TRPV6 Channels

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Contents

Abstract

TRPV6 (former synonyms ECAC2, CaT1, CaT-like) displays several specific features which makes it unique among the members of the mammalian *Trp* gene family (1) TRPV6 (and its closest relative, TRPV5) are the only highly Ca^{2+}-selective channels of the entire TRP superfamily (Peng et al. 1999; Wissenbach et al. 2001; Voets et al. 2004). (2) Translation of *Trpv6* initiates at a non-AUG codon, at ACG, located upstream of the annotated AUG, which is not used for initiation (Fecher-Trost et al. 2013). The ACG codon is nevertheless decoded by

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methionine. Not only a very rare event in eukaryotic biology, the full-length TRPV6 protein existing *in vivo* comprises an amino terminus extended by 40 amino acid residues compared to the annotated truncated TRPV6 protein which has been used in most studies on TRPV6 channel activity so far. (In the following numbering occurs according to this full-length protein, with the numbers of the so far annotated truncated protein in brackets). (3) Only in humans a coupled polymorphism of *Trpv6* exists causing three amino acid exchanges and resulting in an ancestral *Trpv6* haplotype and a so-called derived *Trpv6* haplotype (Wissenbach et al. 2001). The ancestral allele encodes the amino acid residues $C_{197(157)}$, $M_{418(378)}$ and $M_{721(681)}$ and the derived alleles $R_{197(157)}$, $V_{418(378)}$ and $T_{721(681)}$. The ancestral haplotype is found in all species, the derived *Trpv6* haplotype has only been identified in humans, and its frequency increases with the distance to the African continent. Apparently the *Trpv6* gene has been a strong target for selection in humans, and its derived variant is one of the few examples showing consistently differences to the orthologues genes of other primates (Akey et al. 2004, 2006; Stajich and Hahn 2005; Hughes et al. 2008). (4) The *Trpv6* gene expression is significantly upregulated in several human malignancies including the most common cancers, prostate and breast cancer (Wissenbach et al. 2001; Zhuang et al. 2002; Fixemer et al. 2003; Bolanz et al. 2008). (5) Male mice lacking functional TRPV6 channels are hypo-/infertile making TRPV6 one of the very few channels essential for male fertility (Weissgerber et al. 2011, 2012).

Keywords
Calcium selective channel • Non AUG translation start • Prostate cancer • Breast cancer • Male fertility • Polymorphism • Epithelial calcium transport

1 Gene

The *Trpv6* gene is located on chromosome 7q33-q34 (human), chromosome 6 (mouse) and chromosome 4 (rat) in close proximity to its closest relative, *Trpv5* (7q35 in human). The deduced protein sequences comprise ~75 % identical amino acids (Peng et al. 1999, 2000; Hoenderop et al. 1999; Muller et al. 2000; Wissenbach et al. 2001; Hirnet et al. 2003). *Trpv5* and *Trpv6* arose by gene duplication from an ancestral gene, and the pufferfish *Takifugu rubripes*, for example, has only one gene which is slightly more similar to *Trpv6* than to *Trpv5* (Qiu and Hogstrand 2004; Peng 2011). The *TRPV5* gene is mainly expressed in the kidney of mammals, whereas *Trpv6* has a broader expression pattern. Therefore, it was speculated that gene duplication reflects the complex renal situation of land-living animals (Peng 2011). The *Trpv5-6*-like genes can be identified in primitive eukaryotic organisms like the choanoflagellate *Monosiga brevicollis* (King et al. 2008). In general *Trpv5-6*-like genes are not present in prokaryotic organisms,

protocysts, fungi and plants. However, the green algae *Chlamydomonas reinhardtii* and *Volvox carteri* exhibit *Trpv5-6*-like genes, and this may reflect horizontal gene transfer at a comparatively late time point during the evolution of these algae (Merchant et al. 2007). The chromosomal organization of *Trpv6* is conserved among several species. In the mouse genome *Trpv6* spans 15 exons and extends over a region of ~15.7 kb. Depending on the species, the deduced amino acid sequence is in the range of 703–767 amino acids.

1.1 Splice Variants and Polymorphisms

To date there are no splice variants known for human and mouse *Trpv6*. But in humans two alleles of the *Trpv6* gene were identified (Wissenbach et al. 2001): one ancestral variant (*Trpv6a*, red in Fig. 1a, b) and a so-called derived variant (*Trpv6b*, yellow in Fig. 1a, b). The cDNA sequences of the two alleles differ in five bases resulting in three amino acid substitutions with $R_{197(157)}$, $V_{418(378)}$ and $T_{721(681)}$ in the derived variant and $C_{197(157)}$, $M_{418(378)}$ and $M_{721(681)}$ in the ancestral variant. Additional polymorphisms found within the intronic regions of the *Trpv6* gene seem to be coupled to those polymorphisms in the coding region (Kessler et al. 2009). The frequencies of the two *Trpv6* alleles are highly variable between different ethnic groups. The percentage of the ancestral *Trpv6a* allele decreases with increasing distance to the African continent (Akey et al. 2006). In a few South African populations, the *Trpv6a* allele frequency is higher than 50 % of the tested alleles and decreases to less than 5 % in East Asian populations. From these data, it was assumed that a so far unknown selection pressure leads to the higher allele frequency of the *Trpv6b* variant in non-African humans (Akey et al. 2004; Stajich and Hahn 2005; Soejima et al. 2009). The physiological consequence of the polymorphisms is not clear yet. After heterologous expression of the *Trpv6a* and *Trpv6b* cDNAs, a non-significant faster Ca^{2+}-dependent inactivation of TRPV6b channels was noted (Hughes et al. 2008), but in general both channel variants revealed very similar biophysical properties (ion selectivity, reversal potential, Ca^{2+}-dependent inactivation, Mg^{2+} block). After *Trpv6* cRNA injection into *Xenopus laevis* oocytes, current amplitudes (measured as amplitudes of endogenous Ca^{2+}-activated Cl^- currents) (Sudo et al. 2010) or $^{45}Ca^{2+}$ uptake (Suzuki et al. 2008b) were larger for TRPV6a than for TRPV6b. Unfortunately, the latter publication lacks adequate controls. Interestingly, Akey et al. (2004) speculated that strong selection on the *Trpv6* locus is correlated to milk consumption and lactase persistence.

