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Regulation of calcium influx and signaling pathway in cancer cells via TRPV6-Numb1 interaction

암세포에서 TRPV6 와 Numb1 상호작용을 통한 칼슘유입과 신호전달의 조절

2013 년 8 월

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A thesis of the Degree of Doctor of Philosophy

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August 2013

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by
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A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Biomedical Sciences) at Seoul National University College of Medicine

July 2013

Approved by Thesis Committee:

Professor _____Chairman

Professor _____Vice chairman

Professor _____

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Professor _____

ABSTRACT

Introduction: Calcium is a critical factor in the regulation of signal transduction and calcium homeostasis is altered in different human diseases. The level of calcium in cells is highly regulated through a diverse class of regulators. Among them is the Transient Receptor Potential Vanilloid 6 (TRPV6), which is a calcium selective channel that absorbs calcium in the small intestine. TRPV6 is overexpressed in some cancers and exhibits oncogenic potential, but its exact mechanism is still poorly understood. The Numb protein is a cell fate determinant that functions in endocytosis and as a tumor suppressor via the stabilization of p53.

Methods: We used calcium influx assay to measure activity of TRPV6, To validate interaction, I used co-immunoprecipitation (co-IP) and FRET assay. Using site directed mutagenesis, I constructed deletion mutants. Other molecular biology techniques (Western blotting, MTT assay, Cell culture, Surface biotinylation, Transfection, siRNA) were used.

Results: The expression of Numb1 decreased cytosolic calcium concentrations in TRPV6-transfected HEK293 cells. When all the isoforms of Numb were depleted using siRNA in a TRPV6 stable cell line, the levels of cytosolic calcium increased. We observed an interaction between Numb1 and TRPV6 using co-IP. We confirmed this interaction using Fluorescence Resolution Energy Transfer (FRET). We identified the TRPV6 and Numb1 binding site using TRPV6 c-terminal truncation mutants and Numb1 deletion mutants. The binding site in TRPV6 was an aspartic acid at amino acid

residue 716, and that binding site in Numb1 was arginine at amino acid residue 434. A Numb1 mutant, lacking TRPV6 binding activity, failed to inhibit TRPV6 activity. Every isoform of Numb knockdown, using an siRNA-based approach in MCF-7 breast cancer cells, not only showed enhanced TRPV6 expression but also both the cytosolic calcium concentration and cell proliferation were increased. The down-regulated expression of TRPV6 using siRNA increased Numb protein expression; however, the cytosolic influx of calcium and proliferation of the cell were decreased. To examine downstream signaling during calcium influx via TRPV6, we performed western blotting analysis on TRPV6 upregulated cancer cells (MCF-7, PC-3). we showed that influx of calcium via TRPV6 phosphorylates not only GSK3ß, JNK and Erk in MCF-7 but also p38 in PC-3 cells.

Conclusions: Taken together, these results demonstrated that Numb1 interacts with TRPV6 through charged residues and inhibits its activity via the regulation of protein expression. Moreover, we provided evidence for a calcium-regulated cancer cell signaling pathway and that the calcium channel is a target of cancer cells.

* This work is published in Cell Calcium. 2013 Feb;53(2):102-11

Keywords: Calcium, TRPV6, Numb, Cancer

Student number: 2008-21992

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INTRODUCTION

Changes in intracellular free Ca^{2+} play a major role in many cellular processes and deregulation of Ca^{2+} signaling is a feature of a variety of diseases. Every cell expresses a unique complement of components from a calcium signaling toolkit that enables it to generate intracellular calcium signals of a time couse, particular amplitude and intracellular location (22-23). The calcium toolkit is extensive and includes environmental sensors; signal transducers (such as G proteins and phospholipase C isoforms (PLCs)); signal-generating channels such as inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) on intracellular stores and store-operated or second messenger-operated channels on the plasma membrane (for example, ORAI1 and transient receptor potential (TRP) channels respectively); ER-localized calcium storage proteins (such as calreticulin, GRP78 (also known as heat-shock protein 5 (HSPA5)) and calsequestrin); signal terminators that serve to return intracellular calcium levels to pre-stimulation levels, such as the ER- and plasma membrane-localized calcium pumps (SERCA and PMCA, respectively), plasma membrane exchangers (Na^+–$Ca2^+$ exchanger (also known as SLC8A1)), mitochondria and cytosolic buffer proteins; and calcium sensors and effectors such as calmodulin (CaM) and its downstream targets, including CaM kinase (CaMK) and calcineurin (otherwise known as protein phosphatase 2B) and protein kinase C (PKC).

Calcium signaling is linked either directly or indirectly to each of cancer hallmarks. The cancer has eight acquired characteristics: the ability to evade apoptosis, self-sufficiency in growth signaling, insensitivity to anti-growth

signals, the capacity to invade and metastasize, “limitless” replication potential, the promotion of angiogenesis, reprogramming of energy metabolism and evading immune destruction (1). Cancer cells use the same calcium channels, pumps, and exchangers as non-malignant cells. However, there are often key alterations in calcium channels and pumps in cancer cells. Such changes in cancer cells may include the expression of calcium channels or pumps not normally present in non-malignant cells of the same cell type, pronounced changes in the level of expression, altered cellular localization, altered activity through changes in post-translational modification, gene mutations, and changes in activity or expression associated with specific cancer-relevant processes. These changes are often reflected in alterations in calcium influx across the plasma membrane or across intracellular organelles.

The influx of calcium across the plasma membrane into the cell is a key trigger or regulator of cellular processes relevant to tumor progression, including proliferation, migration, and apoptosis. Calcium-permeable ion channels of almost every class have now been associated with aspects of tumor progression. For example, normal keratinocytes differentiate as calcium levels increase whereas transformed keratinocytes show little differentiation at any calcium concentration (2). Even transforming growth factor β, a growth inhibitor for many epithelial cells, requires the Ca^{2+}-binding protein S100A11 for inhibition of keratinocyte growth (3). Telomere erosion through successive cycles of replication normally leads to cellular senescence. To maintain their telomeres, cancer cells upregulate telomerase expression. The calcium-binding protein S100A8 has been shown to mediate calcium-induced

inhibition of telomerase, suggesting that remodelling of calcium signalling, in particular a reduced dependency on calcium for cell-cycle progression, might be important in tumor cell immortality (4). Tumors must acquire a blood supply to grow. calcium is required for hypoxia-induced activation of hypoxia-inducible factor 1 (HIF1), the transcription factor that promotes expression of vascular endothelial growth factor (VEGF) and for VEGF-dependent endothelial cell proliferation (5,6). In addition, secretion of thrombospondin-1 (THBS1), an angiogenesis inhibitor, is controlled by calcium entry through the TRPC4 (transient receptor potential ion channel 4) calcium channel. Renal cell carcinomas exhibit a profound decrease in TRPC4 expression, impaired calcium intake and diminished secretion of THBS1, thus enabling an angiogenic switch during carcinoma progression (7). Metastasis is generally defined as the spread of malignant cells from the primary tumor through the circulation to establish secondary growth in a distant organ. The ability to migrate is a prerequisite for a cancer cell to escape the primary tumor and to enter the circulation (8). Calcium is one of the crucial regulators of cell migration but the sources of Ca^{2+} and the mechanism by which it modulates this process in cancer cells are only now beginning to be understood (9). At the leading edge membrane stretch-activated, transient receptor potential cation channel subfamily M member 7 (TRPM7)-mediated calcium influx coupled to inositol trisphosphate receptor (IP_3R)-mediated calcium release participates in the guidance of the leading edge towards a chemoattractant (10). Calcium influx through TRPV2 promotes migration by induction of key invasion markers, matrix

metalloproteinase (MMP2), MMP9 and cathepsin B (CTSB) (11). At the trailing end calcium influx through L-type voltage-gated calcium channels (VGCCs) regulates contraction through calcium-dependent phosphorylation of contractile proteins (12). Certain types of Cav3 VGCCs and TRP members, TRPC1, TRPM8 and TRPV1 are implicated in the enhanced migration of cancer cells (13-16). Formation of focal adhesions is facilitated by IP_3R-mediated calcium release that is stimulated through surface G protein-coupled receptors (GPCRs) or exchange proteins directly activated by cAMP (EPAC) leading to calcium/calmodulin-dependent protein kinase II (CaMKII)-dependent focal adhesion kinase (FAK) phosphorylation or enhanced actin assembly, respectively, by stromal interaction molecule 1 (STIM1) – calcium release – activated calcium channel protein 1 (ORAI1) – based store – operated calcium entry, resulting in RAS and RAC activation and by secretory pathway Ca^{2+}-ATPase (SPCA2)-ORAI1 complex-mediated constitutive calcium influx. IP_3R-and ryanodine receptor (RYR)-mediated calcium mobilization from the endoplasmic reticulum (ER) stores also promotes cell migration in an S100A4-dependent manner (17-21).

Transient receptor potential (TRP) channels constitute a large and functionally versatile superfamily of cation channel proteins that are expressed in many cell types from yeast to mammals (24). The TRP superfamily contains a growing number of proteins in vertebrates and invertebrates unified by their homology to the product of the Drosophila trp gene, which is involved in light perception in the fly eye (25). On the basis of structural homology, the superfamily can be subdivided into seven main subfamilies: TRPC

(canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin) and TRPN (no mechanoreceptor potential C, NOMPC) (26-27). Among them, in vertebrates, six TRPV channels have been identified. TRPV1 mediates nociception and contributes to the detection and integration of diverse chemical and thermal stimuli (28-29), TRPV2 and TRPV3 open upon heating, activating in the warm and noxious heat range (30-31), TRPV4 plays a role in osmosensing, nociception and warm sensing (32-33), and finally TRPV5 and TRPV6 are highly calcium-selective channels that play a role in Ca^{2+} reabsorption in the kidney and intestine (34-35). It is really notable that among all TRP channels TRPV5 and TRPV6 are highly calcium selective, with P_{Ca}/P_{Na} values exceeding 100; such high calcium selectivity is unique within the TRP superfamily and makes these channels quite distinguishable, especially in calcium-related intracellular pathways.

