A mechanism of action by which TRPV6 promotes epithelial cancers

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The Transient Receptor Potential Vanilloid (6) calcium channel

The transient receptor potential (TRP) channels comprise a large superfamily of cation channels that constitute important cation influx machinery in most vertebrate and invertebrate cell types. They are directly involved in thermo-, mechano-, chemo-, and nociception, responding to a wide variety of different physical and chemical stimuli (Vriens *et al*., 2009). TRP channels are tetrameric, intrinsic membrane proteins with six putative transmembrane (TM) spans and a cation-permeable pore region formed by a short hydrophobic stretch between transmembrane spans 5 and 6. TRPV6, a member of one of seven subfamilies of TRP channels, plays an important role in Ca^{2+} absorption/reabsorption (Vriens *et al*., 2009), and a functional role in cell proliferation and cell survival (Zhuang *et al.*, 2002). Thus, this channel may play a role in rapid tissue renewal.

TRPV6 is not a voltage-gated or receptor-operated channel such as would be found in excitable tissues. Instead TRPV6 (and the closely related TRPV5) acts as the first step of the transcellular pathway which is involved in many processes such as Ca^{2+} absorption in the intestine and re-absorption in the kidney (Peng *et al*., 2011). The TRPV6 channel appears to be constitutively active with its activity tightly regulated by intracellular Ca^{2+} through effects on intrinsic channel structure and interaction with Ca^{2+}-binding proteins (Niemeyer *et al.,* 2001; Peng *et al*., 2011). TRPV6 is localized throughout the gastrointestinal (GI) tract from esophagus to colon, and also present in the distal tubules of the kidney (Zhuang *et al.*, 2002; Nijenhuis et *al.*, 2003). TRPV6 is also expressed in the placenta where it appears to play a role in maternal-fetal Ca^{2+} transport, in the uterus, with a potential role in establishing and maintaining pregnancy and in exocrine organs such as the pancreas, prostate, mammary, salivary and sweat glands (Peng *et al*., 2011). Expression of TRPV6 has also been demonstrated in retina pigment epithelium where it may regulate the Ca^{2+} level in light/dark transitions, and in the inner ear where it may play a role in maintaining the low Ca^{2+} level required for hearing and balance (Peng *et al*., 2011). Additional studies have shown that TRPV6 is dynamically expressed in bone cells, human and murine osteoblast-like cells and osteoblasts (van der Eerden *et al.,* 2012). Studies in mice however, suggest that TRV6, although expressed, is

not crucial for bone mineralization (van der Eerden *et al.,* 2012). A recent study has also shown TRPV6 to be expressed in the epididymal epithelia of the mouse where it mediates functionality of sperm (Weissgerber *et al.,* 2011). TRPV6 has not been detected in the heart (Hoenderop *et al.*, 2001; Hirnet *et al*., 2003) or vasculature (Earley, 2010; Wong and Yao, 2011).

Tissue expression of TRPV6

TRPV6 is up regulated in tissue samples originating from ovary, breast, prostate, thyroid, colon, and pancreatic tumors (Zhuang et al., 2002). In normal tissue the channel is primarily localized to the apical membrane of epithelia (e.g. GI, salivary gland, kidney) where it delivers calcium into the cells.

In mammary adenocarcinoma tissue, immunohistologic analysis showed a clear enhancement in TRPV6 expression over normal tissue, suggesting that it may play some role in tumor development (Zhuang et al., 2002). Bolanz et al. (2008) report that TRPV6 mediates Ca^{2+} uptake in the breast cancer cell line T 47D, with downstream activation of the nuclear factor of activated T-cell transcription factor (NFAT).

TRPV6 is strongly expressed in advanced stages of prostate cancer, whereas there is little to no expression evident in healthy tissue and in benign prostate hyperplasia (Peng et al., 2001, Zhuang et al., 2002; Fixemer et al., 2003). The transcript levels of TRPV6 correlated positively with tumor progression and aggressiveness as indicated by the pathologic stage and Gleason scores of the prostate tumors (Peng et al., 1999; Wissenbach et al., 2001). Analysis of 40 tissue samples showed that TRPV6 transcripts occur in more than 90% of patients with extra-prostatic adenocarcinoma, indicating that patients with TRPV6 positive tumors have a poor prognosis (Fixemer et al., 2003). Studies by Lehn'kyi et al., (2007) show that Ca^{2+} uptake into lymph node carcinoma of the prostate (LNCaP) cells is mediated by TRPV6, with the subsequent downstream activation of NFAT.

TRPV6-mediated Ca^{2+} entry was also shown to be involved in apoptosis resistance of LNCaP cells.