1.2 Initiation of Translation from a Non-AUG Codon

Recently our group showed (Fecher-Trost et al. 2013) that the full-length endogenous human TRPV6 protein is 40 amino acid residues longer at the N terminus as previously thought (Fig. 1c). Translation starts at an ACG codon upstream of the

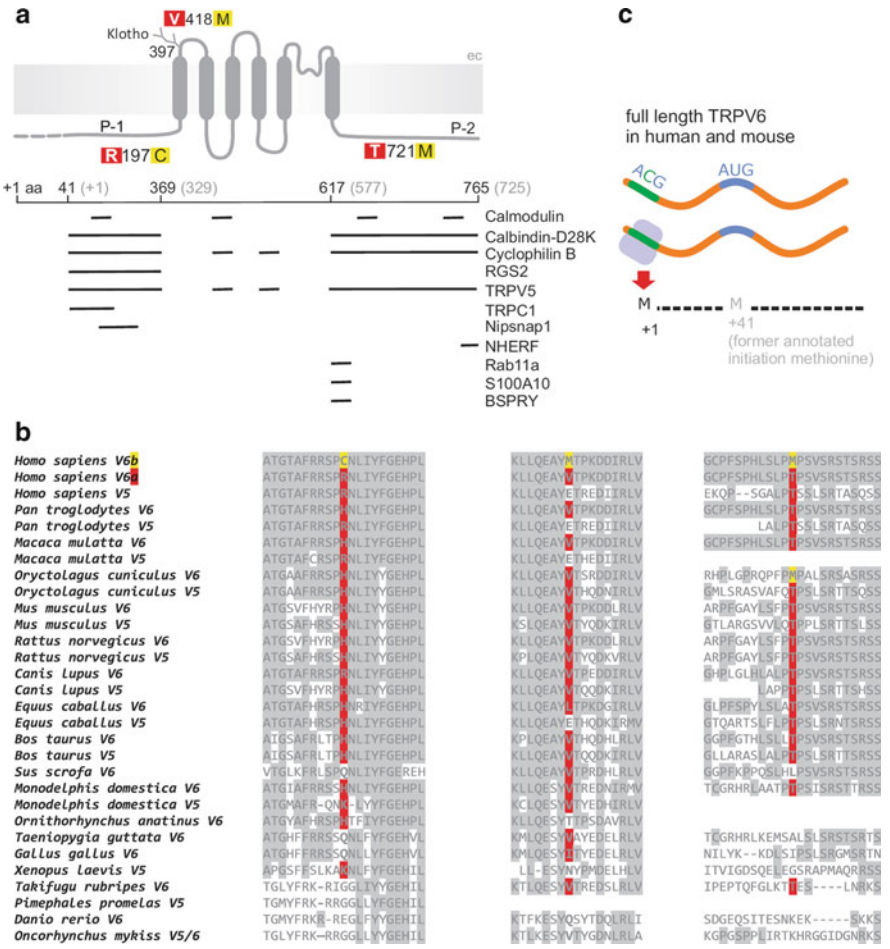

Fig. 1 (**a**) Structure of the TRPV6 channel non-synonymous polymorphisms are indicated: ancestral TRPV6a (*red*) and the derived variant TRPV6b (*yellow*). P-1, phosphorylation sites for *src* kinase (P-1, tyrosine$_{161}$), for PTP1B phosphatase (P-1) and for protein kinase C (P-2, threonine$_{742}$). Below numbering of the amino acids of the full-length TRPV6 (*black*) and the truncated annotated TRPV6 (*grey*). Protein interaction sites are indicated (see also Table 1 and text). (**b**) Multiple alignment of the non-synonymous polymorphism shown in (**a**), among several species (V6) aligned with the corresponding amino acid sequences of TRPV5 (V5). The derived TRPV6b variant (*yellow*) is only present in humans. The amino acid residues in TRPV5 proteins correspond to the amino acids present in TRPV6a. (**c**) Translation initiation of the full-length TRPV6 protein occurs at an ACG which is decoded by methionine and which is localized upstream of the annotated AUG codon

first AUG of the *Trpv6* mRNA. Although ACG codons are normally translated into threonine (T), the translational machinery incorporates methionine (M) instead. Translation of a non-AUG codon into methionine is a rare event, and to our knowledge, only the testis-specific PRPS3 protein (ribose-phosphate pyrophosphokinase 3, NM_175886.2) is also initiated from an ACG codon which

is apparently decoded by methionine (Taira et al. 1990). Alignments of the annotated 5′-untranslated *Trpv6* sequences indicate that in mammals but not in non-mammals, the TRPV6 protein appears to be translated from an ACG codon upstream of the annotated AUG. Accordingly, the murine TRPV6 protein also comprises a longer N terminus, and the initiation triplet is most likely the corresponding ACG codon (Fecher-Trost et al. 2013). Furthermore, analysis of 5′ regions of other *Trpv* genes indicates that translation initiation from an upstream non-AUG codon is an exclusive feature of *Trpv6* which is not met by any other *Trpv* including *Trpv5*.

2 Expression

The murine *Trpv6* is expressed in placenta, pancreas, prostate, epididymis and several parts of the small intestine including duodenum, oesophagus, stomach, colon, kidney and uterus as demonstrated by RT-PCR and Northern blots (Hirnet et al. 2003; Weissgerber et al. 2011, 2012; Lehen'kyi et al. 2012). Compared to mouse there are some differences of the *Trpv6* expression in humans. First, expression of *Trpv6* within small intestine (including duodenum) has not consistently been shown, and second, whereas *Trpv6* is highly expressed in the murine prostate, it is much less expressed in the human prostate; however, it is clearly overexpressed in prostate cancer as shown by Northern blots analysis and in situ hybridization (Wissenbach et al. 2001, 2004; Peng et al. 2001; Fixemer et al. 2003). *Trpv6* appears to be expressed in various cancer cell lines, but direct identification of the TRPV6 protein by mass spectrometry has only been shown in the human breast cancer cell line T47D (Fecher-Trost et al. 2013) and in the human lymph node prostate cancer cell line LNCaP (Fecher-Trost, Wissenbach, and Flockerzi, unpublished data).