The key early work on calcium signaling in cancer was focused on cancers of the prostate gland and more specifically the calcium-permeable ion channel TRPM8 (36). Although now studied predominately in the context of its role as a cold receptor (37-38), TRPM8 was first identified by its overexpression in some prostate cancers (36). Early work demonstrated that both the silencing of TRPM8 and menthol-mediated activation of TRPM8 reduced the viability of LNCaP prostate cancer cells (39). That both activators and inhibitors are proposed as potential therapeutic agents for prostate cancer cells that overexpress TRPM8 is reflective of the duality of the calcium signal (40), whereby calcium is both a key regulator of proliferation and, in the case of

calcium overload, an initiator of cell death. The ability of TRPM8 activation by prostate-specific antigen to inhibit the migration of PC-3 prostate cancer cells now extends the applicability of channel activators as therapeutics beyond just inducers of cancer cell death (41). Further detailed work on TRPM8 in prostate cancer showed androgen-mediated increases in TRPM8 in LNCaP prostate cancer cells (42). This finding provides one of the first examples of hormone-mediated changes in the expression of a calcium-permeable ion channel in a cancer cell line. As discussed below, this has now been seen with other calcium channels and pumps in breast cancers. The contribution of TRPM8 to cancer progression, as we will see for other calcium channels and pumps, may not always involve its classic role. As opposed to the usual plasma membrane localization, endoplasmic reticulum localization of TRPM8 is observed in some prostate cancer cells, with the consequence being reduced levels of endoplasmic reticulum calcium and increased resistance to apoptosis (43). Aside from prostate cancer, overexpression of TRPM8 is also associated with other cancer types, including melanoma and cancers of the pancreas, breast, colon, and lung (36,44,45,46,47). However, the utility of TRPM8 as a target for cancer therapy might be limited and require knowledge of the individual tumor expression of the channel. For example, TRPM8 expression actually appears to decrease as prostate cancer cells transition to androgen independence and increased aggressiveness (44, 48).

Other examples of TRP channels that are overexpressed in multiple cancer types include TRPC3 and TRPC6. TRPC3 is elevated in some breast (49) and

ovarian epithelial tumors, and its silencing reduces ovarian cancer cell line proliferation in vitro and tumor formation in vivo (50). TRPC6 is elevated in cancers of the breast, liver, stomach, and esophagus and in gliomas, and its silencing reduces the proliferation of some esophageal and breast cancer cell lines and glioma cell lines (49, 51, 52). For esophageal and glioma cell lines, these effects are due to G2/M cell cycle arrest (51, 52). The importance of some TRP channels in tumor progression appears to extend beyond the primary tumor. Other group showed that migrating endothelial cells have a greater cytosolic calcium response to the TRPV4 activator 4--phorbol 12,13-didecanoate than non-migrating cells (53). Furthermore, they showed increased expression of TRPV4 in endothelial cells derived from breast cancers compared with those derived from normal tissue, implicating TRPV4 as a possible key component in angiogenesis associated with breast cancers. Other calcium channels have also been associated with angiogenesis. Calcium entry into the cell via some TRP channels may result in localized calcium signals that contribute to cancer cell migration. One example of such a localized event is referred to as calcium flickers, which are highly localized (5-m diameter) and transient (10 ms to 4 s) increases in calcium that control the direction of migration as lung fibroblasts move toward a growth factor (54). calcium flickers during migration are regulated by TRPM7, which may act as a stretch or mechanical sensing channel (54,55). With TRPM7 inhibition, there is a reduction in migration of a number of cancer cell types, including those of the pancreas, lung, and nasopharynx (56–58).

TRPV6 channel cDNA was cloned in 1999 from rat duodenum by expression cloning using Xenopus oocytes (59). TRPV6 is predominantly expressed in epithelia and the organs that mediate transcellular calcium transport such as duodenum, jejunum, colon and kidney, and also in exocrine tissues such as pancreas, mammary gland, sweat gland and salivary gland (60-62). On the basis of electrophysiological analysis, the characteristics of TRPV6 were determined as it is strongly calcium selective compared with other cations (Ca^{2+} >>> Ba^{2+}, Sr^{2+}) and its apparent affinity for Ca^{2+} (Km) is 0.44 mM (59,63). The TRPV6 KO mice exhibit disordered calcium homeostasis, including defective intestinal calcium absorption, increased urinary calcium excretion, deficient weight gain and reduced fertility, suggesting the pivotal role in calcium homeostasis in tissues where this channel is expressed (64). When compared with normal tissue or cells, the expression of TRPV6 mRNA and/or expression of the TRPV6 protein is substantially increased in prostate cancer tissue, and in human carcinomas of the colon, breast, thyroid and ovary (62,65,66,67). In breast cancer, TRPV6 expression is higher in invasive areas, compared to the corresponding non-invasive areas. Moreover, TRPV6 silencing inhibited MDA-MB-231 migration and invasion, and MCF-7 migration (45). However, the mechanism underlying the TRPV6-mediated regulation of cancer progression and its downstream signaling remains unknown.

The Numb protein was first identified in Drosophila (68). As a cell fate determinant that inhibits notch activity via endocytosis (69). However, Numb was observed to show diverse functions besides Notch endocytosis. The

Numb protein is involved in signal transduction pathways (Hedgehog, p53), endocytosis (cargo internalization and recycling), cell polarity determination, and ubiquitination (70). In cancer, Numb functions as a tumor suppressor via the stabilization of p53 (71). Numb forms a tricomplex with p53 and its negative regulator, MDM2. The loss of Numb expression has been observed in mammary and lung cancer (72,73). Since Numb and Notch are crucial in tumorigenesis, it is expected that Notch and Numb modulate the aforementioned processes, in part, by modifying calcium-sensitive signaling pathways. In previous study, it is reported that Numb regulates Notch-dependent expression of TRPC6 (74). However it is hardly understood how Numb gives an influence on calcium homeostatic mechanisms. Using TRPV6 C-terminal as bait in a yeast two-hybrid screening, we have previously shown that Numb1 interacts with TRPV6 via yeast two hybrid screening (82). In our current study, we showed that Numb1 interacts with TRPV6 via a charge-charge interaction that inhibits TRPV6 activity. We further demonstrated the association of Ca2+ with proliferation in cancer cells.

MATERIALS AND METHODS

1. Materials

anti-c-myc (sc-40), anti-GFP (sc-8334) and anti-GSK3ß (sc-7291) antibodies were obtained from Santa Cruz Biotechnology, Inc. The anti-TRPV6 (ACC-036) antibody was obtained from Alomone Labs. Protein G agarose beads (sc-2002) were obtained from Santa Cruz. Anti-AKT (#9272), anti-p-AKT (#4056), anti-p-JNK (#9251), anti-p38 (#9212), anti-p-p38 (#9216), anti-p-GSK3ß (#9336), anti-Erk (#9102), anti-p-Erk (#9106) and anti-Numb (#2756) antibodies were obtained from Cell Signaling.

2. Cell culture and transfection

HEK293 cells were cultivated in DMEM (MCF-7 cells in RPMI) supplemented with 10% heat-inactivated FBS and penicillin (100 U/ml), streptomycin (100 µg/ml) at 37℃ in a 5% CO_2 humidified incubator. All plasmid DNAs used for transfection were prepared using a plasmid midi kit (QIAGEN) following the manufacturer's protocol. The transfection was performed with Fugene-6 according to the manufacturer's instructions.

3. Plasmid constructs

Numb1 cDNA was obtained from OriGene and subcloned into a pcDNA3-myc vector to generate a myc-fused Numb1 construct. The truncation mutants of Numb1, amino acids 9-651, 17-651, 25-651, 35-651, 1-639, 1-613, 1-600 and 1-570 were partially amplified using PCR and then subcloned into the

EcoRI and XhoI restriction sites of pcDNA3-myc. To generate myc-fused Numb1 deletion constructs, isoform 2, 3, and 4, ΔPTB1, ΔPTB2, ΔPTB3, ΔPTB4, Δ176-220, Δ221-270, Δ271-320, Δ321-365, ΔDPF, ΔNPF, Δ541-570, Δ501-540, Δ461-500, and Δ414-460, we used site-directed mutagenesis with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). The full length TRPV6 cDNA was subcloned into the pEYFP-C1 plasmid (Clontech) using the XhoI, and EcoRI restriction enzyme sites. The TRPV6 C-terminal truncation mutants, 1-721, 1-720, 1-719, 1-718, 1-717, 1-716, 1-715, 1-704, 1-680, 1-635, 1-617, 1-591 and 1-577, were PCR amplified and inserted into the same restriction sites of vector pEYFP-C1. All Numb and TRPV6 point mutation constructs were generated using site-directed mutagenesis.

4. Co-immunoprecipitation

HEK293 cells were plated in 60-mm dishes and cotransfected with myc and EYFP-tagged constructs. The Numb constructs were cotransfected with the TRPV6 constructs at a 1:1 ratio. After a 24 h incubation, the cells were harvested and lysed in 500 μl of cell lysis buffer (0.5% Triton X-100, 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA). After clarification through centrifugation at 4℃ for 10 min at 15,000 g, the whole cell lysates were incubated with 1 μg of anti-myc antibody and 25 μl of protein G-agarose beads at 4℃ overnight with gentle rotation. Subsequently, the beads were washed 3 times with wash buffer (0.1% Triton X-100, 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA), and the precipitates were eluted with 30 μl 2x Laemmli buffer and subjected to western blot analysis for anti-myc and anti-GFP immunoreactivity.