Data from studies performed by Soricimed have indicated the presence of highly elevated TRPV6 [messenger ribonucleic acid (mRNA) and/or protein] in all of 17 ovarian cancer tumor biopsies tested (Study No. SBI O 2011 003). Additionally Soricimed has shown elevated TRPV6 mRNA and protein in ovarian cancer cell lines, as well as cell lines from cancers of breast and prostate, and glioblastoma (Study Nos. SBI O 2010 020; SBI O 2010 026)

A recent publication has shed light on greatly elevated production of the TRPV6 channel in breast cancers and links this phenomenon to a genetic genesis: gene amplification has been reported in a series of breast cancer cell lines and in primary tumours (Peters et al., 2012). While healthy tissues have a single copy of *trpv6* the greater the copy number of *trpv6* gene in breast cancer cell lines the more aggressive was the cell line. Breast cancer patients with the greater amplification of the gene, and corresponding elevation of TRPV6 ion channel, had decreased survival compared to patients with low expression of TRPV6. TRPV6 knockdown experiments, which would significantly decrease the calcium flux into the cell, reduced cell proliferation and led to an increase in cells in the G1 phase.

Potential role of TRPV6 on proliferative and apoptotic pathway

Based on existing data, the following mechanism has been proposed by which TRPV6 may influence proliferative and anti-apoptotic pathways (See Figure 1). TRPV6 exists in two forms: a fully glycosylated ion channel (TRPV6-G) and an ion channel with modified oligosaccharides (TRPV6). Deglycosylation of TRPV6-G (i.e., removal of sialic acid residues of the oligosaccharide) increases its residence time in the membrane (Renkema et al., 2008) as well as increasing the activity of the channel (Chang et al., 2005, Lu P et al. 2008a). Klotho, a β-glucuronidase linked to ageing, is believed to play a part in the de-glycosylation of TRPV6 and has been

linked to ovarian cancer progression (Lu et al., 2008a; Lu et al., 2008b). Calcium entering through the TRPV6 binds to the calcium binding protein calmodulin (CaM). This complex activates calcineurin: a CaM/Ca^{2+} activated phosphatase, that de-phosphorylates Nuclear Factor of Activated T-cells (NFAT), a transcription factor, thereby activating it (Crabtree et al., 2002). Active NFAT moves to the nucleus (Masuda et al., 1998) where, with Jun/Fos, it activates a number of intracellular agents including: (i) Membrane Type 1 Matrix Metalloproteinase (MT1-MMP), (ii) Matrix Metalloproteinase-type 2 (MMP-2; Saygili et al. 2009) which is produced and excreted as pro-MMP-2 and cleaved extracellularly by MT1-MMP), (iii) Autotaxin (ATX; Mancini and Toker, 2009) an extracellular phospholipase that activates Growth Factor Receptor (GFR) through its product lysophosphatidyl choline (LPC), (iv) Bcl-2 and BclX anti-apoptotic proteins which inhibit the release of cytochrome c from mitochondria and prevention of apoptosome formation (Gómez et al., 1998), (v) Ca(PO4)x: calcium phosphate mineralization, microcrystals of hydroxapatite which up-regulate production of pro-MMP-2 (Morgan et al., 2001), and (vi) Fibroblast Growth Factor 23 (FGF-23) which, along with soluble Klotho, activates FGF-23 receptors which, in turn, can phosphorylate GSK-3B deactivating it (GSK-3B is a glycogen synthase kinase that re-phosphorylates NFAT, deactivating it and returning it from the nucleus to the cytoplasm (Medici et al., 2008)). Soricimed has demonstrated that there is a shift to the de-glycosylated form of the channel in ovarian, breast, prostate, pancreatic and colon cancer, as well as in glioblastoma cell lines (Study Nos. SBI O 2010 020; SBI O 2010 026). In addition, this form of the channel is the predominant form in tumor xenografts derived from human ovarian tumor biopsies (Study No. SBI O 2011 003). Extensive deglycosylation of TRPV6 was also observed in a number of cancer cell lines (Study No. SBI O 2010 051) and ovarian tumor xenografts (Study No. BPNBI O 2010 012_013). Also, data generated by Soricimed indicate that NFATc2 is present in ovarian and breast cancer cell lines with a shift in the majority of the transcription factor to the activated, dephosphorylated form (Study Nos. SBI O 2010 052; SBI O 2011 021; SBI O 2011 023). Additionally, in an on-going study, Soricimed has identified other isoforms of

the NFAT transcription factor (c1, c3 and c4) in breast, prostate and ovarian cancers (Study No. SBI O 2012 004).