In murine kidney only low levels of mRNA were detected by Northern blots and RT-PCR (Hirnet et al. 2003; Song et al. 2003; Peng 2011), but in immunostains the TRPV6 protein was identified in the apical domain of the distal convoluted tubules, in connecting tubules and cortical and medullary collecting ducts (Nijenhuis et al. 2003; Hoenderop et al. 2003a). In rat and in human kidney, *Trpv6* transcripts are not detectable by Northern blots (Peng et al. 1999; Wissenbach et al. 2001; Brown et al. 2005), but fragments of *Trpv6* transcripts could be weakly amplified in human kidney by RT-PCR (Peng et al. 2000; Hoenderop et al. 2001). Apparently, different age, variability of Ca^{2+} and vitamin D within the food and the hormonal state may confound detection of *Trpv6* expression in kidney (Lee et al. 2004; Hoenderop et al. 2005) and also in duodenum. In summary, data from Northern blots consistently confirm *Trpv6* expression in rat small intestine, in human and mouse placenta, pancreas and to a lower extent in the intestine, in human prostate cancer, in mouse placenta and in mouse epididymis. The *Trpv6* cDNAs were originally cloned from rat intestine (Peng et al. 1999) and human placenta (Wissenbach et al. 2001). Using antibodies which discriminate between corresponding tissues from wild-type and *Trpv6*-deficient mice, TRPV6 proteins have been identified in human placenta (Stumpf et al. 2008; Fecher-Trost

et al. 2013), mouse prostate and epididymis (Weissgerber et al. 2011, 2012); by mass spectrometry TRPV6 proteins were directly identified in placenta and the human cancer cell lines T47D (Fecher-Trost et al. 2013) and LNCaP cells.

2.1 Transcriptional Regulation of *Trpv6* by Vitamin D_3

Intestinal Ca^{2+} absorption (Bronner et al. 1986a, b) depends on two major pathways, a paracellular pathway and a transcellular pathway which can be stimulated by vitamin D_3 (Bronner and Pansu 1999; Bronner 2003; Kellett 2011; Lieben and Carmeliet 2012). The transcellular pathway includes a Ca^{2+} uptake channel at the luminal/apical membrane, soluble cytosolic Ca^{2+}-binding proteins and a Ca^{2+} extrusion mechanism at the basal membrane (Hoenderop et al. 2005; Suzuki et al. 2008c); a similar pathway appears to be relevant in placenta (Brunette 1988; Lafond et al. 1991; Belkacemi et al. 2002, 2003, 2004). Initial findings revealed that the amounts of *Trpv6* transcripts in the rat duodenum detected by Northern blots were not changed by prior treatment of the rats by 1,25-dihydroxyvitamin D_3 or by feeding them a Ca^{2+}-deficient diet (Peng et al. 1999), arguing that *Trpv6* expression is not regulated by vitamin D_3 or calcium deficiency in duodenum. In contrast, treatment of Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line, by 1,25-dihydroxyvitamin D_3 upregulates *Trpv6* expression (Wood et al. 2001). In addition, *Trpv6* transcript levels were decreased in vitamin D receptor-deficient mice (Okano et al. 2004), whereas 1,25-dihydroxyvitamin D_3 supplementation of *25-hydroxyvitamin D_3-1α-hydroxylase*-deficient mice, which have undetectable levels of endogenous 1,25-dihydroxyvitamin D_3 and suffer from hypocalcaemia, resulted in increased expression of TRPV6, calbindin-D9K and PMCA1b and normalization of serum Ca^{2+} (van Abel et al. 2003). Binding sites for 1,25-dihydroxyvitamin D_3 and enhancer regions within the *Trpv6* promoter have been identified and characterized in detail (Pike et al. 2007), and it was concluded that transcellular Ca^{2+} uptake involves TRPV6 channels at the luminal intestinal side as primary Ca^{2+} uptake mechanism. Intracellular Ca^{2+} is then bound to calbindin 9K and transported across the cell to the basolateral side where it is extruded through the plasma membrane Ca^{2+}-ATPase (PMCA1b) and the Na^+/Ca^{2+} exchanger (van Abel et al. 2003). However, this model had to be reconsidered by the finding that duodenal Ca^{2+} uptake is normal in *Calbindin 9K*-deficient mice (Lee et al. 2007). In *Calbindin 9K*-/*Calbindin 28K*-double knockout mice, serum Ca^{2+} levels and bone length were decreased only under a low Ca^{2+} diet (Ko et al. 2009). In the *Trpv6*-deficient (Bianco et al. 2007; Kutuzova et al. 2008) and in the $Trpv6^{D/A/D/A}$ knock-in mouse lines (Weissgerber et al. 2011; Woudenberg-Vrenken et al. 2012), respectively, no functional TRPV6 channels are expressed; intestinal Ca^{2+} uptake was not affected in both lines (Kutuzova et al. 2008; Woudenberg-Vrenken et al. 2012) at a normal Ca^{2+} diet; at a low Ca^{2+} diet, intestinal Ca^{2+} uptake was slightly decreased in the $Trpv6^{D/A/D/A}$ knock-in mice. The finding that 1,25-dihydroxyvitamin D_3 administration increases intestinal Ca^{2+} uptake in *Trpv6*-deficient mice and in

Trpv6/Calbindin 9K-deficient double knockout mice put into question that TRPV6 and calbindin 9K are essential for vitamin D-induced active intestinal calcium transport (Benn et al. 2008). So in summary, it appears that *Trpv6* expression is regulated by vitamin D_3, but the TRPV6 channel is not the essential component of intestinal Ca^{2+} uptake; instead it might rather be involved in mechanisms preventing the loss of Ca^{2+} already present in the organism by reabsorbing Ca^{2+} (see also TRPV6 functions in mouse epididymis and prostate below).

2.2 Transcriptional Regulation of *Trpv6* by Steroid Hormones

Duodenal *Trpv6* (and *Trpv5*) expression was shown to be upregulated in ovariectomized rats after 17β-estradiol supplementation (van Abel et al. 2003), and in *oestrogen receptor*-deficient mice, duodenal *Trpv6* transcripts were reduced (Van Cromphaut et al. 2003). Decreased *Trpv6* transcript levels were also seen in an *aromatase*-deficient mouse line (Oz et al. 2007), a model of oestrogen deficiency. Ca^{2+} uptake of *Xenopus laevis* oocytes injected with the *Trpv6* cRNA (Bolanz et al. 2008) was reduced by the selective oestrogen receptor modulator tamoxifen. Similarly, basal cytosolic Ca^{2+} levels which were higher in the human breast cancer cell line MCF-7 which has been transfected with a *Trpv6* cDNA in comparison with non-transfected cells were also reduced in the presence of tamoxifen (Bolanz et al. 2009). Interestingly, this effect could be also demonstrated in the oestrogen receptor-negative MDA-MB-231 cells (Bolanz et al. 2009). Apparently, *Trpv6* expression may be regulated independently by oestrogen receptors and by tamoxifen. In the human breast cancer cell line T47D, the only breast cancer cell line in which the TRPV6 protein has been directly identified so far (Fecher-Trost et al. 2013), *Trpv6* expression appears to be increased in the presence of estradiol, progesterone and 1,25-dihydroxyvitamin D_3 and to be reduced in the presence of tamoxifen (Bolanz et al. 2008).