5. Immunoblotting

Aliquots (20-60 μg of protein) of the purified cell lysates, obtained with 0.5% Triton X-100 lysis buffer containing protease inhibitor cocktail (Calbiochem) and phosphatase inhibitor Calbiochem), were clarified using centrifugation (15,000 g for 10 min). The supernatants were heated for 1 min under reducing conditions and subsequently subjected to electrophoresis on an 8% SDS acrylamide gel. The proteins were transferred electrophoretically onto nitrocellulose membranes (BioRad), which were previously blocked in 5% (wt/vol) nonfat milk for 1 h, and further incubated with antibodies against TRPV6, Numb, and GAPDH overnight at 4℃. After incubation with horseradish peroxidase-coupled secondary antibodies for 1 hour at room temperature, the signal was detected using a commercial detection system (Thermo Scientific).

6. Measurement of intracellular calcium

The ratiometric measurement of $[Ca^{2+}]_i$ was performed using Fura-2 AM (Molecular probe). The cells were grown in 24-well dishes and loaded with 5 μM of Fura-2 AM for 30 min at 37℃. The Fura-2 fluorescence was measured at a 510 nm emission with a 340/380 nm dual excitation using a DG-4 illuminator. The experiments were performed in an Normal Tyrode solution containing 3.6 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 145 mM NaCl, 2 mM $CaCl_2$, 5 mM Glucose, pH 7.4 .

7. Fluorescence Resolution Energy Transfer

3 FRET images (cube settings for CFP, YFP, and Raw FRET) were obtained from a pE-1 Main Unit to 3 FRET cubes (excitation, dichroic mirror, filter)

through a fixed collimator: CFP (ET435/20m, ET CFP/YFP/mCherry beamsplitter, ET470/24m, Chroma); YFP (ET500/20m, ET CFP/YFP/mCherry beamsplitter, ET535/30m, Chroma); and Raw FRET (ET435/20m, ET CFP/YFP/mCherry beamsplitter, ET535/30m, Chroma). The excitation LED and filter were sequentially rotated, rotation period for each of filter cubes was ~0.5 sec, and all images (three for CFP/YFP/Raw FRET, respectively) were obtained within 1.5 sec. Each of the images was captured on a cooled 10MHz (14 bit) CCD camera (DR-328G-C01-SIL: Clara, ANDOR technology, USA) with an exposure time of 100 ms with 2×2 binning (645×519 pixels) under the control of MetaMorph 7.6 software (Molecular Devices, Japan). To obtain the FRET efficiency of a cell, we used a microscope (IX70, Olympus, Japan) with a 60x oil objective and the three-cube FRET method calculation.

8. Gene silencing using siRNA

For gene silencing, the siRNA was transfected using the RNAi Max Transfection Reagent (Invitrogen), according to the manufacturer's instructions. Numb siRNA was used to target the sequence 5'-CAUAGUUGACCAGACGAUATT-3'. TRPV6 siRNA was used to target the sequence of 5'-GGGAAACACAGUGUUACACTT-3'. All siRNA were synthesized at the Shanghai GenePharma Co. Control siRNA was purchased from Dharmacon Cat.D-001210-01-05. The silencing effects were evaluated using western blot analysis as described above.

9. Statistical analysis

The data were compared by using ANOVA followed by a post hoc test. The values are given as the means $\pm$SEM. The results were considered to be significantly different when $P < 0.05$.

RESULTS

Numb negatively regulates calcium influx activity of TRPV6

The TRPV6 channel is Ca^{2+} selective (Ca^{2+} >>> Ba^{2+}, Sr^{2+}). To measure TRPV6 activity, we used calcium influx assay (75). In TRPV6 transfected HEK293 cells, the calcium influx increased 10 to 15 fold when the extracellular Ca^{2+} changed from 0 to 2 mM or 10 mM compared with mock-transfected cells (Fig. 1A,B). To explore the functional role of Numb1 in TRPV6 activity, we used a loss and gain-of-function approach. First, we established a stable HEK293 cell line, constitutively expressing TRPV6, using G418 selection. The TRPV6 stable cell line displayed similar calcium influx activity compared to trasiently overexpressed cells. Knocking down Numb, resulted in an increase in calcium influx (Fig. 2A,B). The knockdown efficiency was examined using western blotting (Fig. 2A). The data showed that the overexpression of Numb1 decreased calcium levels (Fig. 2C,D). Taken together, these results showed that Numb1 blocked TRPV6 calcium influx activity.

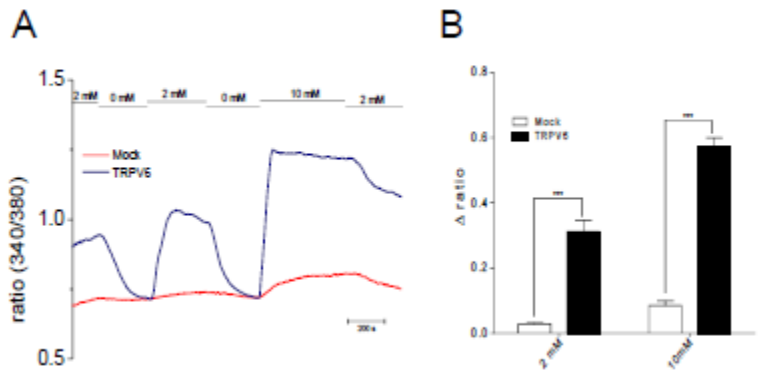

Figure 1. Calcium influx assay for TRPV6 activity

A, HEK293 cells were transfected with YFP-TRPV6 or pEYFP-C1 vector during 24 hr. Cell were loaded with 5 μM Fura-2 AM in NT solution at 37℃ for 30 min. Concentration of calcium was measured after subsequent switches of NT solution, as indicated . B, The average change in fluorescence ratio ± S.E; *P < 0.05, ***P < 0.0001; n=8.

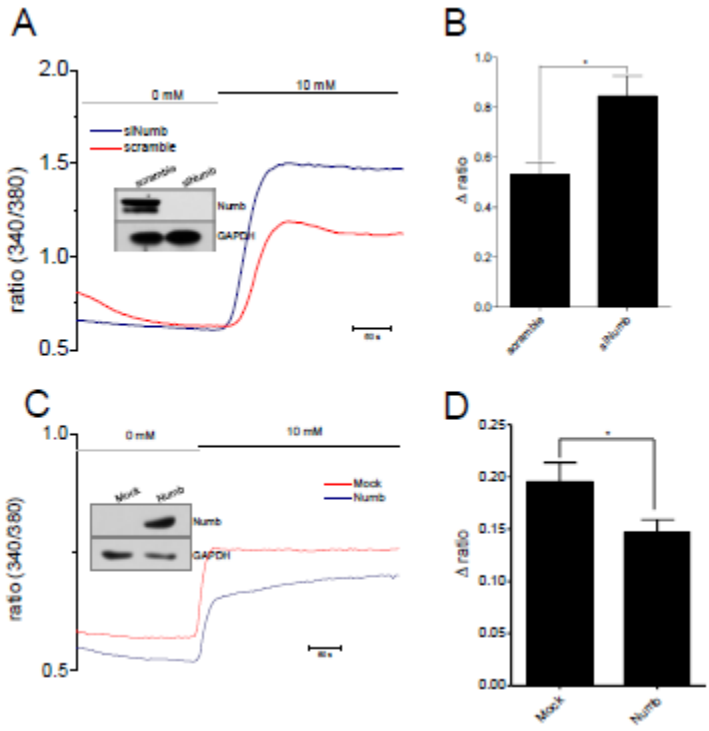

Figure 2. Numb1 inhibits activity of TRPV6

A, HEK293 cells which TRPV6 stably overexpressed were transfected with Numb siRNA. 72 hr after transfection the total cell lysates were examined by western blotting analysis of the protein expression of Numb. GAPDH was used as a loading control. Calcium influx experiment was performed in Numb knockdown cells (n=30). B, The average change in fluorescence ratio ± S.E; *P < 0.05. C, YFP-TRPV6 and Numb-myc, empty vector were co-transfected in HEK293 cells. 24 hr after transfection the total cell lysates were examined by western blotting analysis of the protein expression of Numb. GAPDH was used as a loading control. calcium influx assay was conducted. D, The average change in fluorescence ratio ± S.E; *P < 0.05, n=15.

Validation of Numb as novel binding partner of TRPV6

To validate the yeast two-hyrid identification of Numb as a TRPV6 binding partner, we performed co-immunoprecipitation, confocal microscopy and Fluorescence Resolution Energy Transfer (FRET) analysis. For the co-immunoprecipitation, we cotransfected YFP-tagged TRPV6 and myc-tagged Numb1 in HEK293 cells. After a 24 hr incubation, we conducted an immunoprecipitation experiment using an anti-myc antibody followed by immunoblot analysis with an anti-GFP antibody. We observed an interaction between TRPV6 and Numb1, confirming the reciprocal interaction test (Fig. 3A). Subsequently, we performed a FRET analysis. In the previous report, YFP- tagged TRPV6 was not localized to the plasma membrane, and reason for this phenomenon is not understood (76). Consistent with previous observations, a low FRET efficiency was observed when TRPV6-YFP and Numb1-CFP were cotransfected. We postulated that the activation of TRPV6 would result in its increased trafficking to plasma membrane. ß-glucuronidase is closely related to the klotho analogue, which activates TRPV6 via N-oligosaccharide hydrolysis (75), and ß-ionone also activates TRPV6 through src kinase activation (77). TRPV6 undergoes calcium - dependent inactivation mechanisms, therefore we used BAPTA and BAPTA-AM to chelate Ca^{2+}. After a 24 hr incubation in the presence of the activators, the plasma membrane localization of TRPV6 and FRET efficiency were enhanced (Fig. 3B,C). Because the binding site of TRPV6 is located at the c-terminus, when TRPV6 was tagged at the N-terminus with YFP, the distance between YFP and CFP was far; hence, the FRET efficiency was low (Fig. 4). TRPV5 and

TRPV6 have high sequence homology and are capable of inducing calcium influx (Fig. 5). As both proteins have overlapping interacting partners, we performed an experiment to determine whether TRPV5 also interacted with Numb1. TRPV5 did not show any interaction with Numb1 (Fig. 6A). Numb1 did not influence the activity of TRPV5 (Fig. 6B,C). These findings suggest that Numb1 specifically regulates TRPV6 functions.

