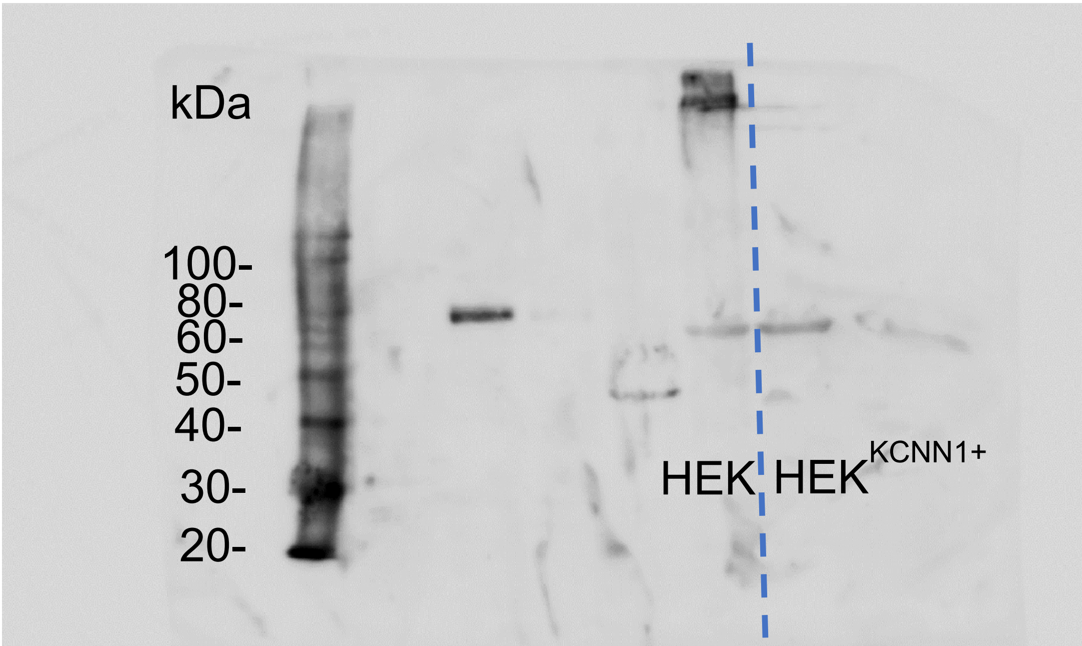
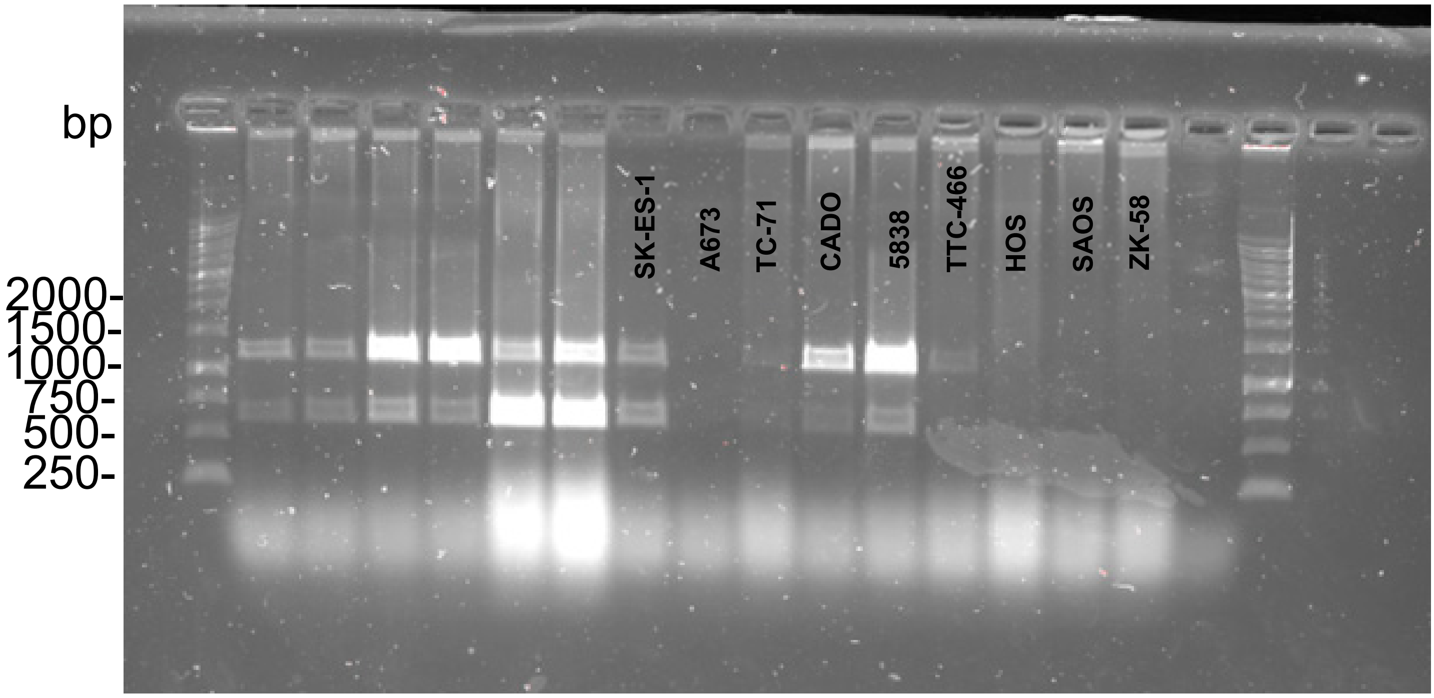
6. **Uncropped Western Blots (Figure S4) and PCR gels (Figure 3)**