Duodenal Ca^{2+} uptake in mice was decreased after prednisolone treatment (Huybers et al. 2007) which resulted in reduced *Calbindin 9K* and *Trpv6* transcript expression in duodenum. Accordingly, it was concluded that reduced bone mineral density as unwanted effect from glucocorticoid treatment could result from downregulation of *Trpv6*. However, 5 days of dexamethasone administration affected bone metabolism, but expression levels of *Trpv6*, *calbindin 9K* and *PMCA1b* were not significantly different from tissues of untreated animals (Van Cromphaut et al. 2007). In contrast, dexamethasone-dependent downregulation of *Trpv6* was reported when five times higher dexamethasone doses had been applied (Kim et al. 2009a, b). Little or no expression of *Trpv6* was found in a variety of human and murine osteoblastic cells using qPCR (Little et al. 2011), indicating that TRPV6 plays no pivotal role in bone mineralization. These data are fully consistent with the data obtained from the $Trpv6^{D/A/D/A}$ mouse line (Weissgerber et al. 2011), which lacks functional TRPV6 channels, and did not show alterations of bone metabolism and bone matrix mineralization (van der Eerden et al. 2012).

In LNCaP, expression of *Trpv6* was shown to be upregulated in the presence of an androgen receptor antagonist whereas dihydrotestosterone reduced expression (Peng et al. 2001), but treatment of these cells did not affect Ca^{2+} entry (Bodding et al. 2003). Additional conditions have been reported to enhance *Trpv6* transcript expression including hypoxic conditions in human placenta (Yang et al. 2013) and developmental upregulation in intestine of neonatal mice at weaning (Song et al. 2003) which appeared to depend on vitamin D_3 and during pregnancy (Lee and Jeung 2007) but still occurs in *vitamin D receptor*-deficient mice (Van Cromphaut et al. 2003).

2.3 Transcriptional Regulation of *Trpv6* by Other Hormones

Intestinal Ca^{2+} absorption has been shown to be upregulated during pregnancy and lactation independent of vitamin D_3 (Boass et al. 1981; Brommage et al. 1990; Halloran and DeLuca 1980) or of the vitamin D receptor (Van Cromphaut et al. 2003). In accordance with these observations, prolactin had been shown to enhance intestinal Ca^{2+} absorption in vitamin D_3-deficient rats, to directly stimulate the transcellular Ca^{2+} transport in duodenal preparations (Charoenphandhu et al. 2001; Pahuja and DeLuca 1981) and to regulate vitamin D metabolism and induction of *Trpv6* mRNA expression (Ajibade et al. 2010).

During pregnancy, an active Ca^{2+} transport through the placenta (Sibley and Boyd 1988) was found to be regulated by the parathyroid hormone-related protein PTHrP (Tobias and Cooper 2004). In *PTHrP*-deficient mice, significant differences in placental Ca^{2+} transport compared to wild-type mice were observed, but the transcript levels of *Trpv6* and plasma membrane Ca^{2+} ATPase (*Pmca*) 1 and 4 were unaltered (Bond et al. 2008; Karaplis et al. 1994) arguing against transcriptional regulation of *Trpv6* and *Pmca* by PTHrP.

3 The Channel Protein Including Structural Aspects

The endogenous human full-length TRPV6 protein consists of 765 amino acids (accession number KF534785; Fecher-Trost et al. 2013) compared to the 725-aa annotated TRPV6 protein, which is truncated. The additional 40-aa sequence shows no similarity to any known protein sequence; nine out of the 40 amino acid residues are proline residues, and they might well constitute motifs involved in protein–protein interaction mediated by SH3 domains. The N terminus contains a series of 6 repeats with similarity to domains found in ankyrin repeat proteins (Wissenbach et al. 2001). Erler et al. (2004) identified ankyrin repeat 3 and 5 as critical components involved in the assembly of functional channel complexes. The crystal structure of the six ankyrin repeat domains (aa 124–345 or aa 84–305 in the truncated version) revealed conserved helical-turn-helix conformations, very similar to those present in TRPV1 and 2, but with a variable long-loop region between ankyrin repeat 3 and 4 (Phelps et al. 2008). Various short intracellular C-terminal

fragments of TRPV6 (and TRPV5) were used for circular dichroism (CD) and NMR spectroscopy in the presence and absence of calmodulin, and the results obtained suggest an association of calmodulin and the TRPV6 C terminus (Kovalevskaya et al. 2011, 2012).

TRPV6 proteins most likely co-assemble to homotetrameric channels. Heterotetrameric TRPV6/TRPV5 channels have been reported after co-expression of the respective cRNAs in oocytes (Hoenderop et al. 2003b), but the in vivo expression pattern of both genes does hardly overlap. An exception might be the kidney, where both genes are expressed, but *Trpv5* expression is much more abundant than *Trpv6* (van Abel et al. 2005). TRPV6 proteins are glycosylated at Asn 397 (357) which is located within the extracellular S1–S2 linker (Hirnet et al. 2003; Chang et al. 2005). The beta-glucuronidase klotho stimulates TRPV6 (and TRPV5) channel activity by sugar hydrolysis at the Asn 397 glycosylation site which is conserved in the TRPV5 protein (Chang et al. 2005). The higher expression of klotho within kidney tissue might be an indicator that in vivo the target for klotho-dependent regulation is TRPV5 (Kuro-o et al. 1997).

4 TRPV6-Interacting Proteins

Studies were performed using yeast two-hybrid screens, pull-down assays and antibody-based affinity purifications from primary tissues and HEK cells expressing the TRPV6 cDNA to identify proteins which are transiently or stably associated with TRPV6. A few proteins have been identified including calmodulin, klotho, S100A10-annexin 2, the PDZ domain-containing protein Na^+/H^+ exchanger regulatory factor 4 (NHERF4) and Rab11a which regulate the trafficking, plasma membrane anchoring and activity of the TRPV6 channel. The TRPV6-binding sites for these proteins have been mapped within extracellular and intracellular linkers of transmembrane regions and within the cytosolic C- and N-termini of TRPV6 (Fig. 1a, Table 1).