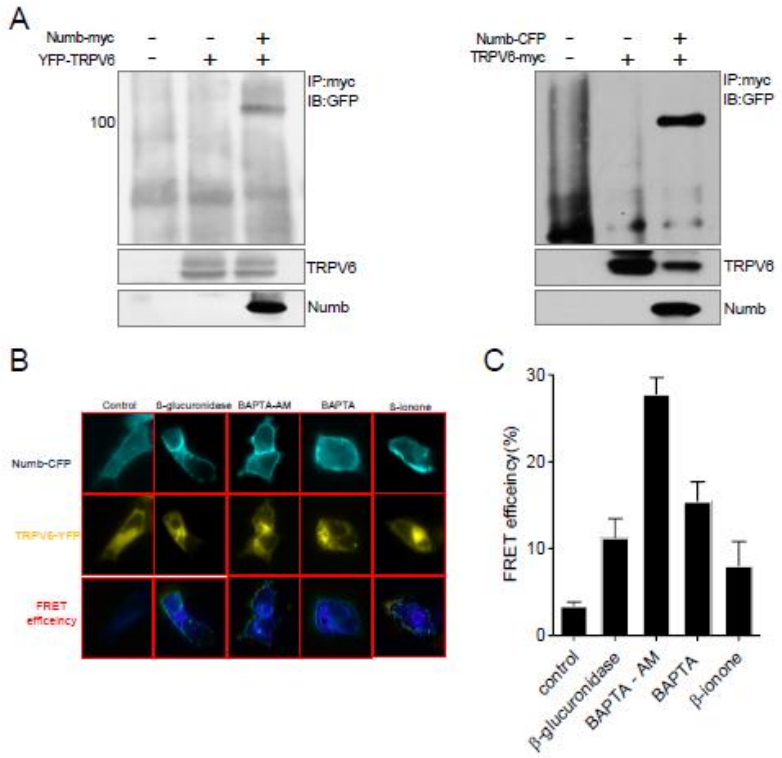

Figure 3. Numb1 interacts with TRPV6

A, HEK293 cells were transiently co-transfected YFP-TRPV6 and Numb1-myc or empty vector. After 24 hr incubation, the expressed fusion proteins were immunoprecipitated from cell lysates (300 µg) using myc antibody (1 µg) and detected by western blotting using a GFP antibody. Total protein of TRPV6 was detected using GFP antibody and Numb1 was detected using myc antibody. TRPV6-myc and Numb-CFP plasmids were used for reciprocally validation. B, Fluorescence was measured in HEK293 cells TRPV6-YFP and Numb1-CFP. TRPV6 activator (BAPTA 1 µM, BAPTA-AM 1 µM, ß-glucuronidase 400 U/ml, ß-ionone 100 µM) incubation time was 24 hr. C, Represents the mean ±S.E. for change in FRET ratio signals.

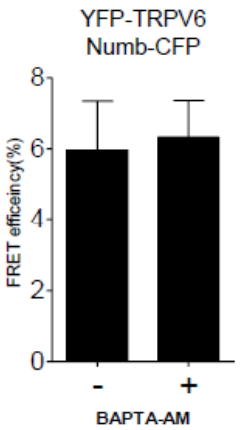

Figure 4. FRET efficiency of TRPV6 and numb1

Fluorescence was measured in HEK293 cells YFP-TRPV6 and Numb-CFP. BAPTA-AM 1 μM incubation time was 24 hr. Represents the mean ±S.E. for change in FRET ratio signals.

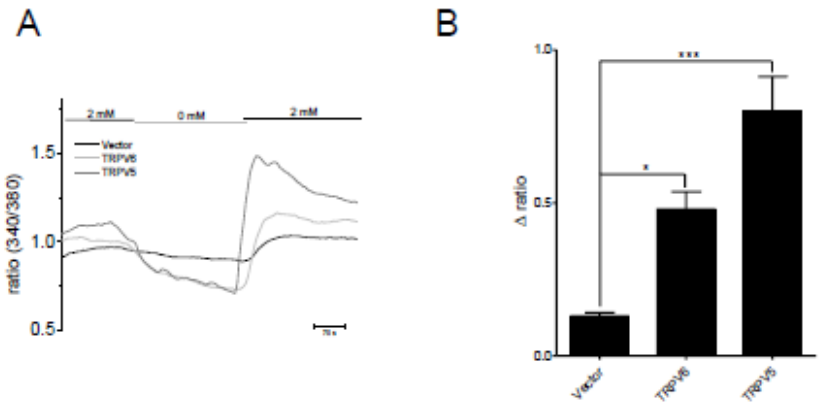

Figure 5. Calcium influx assay of TRPV5 and TRPV6

A, represents the changes in the ratio of fura-2 fluorescence upon addition of NT solution containing 2 mM $CaCl_2$ in YFP-TRPV5, YFP-TRPV6 transfected HEK293 cells (n=15). B, The average change in fluorescence ratio ±S.E.

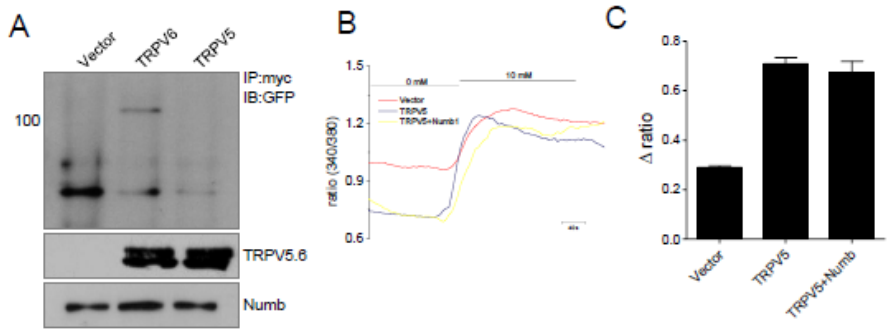

Figure 6. Interaction of TRPV5 and Numb1

A, YFP-TRPV5,YFP-TRPV6,pEYFP-C1 and Numb1-myc were cotransfected in HEK293 cells. Immunoprecipitation was performed with myc antibody followed by immunoblotting with GFP antibody. Total proteins of TRPV5,V6 were detected using GFP antibody and total protein of Numb1 was detected using myc antibody. B, represents the changes in the ratio of fura-2 fluorescence upon addition of NT solution containing 10 mM $CaCl_2$ in YFP-TRPV5, Numb1-myc transfected HEK293 cells (n=15). C, The average change in fluorescence ratio ±S.E.

Identification of TRPV6 binding site 716 Asp

Because function is highly related to structure, mapping the binding site is important. The specific binding region of TRPV6 and its interacting partner have not been previously determined (78). To identify the specific TRPV6 binding site, we designed c-terminal truncation constructs (Fig. 7). The function of these mutants was evaluated using a calcium influx assay (Fig. 8). YFP-TRPV6 truncation mutants and the Numb1-myc plasmid were coexpressed in HEK293 cells, and the cells were subjected to co-immunoprecipitation. First, we conducted an experiment using broadly cut TRPV6 mutants, 1-577, 1-591, 1-617, 1-635, 1-680, 1-704, and WT (Fig. 9A). The amino acid region 705-725 was identified, and this region was further segmented. Amino acids residues 716-720 of TRPV6 were critical for the binding (Fig. 9B), and serially deleted mutants of this region were used in the experiment. We discovered that aspartic acid 716 is essential for binding to Numb1 (Fig. 9C,D). Because aspartic acid is negatively charged, we postulated that the two proteins would form an ionic bond. Therefore, we generated D716A and positively charged D716K mutants, and subsequently tested these mutants using co-immunoprecipitation. The D716A mutant showed a weaker interaction compared to WT, and much weaker interaction was detected in D716K mutant (Fig. 9E). The D716A and D716K mutants had calcium influx activity similar to WT (Fig. 8). It is noticeable that charge plays a key role in the interaction between TRPV6 and Numb in that D716K shows weaker interaction with Numb than D716A.

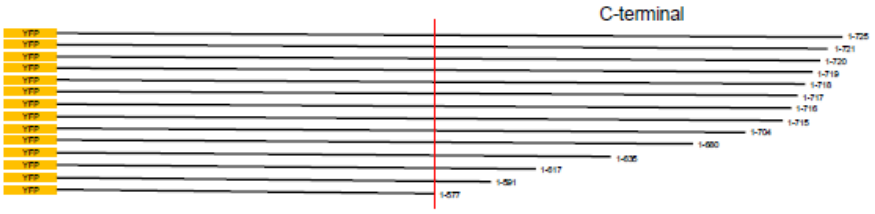

Figure 7. TRPV6 c-terminal deletion mutants

Schematic diagram of TRPV6 C-terminal truncation mutants.

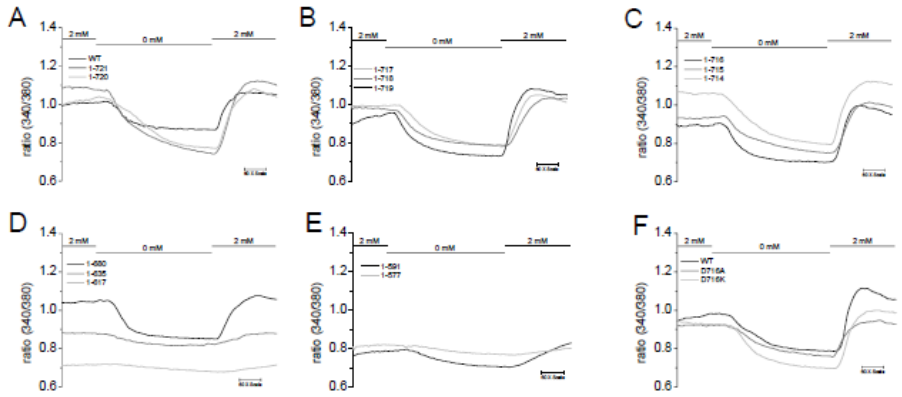

Figure 8. Calcium influx assay of TRPV6 mutants

A-F, HEK293 cells were transfected with YFP-TRPV6 mutants during 24 hr. Cell were loaded with 5 μM Fura-2 AM in NT solution at 37℃ for 30 min. Concentration of Ca^{2+} was measured after subsequent switches of NT solution, as indicated (n=5).