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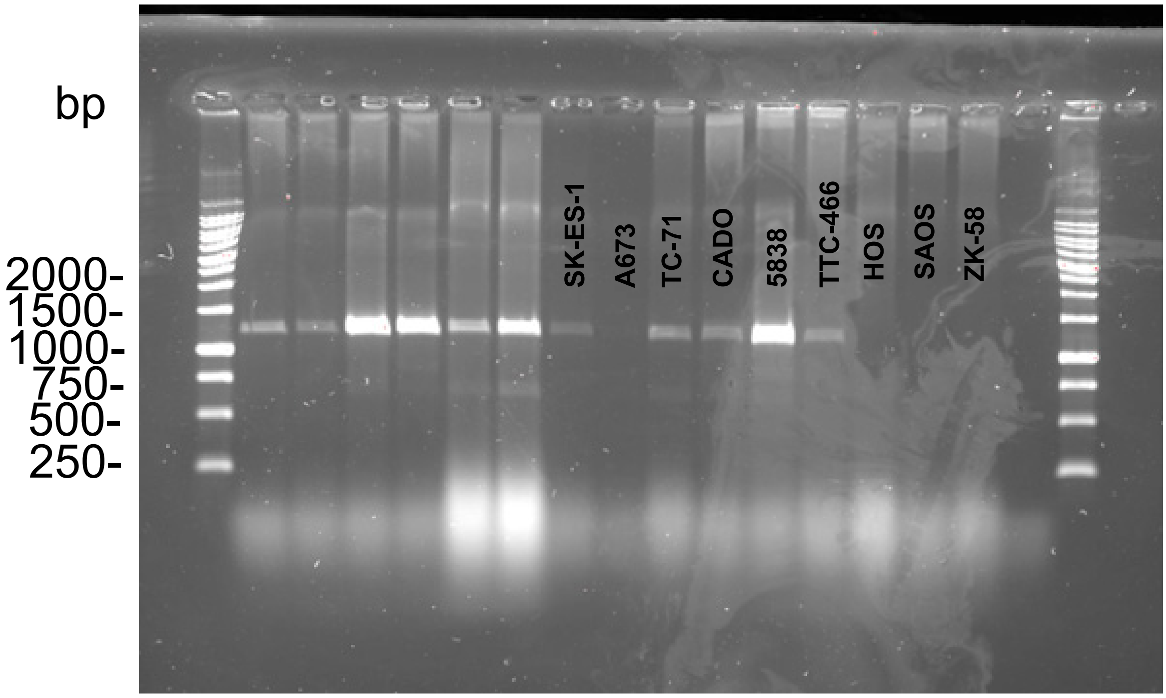
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