Calmodulin is known to regulate TRPV6 channel activity, and strong evidence exists for a functional high-affinity calmodulin association at the C terminus of human/mouse TRPV6 between aa 735 and 756 (Niemeyer et al. 2001; Hirnet et al. 2003; Derler at al. 2006; Cao et al 2013). The calcium-dependent calmodulin binding to the C terminus facilitates channel inactivation and is counteracted by protein kinase C-mediated phosphorylation within the calmodulin-binding site at threonine residue 742 (Niemeyer et al. 2001). Stumpf and co-workers used the high-affinity binding of TRPV6 for calmodulin to copurify TRPV6 and interacting proteins from human placenta with a calmodulin column (Stumpf et al. 2008).

The C-terminal high-affinity calmodulin-binding site is also sensitive for phosphatidylinositol 4,5-bisphosphate (PIP2) binding (Zakharian et al. 2011; Cao et al 2013), which interferes with the binding of calmodulin and inversely regulates TRPV6 activity, but presumably not through a direct competition mechanism. The latter study shows a complex interplay between calmodulin and PIP2 and

Table 1 TRPV6-interacting proteins

Protein	Putative function	References
Calmodulin	Channel inactivation/modulation	Niemeyer et al. (2001), Hirnet et al. (2003), Lambers et al. (2004), Derler et al. (2006), Stumpf et al. (2008), Kovalevskaya et al. (2012)
	Competitive PIP2/calmodulin regulation	Cao et al. (2013), Zakharian et al. (2011)
Klotho	Activation through increase of plasma membrane level	Chang et al. (2005)
Calbindin D28K	Binding	Lambers et al. (2006)
BSPRY	Binding	van de Graaf et al. (2006b, c)
PKC	Competitive regulation of calmodulin-dependent inactivation	Niemeyer et al. (2001)
PTBIP, SRC	Channel inhibition	Sternfeld et al. (2007)
Cyclophilin B	Channel activation	Stumpf et al. (2008)
NHERF	Protein transport to plasma membrane, activation	Kim et al. (2007)
Rab11a	Protein transport to plasma membrane, channel activation	van de Graaf et al. (2006a)
Nipsnap1	Channel inhibition	Schoeber et al. (2008)
RGS2	Channel inhibition	Schoeber et al. (2006)
S100A10	Increase in plasma membrane level, activation	Borthwick et al. (2008), van de Graaf et al. (2003)
TRPC1	Suppression of plasma membrane targeting, inhibition of TRPV6 activity	Schindl et al. (2012), Courjaret et al. (2013)
TRPV5	Heterooligomerization, binding	Hellwig et al. (2005), Hoenderop et al. (2003a), Semenova et al. (2009)

demonstrates that calcium, calmodulin and the depletion of PIP2 contribute to the inactivation of TRPV6 channels.

Three additional calmodulin-binding sites have been reported (Lambers et al. 2004), N- and C-terminal and within the intracellular S2–S3 linker (see Fig. 1), but their functions have not been confirmed (Derler at al. 2006; Cao et al 2013). In addition, the presumed N-terminal calmodulin-binding site forms a part of the ankyrin repeat core domain and is therefore unlikely to serve as calmodulin interaction site (Phelps et al. 2008). In line with this, Derler and co-workers show that the N terminus is not involved in channel inactivation by calmodulin (Derler at al. 2006).

Hydrolysis of the extracellular N-linked sugar residues at asparagine 397 by the glucuronidase klotho (see above) entraps the channel proteins in the plasma membrane (Chang et al. 2005) and, as a consequence, results in a higher channel activity. Klotho is the first mammalian proteo-hormone, which binds to TRPV6 from the extracellular side. Replacing the asparagine residue by a glutamine and thereby

creating a glycosylation-deficient TRPV6 mutant abolished klotho-dependent channel activation. The stimulatory effect of klotho is restricted to TRPV5 and TRPV6 and not detectable for TRPV4 and TRPM6, which are also expressed in the kidney (Lu et al. 2008).

In summary, some TRPV6-interacting proteins, like calmodulin or klotho, have been investigated by several studies, and it has been shown consistently that they modulate/regulate channel activity, whereas most of the other candidate proteins were only reported with little or without biochemical or functional characterization (see also http://trpchannel.org/summaries/TRPV6).

5 Biophysical Properties of TRPV6 Channels

TRPV6 (and TRPV5) represent Ca^{2+}-selective ion channels (Peng et al. 1999; Nilius et al. 2000; Vennekens et al. 2000; Wissenbach et al. 2001; Hirnet et al. 2003). Under physiological conditions, TRPV6 conducts only Ca^{2+} ions, but in the absence of divalent cations, the channel conducts monovalents such as Na^{+} (Vennekens et al. 2000; Nilius et al. 2001; Wissenbach et al. 2001; Voets et al. 2003). Interestingly, Na^{+} currents do not inactivate, whereas Ca^{2+} currents quickly inactivate indicating a Ca^{2+}-dependent inactivation mechanism (Hoenderop et al. 2005). Another mechanism of inactivation includes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Thyagarajan et al. 2008). Al-Ansary and co-workers mapped an ATP binding site in the N-terminal region of the TRPV6 protein which indicates that the channel is regulated by the cytosolic ATP concentration (Al-Ansary et al. 2010). The channel permeability was estimated to be about 100 times more selective for Ca^{2+} than for Na^{+}, and the single aspartate residue D581 in the murine TRPV6 (D541 in the truncated protein) and D582 in the human TRPV6 (D542 in the truncated protein) were identified to be part of the selectivity filter (Nilius et al. 2001, 2003; Voets et al. 2003, 2004; Vennekens et al. 2008). In the absence of Ca^{2+}, TRPV6 also conducts Mg^{2+} and the initially large currents quickly inactivate (Voets et al. 2001, 2003). Depending on the membrane potential, Mg^{2+} ions block and unblock the channel pore (Voets et al. 2003) and influence the inward rectification behaviour of TRPV6. Some inward rectification also appears in the absence of Mg^{2+} and seems to be an intrinsic property of TRPV6 (Hoenderop et al. 2005). TRPV6 and TRPV5 conduct Ba^{2+}, but the permeability and inactivation properties of Ba^{2+} currents are different between TRPV6 and TRPV5 although the pore sequences are identical (Nilius et al. 2002; Hoenderop et al. 2005). Also Cd^{2+} and Zn^{2+} can permeate and contribute to the toxicity of these heavy metal ions (Kovacs et al. 2013). Interestingly, Ba^{2+}-dependent current properties seem to be determined by residues in the transmembrane regions 2 and 3 indicating that structures quite distant to the pore region may influence pore properties as well as the inactivation behaviour of the channel.