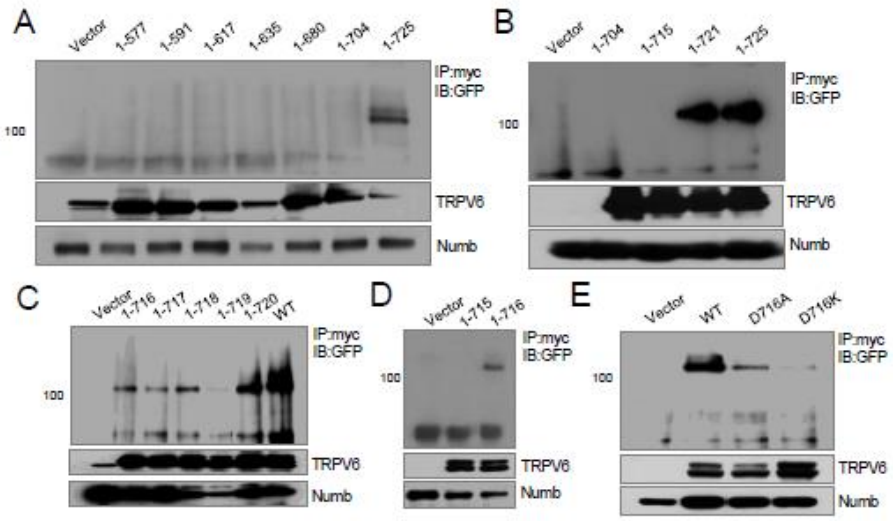

Figure 9. Binding site mapping of TRPV6

A-E, Full-length or truncated YFP-TRPV6 mutants and Numb-myc were transfected in HEK293 cells. Lysates (300 μg) were immunoprecipitated with antibody directed against myc (1 μg) and analyzed by immunoblotting with GFP antibody. Total proteins of TRPV6 mutants were detected using GFP antibody and Numb was detected using myc antibody.

Identification of Numb binding site 434 Arg

Next, we mapped the binding site of Numb1. To determine the Numb1 binding site, we created Numb1 deletion constructs (Fig. 10). Numb1 consists of 651 amino acids that contains phospho-tyrosine binding (PTB), proline rich (PRR) domains, Asn-Pro-Phe (NPF) and Asp-Pro-Phe (DPF) motifs. Numb has four isoforms and each isoform has distinct functions (70). TRPV6 is capable of binding to each isoform. Because isoform4 has low transfection efficiency, it has weak band compared to other isoforms. That is, TRPV6 does not tend to be isoform-specific (Fig. 11A). The NPF and DPF motifs are relevant to endocytosis. As TRPV6 is a membrane protein, Numb1 might be involved in endocytosis. The results from the protein binding experiment, showed that the NPF and DPF deletion mutants interacted with TRPV6 (Fig. 11B), and TRPV6 surface expression was not changed when Numb1 was overexpressed (Fig. 11C). The PTB domain is a critical region for determining Numb1 function. Diverse proteins, such as ubiquitinase regulate the function of Numb1 via the PTB domain. The PTB deletion mutants of Numb1 interacted with TRPV6 in a manner similar to WT (Fig. 11D). We conducted further mapping experiments using C and N-terminal truncation mutants. However, we observed that these mutants were all able to interact with TRPV6; therefore, generated additional mutants with deletions in the left region of Numb1 (Fig. 11E,F,G). We observed that the Δ414-460 mutant did not show any interaction with TRPV6; surprisingly, this mutant did not express total protein (Fig. 12A). Due to unexpected finding of the non-binding region, we decided to focus on this site for further testing. As TRPV6

interacts with Numb1 through its negatively charged residue, we substituted all positively charged residues with Ala. Among the 5 Ala mutants R434A did not bind to TRPV6 (Fig. 12B). Similar to previous data, R434A total protein was not expressed. Next, we generated a R434E mutant and conducted co-immunoprecipitation experiments. Although the R434E total protein was detected, this mutant did not show appreciable binding to TRPV6 (Fig. 12C). These results suggested that Numb1 forms a complex with TRPV6 via a charge-charge interaction. However, the reduced expression of the Δ414-460 and R434A mutants is not understood. We further demonstrated that the R434E mutant did not significantly influence on TRPV6-mediated calcium influx (Fig. 13A,B,C), suggesting that the inhibitory function of Numb1 is only effective in a proper complex with TRPV6. Next we focused on the phosphorylation of Numb1. The protein kinase aPKC and CaMK regulate the interaction of Numb1 with AP-2 through phosphorylation (79-82). The aPKC phosphorylation sites on Numb1 are Ser 7 and 276, and the CaMK phosphorylation sites are Ser 276 and 295. Thus, to assess whether Numb1 binds to TRPV6 in a phosphorylation-dependent manner, we conducted co-immunoprecipitation using phosphor-site mutants of Numb1, in which Ser 7, 276, and 295 were replaced with alanines or aspartates. The single phospho-site mutants exhibited no changes compared with WT (Fig. 14A,B). In contrast, the double phospho-site mutants S276A/S295A did not interact with TRPV6. However, the phosphomimetic double mutant S276D/S295D interacted well with TRPV6 (Fig. 14C). The S276A/S295A mutant did not

produce total protein similar to the Δ414-460 and R434A mutants. These phenomena require further studies.

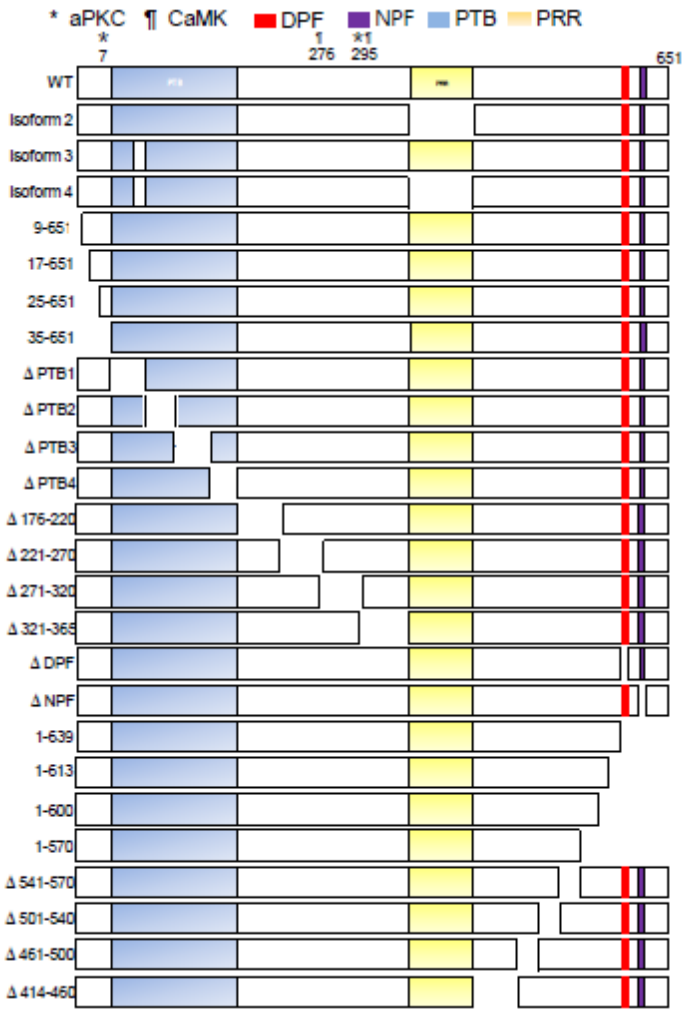

Figure 10. Deletion mutants of Numb1

Schematic diagram of Numb deletion mutants.

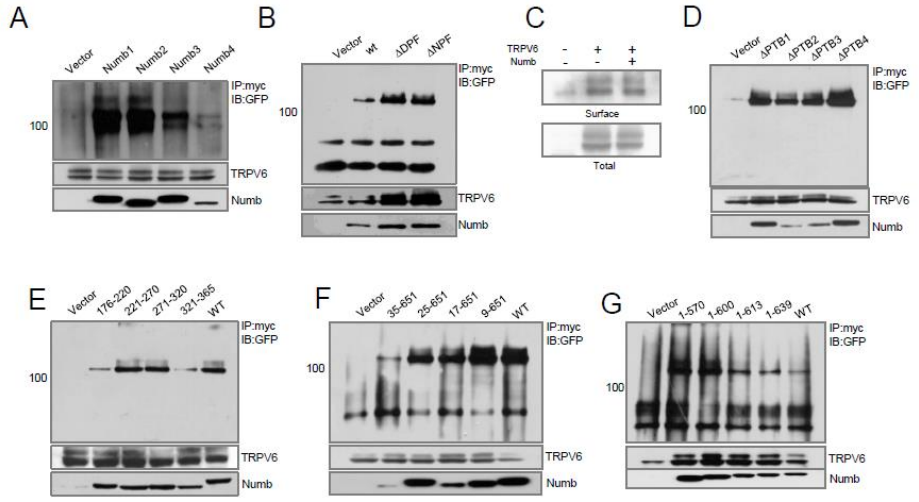

Figure 11. Binding site mapping of Numb1

A,B,D,E,F,G, Full-length or deleted Numb-myc mutants and YFP-TRPV6 were transfected in HEK293 cells. Immunoprecipitation was conducted with myc antibody (1 μg) and visualized by western blotting with GFP antibody. Total proteins of Numb mutants were detected using myc antibody and TRPV6 was detected using GFP antibody. C, HEK293 cells were transfected YFP-TRPV6 and Numb-myc. After 24 hr, surface biotinylation assay was performed.