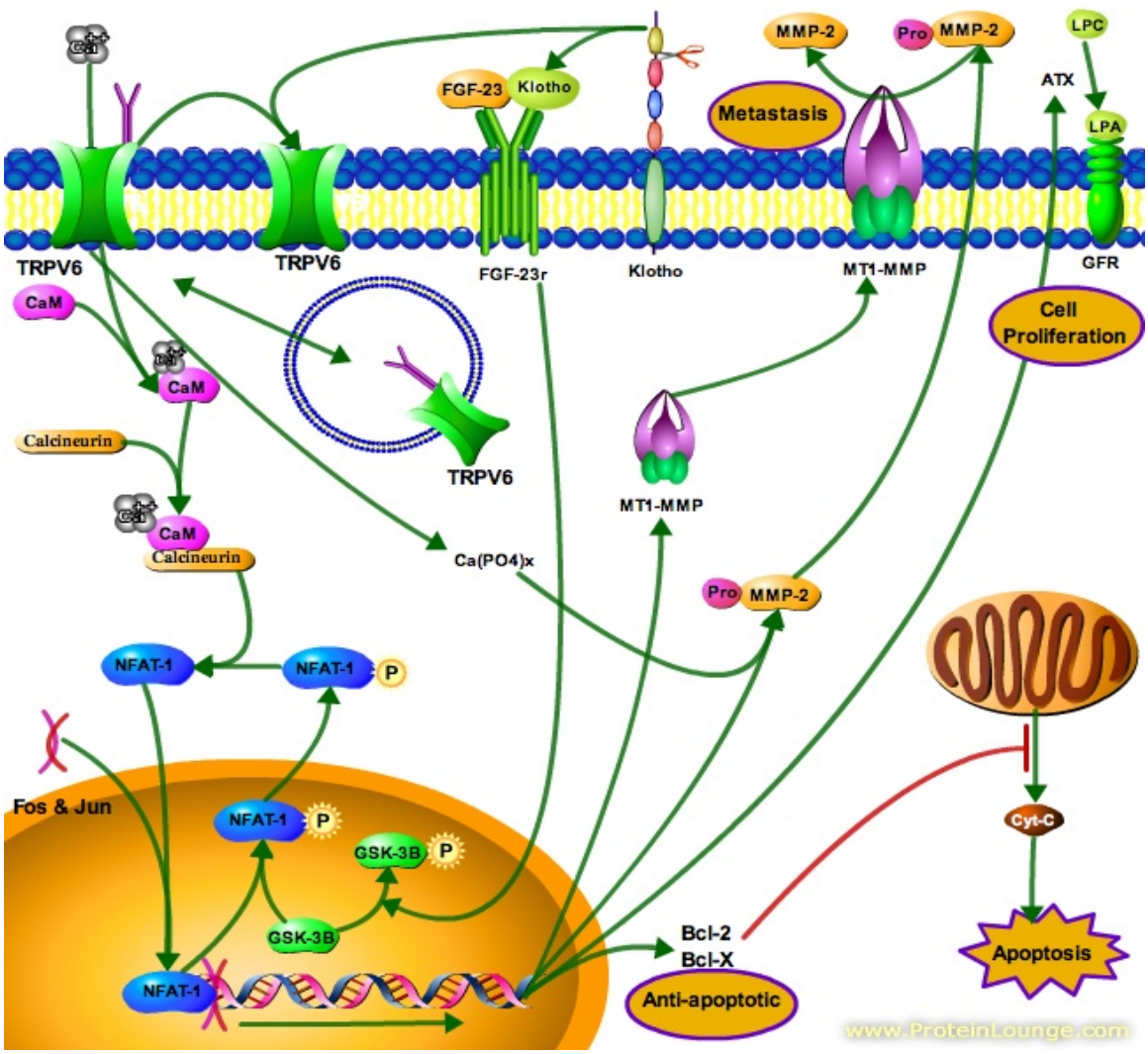

Figure 1: Diagramatic representation of the role of TRPV6 in cell proliferation and inhibition of apoptosis. Legend: TRPV6 [the 6th member of the transient receptor potential cation channel, vanilloid family], CaM [calmodulin], FGF-23 [Fibroblast Growth Factor 23], MMP-2 [Matrix Metalloproteinase-type 2], ATX [autotaxin], LPC [lysophosphatidylcholine], LPA [lysophosphatidic acid], Ca(PO_4) [calcium phosphate], NFAT [nuclear factor of activated T-cell transcription factor], GSK-3B [a glycogen synthase kinase that re-phosphorylates NFAT, deactivating it and returning it from the nucleus to the cytoplasm], Fos [DNA binding domain], Jun [DNA binding domain]

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