The single channel conductance of TRPV6 was estimated to be 40–70 pS, and by cysteine scanning the pore width was calculated to be 5.4 Å (Hoenderop et al. 2001, 2005; Voets et al. 2004). Apparently, most of the electrophysiological data

described were obtained from HEK293 cells overexpressing the truncated Trpv6 cDNA. The comparison of the biophysical properties of channels obtained after expressing the full-length cDNA or the truncated cDNA yields very similar results so far (Fecher-Trost et al. 2013). However, the full-length TRPV6 is more efficiently translocated to the plasma membrane, and five times more of the truncated TRPV6 protein is required to produce similar current amplitudes (Fecher-Trost et al. 2013).

Another yet unsolved problem is that in acutely isolated primary cells which do endogenously express Trpv6 transcripts like pancreatic acinar cells, TRPV6-like currents could not be recorded although the protocols applied yield impressive currents in HEK293 cells expressing the *Trpv6* cDNA. Instead, in primary cells and tissues, $^{45}Ca^{2+}$ uptake measurements are the method of choice to monitor TRPV6 activity. Using this method TRPV6 activity was identified in epididymal epithelia (Weissgerber et al. 2011, 2012).

6 Pharmacology of TRPV6 Channels

Xestospongin C, a natural isolate from a sponge (Vassilev et al. 2001), inhibits TRPV6 in the lower μM range. Ruthenium red also blocks TRPV6 but works more potently on TRPV5 (Hoenderop et al. 2001). Some antifungal drugs such as econazole and miconazole inhibit TRPV5 and TRPV6 (Hoenderop et al. 2001). A newly synthesized derivative of a substance called TH-1177 inhibits TRPV6 currents with an IC50 for TRPV6 of 0.44 μM (Haverstick et al. 2000; Landowski et al. 2011). This compound is five times more effective on TRPV6 than on TRPV5. 2-APB (2-aminoethoxydiphenyl borate), a rather non-selective TRP channel blocker/activator, was shown to block TRPV6 but surprisingly not TRPV5 (Kovacs et al. 2012). Recently a peptide, soricidin, derived from short-tailed shrew (Bowen et al. 2013) was shown to exhibit an analgesic effect and to suppress growth of some tumour cells. It was shown that the C-terminal part of the peptide is sufficient for growth inhibition, and the authors demonstrate that one target of this peptide is TRPV6: TRPV6 currents in HEK293 cells are partially blocked with an IC50 in the low nM range. Apparently, soricidin represents the most potent TRPV6 channel blocker [see also Owsianik et al. (2006) and Vennekens et al. (2008)].

7 Physiological Functions of TRPV6

Since the identification and the cloning of the cDNAs and the initial expression studies, both TRPV6 and TRPV5 have been implicated in epithelial Ca^{2+} uptake. In agreement with this function, the *Trpv6* gene was shown to be expressed in placenta, pancreas, salivary gland and several parts of the small intestine including duodenum by Northern blots and in situ hybridization (Peng et al. 1999; Wissenbach et al. 2001; Hirnet et al. 2003). Using antibodies for TRPV6, immunostaining was demonstrated in enterocytes of the intestinal villi in the

murine duodenum with predominant expression at the apical cell membrane at the tips of the villi (Little et al. 2011) as well as in exocrine organs including pancreas, prostate and mammary gland (Zhuang et al. 2002). In addition, the TRPV6 protein was detected within the mouse kidney at the apical domain of the late distal convoluted tubule, the connecting tubule and the cortical and medullary collecting ducts (Nijenhuis et al. 2003). Several groups demonstrated the expression of TRPV6 in the proximal part of duodenum (Zhuang et al. 2002; van de Graaf et al. 2003; Walters et al. 2006; Huybers et al. 2007) in line with a role for TRPV6 in intestinal Ca^{2+} absorption, but others did not detect *Trpv6* expression in duodenum and kidney (Wissenbach et al. 2001).

Appropriate controls for antibody specificity including immunostains from knockout mice strongly support results in general, but have not been described in most studies referred to above. One of the few exceptions is the demonstration that TRPV6 proteins are expressed in the apical membrane of the murine epididymal epithelium and prostatic epithelium (Weissgerber et al. 2011, 2012). It should be mentioned here that expression of *Trpv6* transcripts in human prostate is low or even not detectable depending on the method (Northern blot, in situ hybridization or PCR) but is upregulated in human prostate cancer (see below).

7.1 TRPV6 Channels in Bone

Laser scanning microscopy revealed that TRPV6 proteins—as well as TRPV5—are expressed at the apical domain of murine osteoclasts cultured on cortical bone slices predominantly at the bone-facing site (van der Eerden et al. 2005). However, TRPV6 activity did not compensate the reduced bone resorption occurring in mice lacking *Trpv5* (Hoenderop et al. 2003c). Additional immunohistochemical analysis revealed only weak staining for TRPV6 in osteoblasts and no staining in cortical or trabecular osteocytes nor in the growth plate (Little et al. 2011), whereas *Trpv6* mRNA was detectable in the murine bone (Nijenhuis et al. 2003) with considerable expression in the bone marrow.

7.2 TRPV6 Channels in Placenta

The *Trpv6* mRNA was detected in syncytiotrophoblasts (Wissenbach et al. 2001) and uterus (Moreau et al. 2002) suggesting a role for basal Ca^{2+} influx in placental cells and from there to the foetus. Whereas the placental Ca^{2+} transport and the involved proteins are not completely understood, so far TRPV6 protein was decreased in placenta from women suffering from pre-eclampsia compared to healthy placental tissue (Hache et al. 2011). Pre-eclampsia (PE) is a multisystemic disorder that represents a major factor for maternal and perinatal mortality, and it affects 7–10 % of pregnancies worldwide (Sibai 2005; Walker 2000). In mice, *Trpv6* is highly expressed in the extraplacental yolk sac (the foetal side of the placenta) and only weakly expressed on the maternal side of the placenta during the last

trimester of gestation, but *Trpv6* transcript expression was strongly upregulated from embryonic day 15 to day 18 (Suzuki et al. 2008a) indicating involvement in the increase of maternal-foetal Ca^{2+} transport which supports foetal bone mineralization.