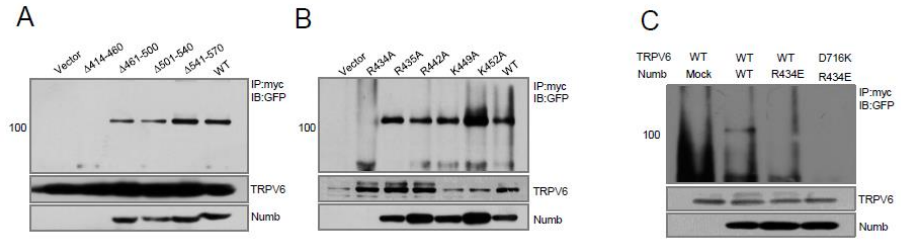

Figure 12. Binding site mapping of Numb1

A-C,Full-length or deleted Numb-myc mutants and YFP-TRPV6 were transfected in HEK293 cells. Immunoprecipitation was conducted with myc antibody (1 μg) and visualized by western blotting with GFP antibody. Total proteins of Numb mutants were detected using myc antibody and TRPV6 was detected using GFP antibody.

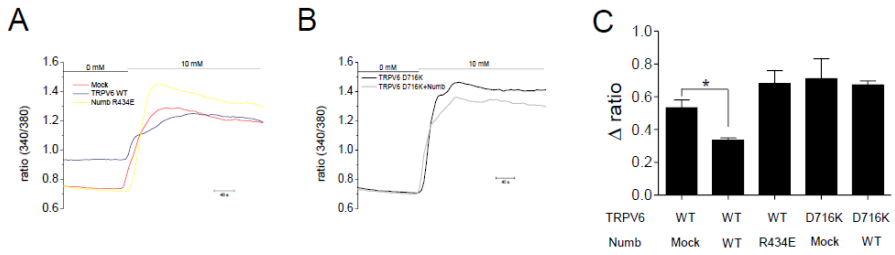

Figure 13. Calcium influx assay of mutants

A-B, Calcium influx assay was performed in transfected HEK293 cells (n=7).

C, The average change in fluorescence ratio ±S.E; *P < 0.05.

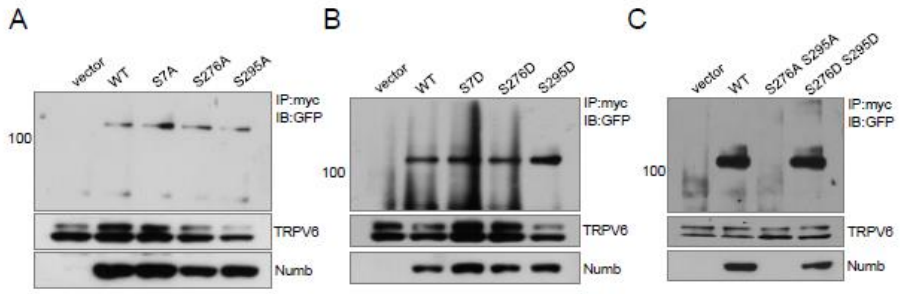

Figure 14. Interaction of TRPV6 and Numb phosphorylation mutants

A-C, HEK293 cells were transfected YFP-TRPV6 and Numb-myc mutants. Lysates were immunoprecipitated with antibody directed against myc and analyzed by immunoblotting with GFP antibody.

Numb regulates the level of intracellular Ca^{2+} and cell proliferation via TRPV6 in MCF-7 cells

It was recently reported that TRPV6 is upregulated in prostate and breast cancers (45). Numb has been suggested to play a significant role as a tumor suppressor in breast cancer (71). To determine the functional significance of the novel interaction between Numb and TRPV6 under physiological conditions, we performed experiments using MCF-7 cells. First, we tested the antibody specificity of TRPV6 (Fig. 15A). We observed Numb interacted with TRPV6 endogenously (Fig. 15B). The knockdown of Numb, using siRNA, increased TRPV6 protein expression (Fig. 15C). Because the interaction of Numb and TRPV6 was strong, we hypothesized that these proteins control each other. However, when we knocked down TRPV6, we observed an increase in Numb protein expression (Fig. 15D). These data indicate that Numb and TRPV6 regulate the protein stability and degradation of each other. Using previous data, we could easily explain the low protein expression of the Numb mutants. The increased TRPV6, as a consequence of Numb knockdown, resulted in the elevation of cytosolic Ca^{2+} and the concomitant stimulation of cell proliferation (Fig. 16A,B,C). On the other hand, however, when TRPV6 knockdown was performed using siRNA, it was observed that both cytosolic Ca^{2+} and proliferation decreased (Fig. 16D,E,F). Taken together, these data suggest that Numb acts as a tumor suppressor via cytosolic Ca^{2+} regulation.

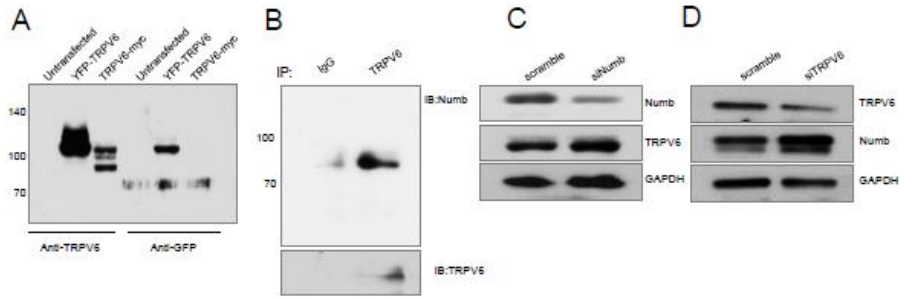

Figure 15. Knockdown of TRPV6 and Numb increases protein reciprocally

A, HEK293 cells were transfected YFP-TRPV6 and TRPV6-myc plasmids and analyzed by immnoblotting with anti-TRPV6, anti-GFP. B, Co-immunoprecipitation of TRPV6 and Numb. Whole cell lysates from MCF-7 cells were prepared, and immunoprecipitation was performed with anti-TRPV6 followed by immnoblotting with antibodies against Numb. C, The effects of Numb knockdown on protein levels of TRPV6 and Numb itself, compared to GAPDH. D, The effects of TRPV6 knockdown on protein levels of Numb and TRPV6 itself, compared to GAPDH.

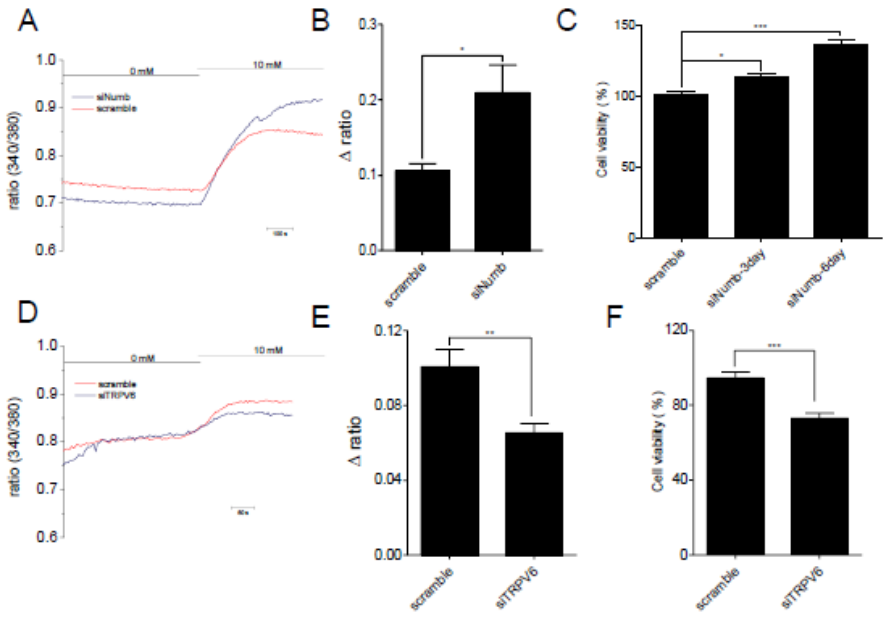

Figure 16. Numb regulates the level of intracellular Ca^{2+} and cell proliferation via TRPV6 in MCF-7 cells

A, The effects of Numb silencing on Ca^{2+} influx into MCF-7 cells (n=40). B, The average change in fluorescence ratio ±S.E; *P < 0.05. C, Cell proliferation of MCF-7 cells measured using MTT assay. MCF-7 cells were plated in 24-well plate and transfected siRNA for 72 hr. After 72 hr, cells were re-plated in 24-well plate on 20% confluency and transfected siRNA for 72 hr again. *P < 0.05, ***P < 0.0001; n=5. D, The effects of TRPV6 silencing on Ca^{2+} influx into MCF-7 cells (n=72). E, The average change in fluorescence ratio ±S.E; **P < 0.005. F, Cell proliferation of MCF-7 cells measured using MTT assay. MCF-7 cells were plated in 24-well plate and transfected siRNA for 72 hr. ***P < 0.0001; n=5.

Calcium influx via TRPV6 induces GSK3ß and MAPKinase activation

Ca^{2+} stimulates various intracellular signaling pathways. We demonstrated that Numb regulates Ca^{2+} influx and cell proliferation via TRPV6. It is known that nuclear factor of activated T-cell (NFAT) are a family of transcription factors that are activated through Ca^{2+} influx in LNCaP cells (83). However, the signaling pathways that are initiated after Ca^{2+} influx are poorly understood. We conducted a Ca^{2+} influx experiment, and identified intracellular signaling pathways involved in cell proliferation in TRPV6-overexpressed cancer cells (MCF-7, PC-3). In MCF-7 cells, Ca^{2+} influx stimulated the GSK3ß, AKT, JNK, and Erk pathways (Fig. 17A). To confirm if it is TRPV6-specific, TRPV6 protein was depleted, and the activation of GSK3ß,JNK,Erk got weaker compared to control test (Fig. 17B). The GSK3ß, JNK, and p38 pathways were activated TRPV6-specific in PC-3 cells (Fig. 18A,B). These data suggest that GSK3ß, MAPKinase, and AKT are novel pathways involved in TRPV6 mediated cell proliferation.

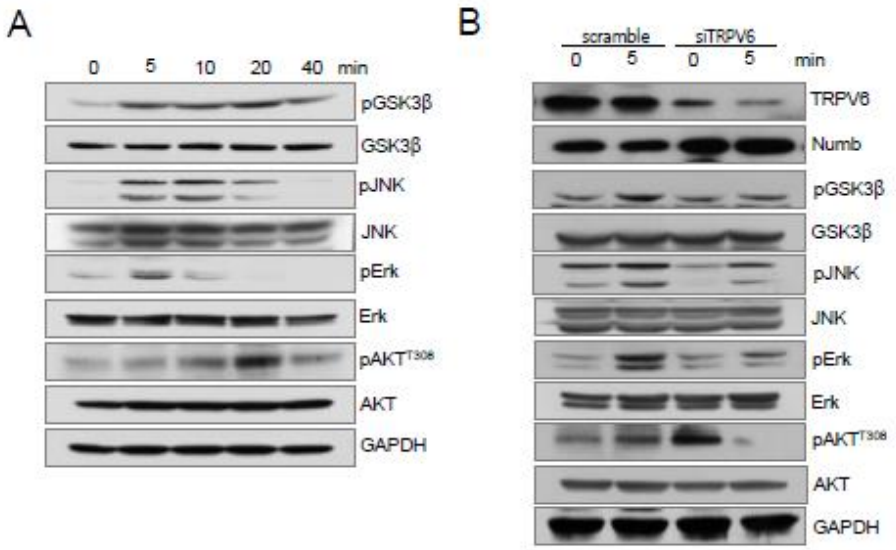

Figure 17. Signaling pathway after calcium influx in MCF-7 cells

Western blotting analysis signaling molecules during Ca^{2+} influx. MCF-7 cells treated with NT containing 0 mM Ca^{2+} for 5 min and changed NT containing 10 mM Ca^{2+}. Cells were collected at the indicated time. TRPV6 siRNA transfected for 72 hr.

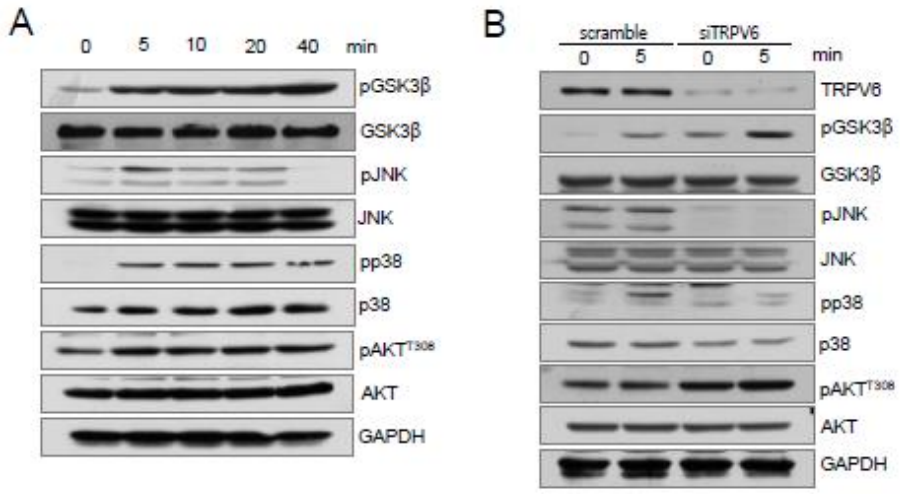

Figure 18. Signaling pathway after calcium influx in PC-3 cells

Western blotting analysis signaling molecules during Ca^{2+} influx. PC-3 cells treated with NT containing 0 mM Ca^{2+} for 5 min and changed NT containing 10 mM Ca^{2+}. Cells were collected at the indicated time. TRPV6 siRNA transfected for 72 hr.

DISCUSSION

Currently, 18 TRPV6-binding proteins have been identified (78). TRP proteins mostly assemble to homomeric channels but can also heteromerize. TRPV5 interacts TRPV6 and they can generate a pleiotropic set of functional heterotetrameric channels with different Ca^{2+} transport kinetics (79). TRPC1 has interaction of TRPV6 that negatively regulates calcium influx in HEK293 cells (80). Cyclophilin B, Klotho, NHERF4, Rab11a and S100A10 proteins are associated with TRPV6 and activate function of TRPV6 (81,75,82,83,84). Calmodulin, Nipsnap1, PTP1B, and RGS2 inhibit activity of TRPV6 (85-88). However, interacting protein of TRPV6 have yet to be studied in cancer cells.

In this study, We showed that Numb1 protein is a novel binding partner of TRPV6. Using a HEK293 cell transfection system, We demonstrated that Numb1 inhibits TRPV6 activity via calcium influx experiments. TRPV6 expression correlates with tumor grades in many tissues, which inspires the idea of this channel as encoded by a possible oncogene. Numb antagonizes Notch signaling through multiple mechanisms. For example, Numb binds Notch and increases its ubiquitination and degradation through interactions with amyloid precursor protein (APP) or the HECT (homologous to E6-AP carboxy terminus) domain ubiquitin ligase, Itch. Numb also increases endocytic trafficking of the Notch receptor . In addition, Numb may regulate differentiation and proliferation through Notch-independent mechanisms such as stabilizing p53 or promoting the Itch-dependent degradation of the Sonic hedgehog pathway transcription factor, Gli1. In drosophila and mice,

disruption of asymmetric cell division leads to neural overgrowth. In humans, Numb restrains the growth of breast cancer cells by inhibiting Notch and stabilizing p53. In addition, downregulation of Numb by musashi 2 promotes the development of blast crisis in leukemia. These observations have led to the suggestion that Numb is a tumor suppressor. Thus, inhibition of TRPV6 by Numb can be considered as another function of tumor suppressor. We validated the interaction of TRPV6 and Numb1 using co-IP, and FRET. Interactions between proteins play pivotal roles in the regulation of genes, and in recent years, it has become quite evident that aberrant protein interactions are the underlying cause for many diseases. Many of the key protein-protein interactions are known to participate in disease-associated signaling pathways, and represent novel targets for therapeutic intervention. Thus, small molecule inhibitors can be designed to disrupt protein-protein interactions. several small molecule inhibitors have been identified and developed that disrupt protein-protein interactions, e.g. small-molecule inhibitors of the MDM2-p53 tumor suppressor protein interaction involved in cancers (89). In spite of the general understanding that protein-protein interactions occur over a large surface area, studies have shown that many protein-protein interfaces contain compact, centralized regions of residues, usually termed ‘hot spots’ that are crucial for these interaction, and in many cases one hot spot is involved in binding to multiple protein partners through different structural motifs. Therefore, identification of these spots can provide a potential target for intervention aimed at interacting surfaces, and subsequently developing and designing small molecules with therapeutic potentials. All TRP channels share a similar

six-transmembrane-segment ion transport domain flanked by intracellular N- and C-terminal domains, and are expected to function as tetramers. The N-terminal of the TRPA, TRPC, TRPN and TRPV channels contain between 3 and 31 ankyrin repeat domain (ARD), which are 33-amino acid residue motifs often involved in protein-protein interactions. The mammalian TRPV subfamily is composed of six members that contain 6 ARD in their N-terminal cytosolic domain. TRPV5 and TRPV6 share approximately 75% sequence similarity and are capable of associating with each other; heterotetramers show intermediate electrophysiologic properties, depending on the ratio of TRPV5 to TRPV6. Homo- and heterotetramerization is dependent on residues in all three regions of the protein, both the N-and C-terminal cytosolic domains and the transmembrane domain. Two ankyrin repeats of the TRPV6-ARD (repeats 3 and 5; residues 116–140 and 192–230, respectively) and ankyrin repeat 1 of TRPV5 (residues 64–76) have been implicated in channel assembly. Furthermore, the C-terminal regions of both TRPV5 and TRPV6 are important for binding proteins involved in plasma membrane localization. The small GTPase Rab11a targets both TRPV5 and TRPV6 to the plasma membrane using residues 595–601 in TRPV5, and the corresponding residues (600–607) in TRPV6; and residues 596–616 in TRPV5 (or 601– 621 in TRPV6) are important for binding the PDZ domain-containing protein NHREF4, which may be involved in plasma membrane retention of the two channels. The C-terminal region also contains the TRP-domain, a short hydrophobic segment found in most TRP channels. The activity of TRPV6, but not TRPV5, is inhibited by direct binding of regulator of G protein

signaling 2 (RGS2) to the TRPV6 N-terminal cytosolic domain. The Ca^{2+}-sensing protein calmodulin (CaM) also inhibits TRPV6. CaM binding to and inhibition of TRPV6 requires both the N- and C-terminal cytosolic domains and the transmembrane domain. The activity of CaM on TRPV6 is opposed by Protein Kinase C (PKC), which is known to phosphorylate the C-terminal domain of TRPV6 (90). We show that Numb does not interact with TRPV5, but it is still unclear if interaction occurs in heteromeric construction. Though building construct was challenging, it is necessary to confirm the interaction in heteromeric state. The modular structure of Numb makes it an adaptor protein interacting with several molecules thereby regulating its multiple cell functions. PTB domain is partially involved in cell membrane localization, contributing to the role of Numb in the control of endocytosis. Interestingly, a short amino acid insert in the PTB region (PTBi), which characterizes the p66 and p72 splice isoforms is responsible for targeting Numb to cortical cell membrane possibly via interaction with acidic membrane phospholipids (PI(4)P and PI (4,5)P_2). Interestingly, Numb movement from cell membrane to the cytosol is promoted by G-coupled Receptors likely via PLC dependent PIP_2 hydrolysis. Whereas the PTBi is responsible for cell membrane localization, the C-terminus of Numb is required for the interaction with several components of the endocytic machinery. DPF and NPF motifs of all mammalian Numb isoforms and Numbl as well as dNumb, are responsible for binding the clathrin adaptor α-adaptin and various proteins of the Epsin15 Homology Domain family involved in both clathrin-dependent and -independent endocytosis. Therefore, by these motifs, Numb is an endocytic