8 Lessons from Knockouts

At present, three independent mouse lines exist with targeted germ line mutations within the *Trpv6* gene (Bianco et al. 2007; Weissgerber et al. 2011). One *Trpv6*-deficient line was generated using a classical knockout strategy replacing exons 9–15 and exons 15–18 of the adjacent *Ephb6* gene by a neomycin resistance cassette (Bianco et al. 2007). In an independent approach, a Cre-loxP strategy was used to delete exons 13–15 of *Trpv6* encoding part of transmembrane domain 5, the pore, the transmembrane domain 6, the cytosolic C terminus and exons 17–18 of the *Ephb6* gene (Weissgerber et al. 2012). For homologous recombination, genomic sequences of appropriate length are required within the gene-targeting vector, and this is the reason that deletion of the exons 13–15 of the *Trpv6* gene also affected parts of the closely adjacent *Ephb6* gene. In a third mouse line, a single aspartate (D) residue at position 581 (541 in the truncated protein) within the pore region was replaced by an alanine (A) residue ($Trpv6^{D/A/D/A}$). This residue is a critical constituent of the TRPV6 selectivity filter, and replacement by an alanine (D/A) completely abolished the Ca^{2+} permeability (Nilius et al. 2001; Voets et al. 2004; Weissgerber et al. 2012).

Bianco et al. (2007) reported that their $Trpv6^{-/-}$ mice were viable but show decreased intestinal Ca^{2+} absorption, lower femoral bone mineral density, lower body weight, growth retardation and reduced fertility of homozygous males and females, and 80 % of all mice developed alopecia and dermatitis (Table 2). Serum Ca^{2+} levels were normal, but under Ca^{2+}-restricted conditions, the $Trpv6^{-/-}$ mice developed hypocalcaemia. Furthermore, the total Ca^{2+} levels in the serum and amniotic fluid of $Trpv6^{-/-}$ foetus were significantly lower than those in wild-type foeti (Bianco et al. 2007; Suzuki et al. 2008a). Analysis of active intestinal Ca^{2+} transport revealed no difference between $Trpv6^{-/-}$ and wild-type mice on a standard diet (Benn et al. 2008).

The analysis of the $Trpv6^{-/-}$ and the $Trpv6^{D/A/D/A}$ mice from Weissgerber et al. (2011, 2012) demonstrated that homozygous males but not females from both mouse lines showed severely impaired fertility despite normal copulation behaviour. Furthermore, the spermatozoa showed markedly reduced motility, fertilization capacity and viability. Northern blot and immunohistochemical analysis demonstrated that *Trpv6* was expressed in the apical membrane of the epididymal epithelium but not in spermatozoa or the germinal epithelium. The Ca^{2+} concentration within the fluid of the cauda epididymis was significantly increased in both mouse lines $Trpv6^{D/A/D/A}$ and $Trpv6^{-/-}$ compared to wild-type controls and led to reduced sperm viability. These results indicate that appropriate Ca^{2+} concentration is essential for the development of viable and fertilization-competent spermatozoa. In addition, deletion of the *Trpv6* gene (Weissgerber et al. 2012) did not further

Table 2 Comparison of *TRPV6*$^{D/A/D/A}$ and *Trpv6*-deficient mouse lines

Analysed parameter	*Trpv6*$^{D/A/D/A}$ (Weissgerber et al. 2011)	*Trpv6*$^{-/-}$ (Weissgerber et al. 2012)	*Trpv6*$^{-/-}$ (Bianco et al. 2007)
Femoral bone mineral density	No difference	Not analysed	Decreased
Body weight	No difference	No difference	Decreased in homozygous males and females
Growth retardation	Not detectable	Not detectable	Decreased in homozygous males and females
Fertility defect	Homozygous males	Homozygous males	Homozygous males and females
Sperm motility	Impaired	Impaired	Not analysed
Sperm viability	Impaired	Impaired	Not analysed
Ca^{2+} homeostasis			
On normal calcium diet:			
–Intestinal Ca^{2+} absorption	No change	Not analysed	Decreased
–Serum PTH and 1,25-dihydroxyvitamin D	Not analysed	Not analysed	Elevated
–Urine osmolality	Not analysed	Not analysed	Altered
–Ca^{2+} excretion	No change	No change	Increased
–Polyuria	No change	No change	Increased
–Serum Ca^{2+} level	No change	No change	No change
On Ca^{2+} restricted diet:			
–Serum Ca^{2+} level	Unaltered	Not analysed	Decreased (hypocalcaemia)
–Intestinal Ca^{2+} uptake	Decreased	Not analysed	Decreased
Alopecia	Not detectable	Not detectable	Yes
Dermatitis	Not detectable	Not detectable	Yes

aggravate the phenotype observed in *TRPV6*$^{D/A/D/A}$ mice (Weissgerber et al. 2011), arguing against residual channel activity of the mutated TRPV6$^{D/A}$ protein. TRPV6 proteins were also identified in the apical epithelial membranes of prostatic epithelium, and *Trpv6* deletion results in Ca^{2+} precipitates within the enlarged prostatic ducts. Van der Eerden et al. (2011) showed that *Trpv6*$^{D/A/D/A}$ mice do not have altered bone mass, and other morphological parameters such as trabecular and cortical bone microarchitecture were similar compared to wild-type mice. However, bone size was affected as shown by reductions in femoral length as well as femoral head, cortical bone and endocortical bone volumes. Intestinal Ca^{2+} uptake under a Ca^{2+}-restricted diet was significantly impaired in *Trpv6*$^{D/A/D/A}$ mice compared to wild-type mice (Woudenberg-Vrenken et al. 2012) demonstrating a specific role of TRPV6 in transepithelial Ca^{2+} absorption under conditions of limited Ca^{2+} supplies.

Differences in Ca^{2+} absorption, weight gain, hair coat or fertility of homozygous females (Bianco et al. 2007) were not observed in the *Trpv6*$^{-/-}$ or *Trpv6*$^{D/A/D/A}$ mice generated by Weissgerber et al. (2011, 2012; Table 2). The reason for the discrepancies between the *Trpv6*-deficient mouse lines is not known, but different exons were deleted in either strategy and differences in the genetic background between the mouse lines cannot be excluded. In addition, the neo-cassette introduced for gene targeting has been removed from the mouse lines of Weissgerber et al. (2011, 2012) before phenotyping. If this foreign DNA remains in the genomic locus, it may influence the expression of neighbouring genes. Because it contains cryptic splice sites, its persisting presence can also fortuitously create a hypomorphic allele. Finally, the number of animals used to characterize a phenotype is crucial; for example, weight gain was studied with four animals (Bianco et al. 2007) and 19–31 animals (Weissgerber et al. 2011).

9 Roles in Hereditary and Acquired Disease

Changes of *Trpv6* transcript expression have been shown in various transgenic mouse models of human diseases (Table 3) but whether these changes occur in humans and whether there is a link between the changes of transcript expression and the pathophysiology of these diseases have only preliminarily been investigated. For example, Lowe disease patients carry mutations within the *phosphatidylinositol bisphosphate (PIP2) 5-phosphatase* gene resulting in increased levels of the substrate phosphatidylinositol 4,5-bisphosphate (PIP2), and this lipid accumulates in the renal proximal tubule cells of these patients (Devuyst and Thakker 2010). PIP2 positively regulates the activity of TRPV6 (Thyagarajan et al. 2008; Zakharian et al. 2011; Wu et al. 2012; Cao et al 2013) and might therefore contribute to the intestinal hyperabsorption of Ca^{2+} in these patients.

From the very beginning, it became apparent that *Trpv6* transcripts are overexpressed in cancerous tissue and in cell lines derived thereof (Table 4, and expression profiles HG-U95A and GDS1746 available at http://www.ncbi.nlm.nih.gov/geoprofiles). In prostate cancer, *Trpv6* transcripts are overexpressed and the expression pattern correlates with the aggressiveness of the disease (Wissenbach et al. 2001, 2004; Peng et al. 2001; Fixemer et al. 2003). Likewise, in the human lymph node prostate cancer cell line LNCaP, upregulation of *Trpv6* transcripts by vitamin D treatment enhanced proliferation rate and resistance to apoptosis (Lehen'kyi et al. 2007, 2011, 2012). The proliferation rate of HEK293 cells expressing the *Trpv6* cDNA was also enhanced (Schwarz et al. 2006). Nude mice in which HEK293 cells stably expressing *Trpv6* were injected subcutaneously as xenografts developed tumours (Wissenbach 2013), whereas control mice injected with non-transfected HEK293 cells or with HEK293 cells stably expressing *Trpc4* did not. As described above, soricidin (Bowen et al. 2013), a peptide isolated from the venom of the short-tailed shrew, inhibits TRPV6 currents. Tumours derived from injection of the ovarian cancer cell line SKOV-3 and the cell line DU145

Table 3 *Trpv6* expression in animal models of human disease

Animal model: disease	Link to *Trpv6*	References
Slc26a4 knockout mouse: animal model for Pendred syndrome	Expression of *Trpv6* (and *Trpv5*) in the cochlea (immunohistochemistry)	Wangemann et al. (2007)
$TNF_{\Delta ARE}$ mouse: animal model of Crohn's-like disease	Transcript ↓(qPCR, duodenum)	Huybers et al. (2008)
(*Clcn5* knockout mouse: animal model of Dent disease)	(Mutations of *OCRL* cause Lowe syndrome and Dent disease) Co-expression of *Trpv6* (immunohistochemistry) and the oculocerebrorenal syndrome of Lowe (*OCRL*) gene product, the phosphatidyl inositol 4,5-bisphosphate 5-phosphatase in rat Intestinum. In *Xenopus laevis* oocytes endogenous OCRL suppressed TRPV6-mediated Ca^{2+} uptake	Wu et al. (2012)
$Slc12A3_{truncated}$ knock-in mouse: animal model of Gitelman syndrome	*Trpv6* transcript ↑(qPCR kidney)	Yang et al. (2010)
Hypercalciuric stone-forming (GHS) rat: animal model for kidney stones	*Trpv6* transcript ↑(qPCR, kidney)	Frick et al. (2013)

Table 4 *Trpv6* expression in malignant human tissues and cancer cell lines

Malignancy	Method	References
Prostate cancer	RT-PCR, Northern, ISH	Fixemer et al. (2003), Peng et al. (2001), Wissenbach et al. (2001, 2004)
LNCaP	Northern, Western	Bodding et al. (2003), Peng et al. (2001)
DU-145	RT-PCR	Lehen'kyi et al. (2011), Peng et al. (2001)
PC3	RT-PCR	Peng et al. (2001)
Breast cancer	RT-PCR, immunostaining[a]	Bolanz et al. (2008), Zhuang et al. (2002)
MCF7	RT-PCR, Western blot[a]	Bolanz et al. (2009)
T47D	MS/MS, RT-PCR	Bolanz et al. (2008), Fecher-Trost et al. (2013)
Colon cancer	Immunostaining[a]	Zhuang et al. (2002)
SW480	Northern	Peng et al. (2000)
LS-180	RT-PCR	Zheng et al. (2012)
Caco	RT-PCR, Western[a]	Fleet et al. (2002), Taparia et al. (2006)
Ovarian cancer	Immunostaining[a]	Zhuang et al. (2002)
SKOV-3	Peptide staining of xenograft	Bowen et al. (2013)
Thyroid cancer	Immunostaining[a]	Zhuang et al. (2002)
Endometrial cancer	ISH	Wissenbach and Niemeyer (2007)
Leukaemia	–	–
K-562	*Northern*	*Peng et al. (2000), Semenova et al. (2009)*

[a]No data available on antibody specificity

derived from brain metastases of prostate cancer could be stained with soricidin-derived peptides in vivo (Bowen et al. 2013) apparently through labelling TRPV6. In addition to prostate cancer, *Trpv6* transcripts are overexpressed in breast cancer and breast cancer-derived cell lines (Zhuang et al. 2002) as well as in oestrogen receptor-negative breast cancer tumours (Peters et al. 2012). The authors also showed that overexpression of *Trpv6* most likely results from genomic amplification of the *Trpv6* gene. Gene amplification of *Trpv6* was also found in prostate tumours (Kessler and Wissenbach, unpublished). In summary, the data indicate that overexpression of *Trpv6* is involved in carcinogenesis of several human cancers. They demonstrate the importance of TRPV6 which not only represent a marker for prostate cancer progression but may serve as a target for therapeutic strategies in the malignancies listed above.

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