adaptor with specialized functions in the regulation of a number of signaling pathways such as EGF, transferrin and Notch receptors (70). We found that TRPV6 binds to novel region in Numb, not in the known domain through binding site mapping of deleted constructs. We showed a electrostatic interaction between TRPV6 and Numb1 through mutagenesis of specific residues and co-IP. Specifically, Asp716 of TRPV6 and Arg434 of Numb1 are involved in the association between TRPV6 and Numb1. This electrostatic interaction may play a role in protein stability and oligomerization (91). Thus we hypothesized that the interaction of TRPV6 and Numb1 influences their protein. To validate our hypothesis under physiological conditions, we examined the endogenous TRPV6 and Numb protein levels in MCF-7 cells. We showed that CaMK is an essential factor in the association of TRPV6 with Numb1. Numb1 is a substrate of aPKC and CaMK (92-95). Although the aPKC-mediated phosphorylation of Numb1 has been well characterized, the function of CaMK phosphorylation is poorly understood. We showed that the CaMK-mediated phosphorylation of Numb1 is required for binding to TRPV6. This result suggests that increasing Ca^{2+} in the cytosol facilitates the CaMK-mediated phosphorylation of Numb1 and its subsequent inhibition of TRPV6-mediated Ca^{2+} influx. Interestingly, we identified that TRPV6 also regulates Numb1. Numb1 is a cell fate determinant that influences the asymmetric cell division of stem cells in the development stage (96). The influence of calcium signaling on Numb1 activity has not been clarified. However, this study provides clue that TRPV6 is involved in the stem cell development stage. More studies are required to fully understand this phenomenon.

Glycogen synthase kinase 3ß (GSK3ß), a multifunctional serine/threonine kinase found in all eukaryotes, had been initially identified as a key regulator of insulin-dependent glycogen synthesis. It is now known that GSK3ß functions in diverse cellular processes including proliferation, differentiation, motility and survival. Aberrant regulation of GSK3ß has been implicated in a range of human pathologies including non-insulin-dependent diabetes mellitus, cardiovascular disease, some neurodegenerative diseases, and bipolar disorder. The role of GSK3ß in tumorigenesis and cancer progression remains controversial; it may function as a tumor suppresso for certain types of tumors, but promotes growth and development for some others. Stimulation of pGSK3ß (Tyr216) is reported to be mediated by alterations in intracellular calcium levels and a calcium-dependent tyrosine kinase, proline-rich tyrosine kinase 2 (PYK2), or by Fyn, a member of the Src tyrosine family. pGSK3ß (Tyr216) is also subject to the regulation of mitogen-activated protein kinase kinase (MEK1/2) . One of the most well-known substrates of GSK3ß is ß-catenin, and GSK3ß is an important regulator of the Wnt/ß-catenin signaling pathway. Wnt signaling inactivates GSK3ß and prevents it from phosphorylating ß-catenin, thus stabilizing ß-catenin in the cytoplasm. As ß-catenin accumulates, it translocates into the nucleus where it binds to TCF/LEF and dramatically increases their transcriptional activity. Genes up-regulated by TCF/LEF include proto-oncogenes, such as c-myc and cyclin-D1, and genes regulating cell invasion/migration, such as MMP-7. GSK3ß regulates the levels as well as intracellular localization of p53. GSK3ß forms a complex with nuclear p53 to promote p53-induced apoptosis. GSK3ß

directly modulates the activity of transcription factors, activator protein 1 (AP-1) and nuclear factor-kB (NF-kB) (97). Mitogen-activated protein kinases (MAPKs) are signalling components that are important in converting extracellular stimuli into a wide range of cellular responses. The ERK1 and ERK2 are activated by mitogens and were found to be upregulated in human tumors. Two other major MAPK pathways, the Jun N-terminal kinase (JNK) and p38 MAPK pathways, which are also called stress-activated protein kinase pathways, are also often deregulated in cancers. JNKs and p38 are activated by environmental and genotoxic stresses and have key roles in inflammation, as well as in tissue homeostasis, as they control cell proliferation, differentiation, survival and the migration of specific cell types. Downstream targets, including transcription factors and other effectors, which determine a range of biological responses from cell proliferation, survival, differentiation and migration to inflammation and cancer. Many genes are directly regulated by these transcription factors, including genes that encode p21, 14-3-3, protein phosphatase 1D (PPM1D), GADD45 and some Bcl-2 family members by p53, immediate early gene products such as FOS by ELK1, GADD45, dual-specificity phosphatases (DUSPs), cyclin D and JUN by activating transcription factor 2 (ATF2), interleukin-6 (IL-6) and cyclooxygenase 2 (COX-2) by C/EBP, and DUSP1 and IL-10 by cAMP-responsive element binding protein (CREB) (98). Akt recognizes and phosphorylates the consensus sequence RXRXX(S/T) when surrounded by hydrophobic residues. As this sequence is present in many proteins, numerous Akt substrates have been identified and validated. These substrates control

key cellular processes such as apoptosis, cell cycle progression, transcription, and translation. For instance, Akt phosphorylates the FoxO subfamily of forkhead family transcription factors, which inhibits transcription of several proapoptotic genes, e.g. Fas-L, IGFBP1 and Bim. Additionally, Akt can directly regulate apoptosis by phosphorylating and inactivating proapoptotic proteins such as Bad, which controls release of cytochrome c from mitochondria, and apoptosis signal-regulating kinase-1, a mitogen-activated protein kinase kinase involved in stress-induced and cytokine-induced cell death. In contrast, Akt can phosphorylate IkB kinase, which indirectly increases the activity of nuclear factor kB and stimulates the transcription of prosurvival genes. Cell cycle progression can also be affected by Akt through its inhibitory phosphorylation of the cyclin-dependent kinase inhibitors, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, and inhibition of GSK-3ß by Akt stimulates cell cycle progression by stabilizing cyclin D1 expression (99). We showed that influx of calcium via TRPV6 phosphorylates not only GSK3ß, JNK and Erk in MCF-7 but also p38 in PC-3 cells. MAPKinase and GSK3ß are well-known pathway for the association between cell proliferation and calcium. Collectively, We confirmed that aberrant expression of TRPV6 in breast and prostate cancer interrupts calcium homeostasis and activates cell proliferation via MAPKinase and GSK3ß signaling in cells.

In summary, we identified Numb1 as a novel regulator of TRPV6, and the results presented in this study Numb1 increase our understanding of the biological function of calcium signaling in cancer cells and development.

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국문 초록

서론: 칼슘은 세포신호전달에 중요한 요소이며 다양한 질병에서 항상성이 변화되어 있다. 세포내 칼슘은 다양한 조절인자에 의해 항상성을 유지하고 있다. 여러 조절인자중에서 Transient Receptor Potential Vanilloid 6 (TRPV6)는 칼슘을 선택적으로 투과시키는 이온채널로서 소장의 칼슘 흡수에 중요한 역할을 한다. TRPV6는 여러암에서 과발현된다고 보고되고 있으며 발암유전자로서의 가능성이 대두되고 있지만 정확한 기전은 아직 연구되어 있지 않다. Numb 은 세포운명 결정인자로서 p53 을 안정화시키는 암억제유전자로 알려져 있다.

방법: TRPV6 의 활성을 측정하기 위한 전기생리학적인 칼슘유입분석법을 사용하였으며 단백질 상호작용을 연구하기 위하여 상호면역침전방법과 FRET 분석법을 사용하였으며 MTT 시약을 이용하여 세포성장을 측정하였다.

결과: HEK293 세포주에서 TRPV6 와 Numb 을 과발현시켰을때 TRPV6 의 활성이 저해되는것을 관찰하였고 TRPV6 가 과발현된 HEK293 세포주에서 Numb 의 발현을 siRNA 로 줄였을때 TRPV6 의 활성이 증가하는것을 확인하였다. 상호면역침전과 FRET 분석을 통해 두 단백질이 상호작용하는것을 확인하였다. 두

단백질의 결합부위를 찾기 위하여 각각의 부위가 없는 돌연변이를 제작하였으며 이 돌연변이를 이용하여 상호면역침전실험을 수행한 결과 TRPV6 의 716 번째 아미노산인 Aspartic acid 와 Numb 의 434 번째 아미노산인 Arginine 이 결합에 핵심적인 부위인것을 확인하였고 두 단백질사이의 결합이 활성조절에 중요한것을 관찰하였다. 유방암 세포주인 MCF-7 에서 Numb 의 발현이 TRPV6 의 활성을 조절하여 세포내 칼슘유입을 조절하며 세포성장에 영향을 미치는 결과를 확인하였다. 전립선암세포주(PC-3)와 MCF-7 세포주에서 TRPV6 에 의한 칼슘유입이 MAPKinase, GSK3ß 신호전달과정을 활성화시켜 세포성장을 조절하는 것을 Western blotting 실험으로 확인하였다.

결론: 실험결과를 통해 Numb 이 TRPV6 와 상호작용하여 활성을 조절하는 새로운 인자인것을 밝혔으며 암세포에서 세포내 칼슘과 세포성장을 조절하는것을 확인하였다. 더욱이 칼슘유입에 의한 암세포내 세포성장과 관련된 신호전달과정을 확인함으로써 칼슘채널이 암 치료의 대상이 될 수 있는 증거를 제시하였다.

* 본 내용은 미국 세포칼슘 학회지 (Cell Calcium, 2013 Feb;53(2):102-11) 에 출판 완료된 내용임

주요어: 칼슘, TRPV6, Numb, 암
학번: 2008-21992