Chapter 1

General introduction

The fabulous tale of TRPM7:
“In the book of science it tells the year 2001 as the time of my arrival. I have gone by many names since, until the scientific community agreed on TRPM7. Only few know me for what I am.....or.....do they really? Whatever one might know is what I am according to others; A myth? A legend? Just another stretch of coded sequence? Yet another gene, or protein? Unique? Complex? Interesting? Essential? Biologically relevant? Or even the most important protein in the world?”

Preface
The interest of our group into the Ca²⁺-permeable divalent cation channel TRPM7 started with the observations made by Frank van Leeuwen on phospholipase C (PLC)-mediated cell flattening and adhesion in neuroblastoma cells. Stimulation of these cells with low doses of Bradykinin caused a prominent increase in cell spreading and cell adhesion that was associated with increased phosphorylation of the myosin II heavy chain (MHC-II) (van Leeuwen et al., 1999). In Dictyostelium, MHC-II phosphorylation is mediated by so-called α-kinases, which had not been identified before in vertebrates. In an effort to identify the putative mammalian homologue of those enzymes Frank van Leeuwen picked up a partial clone in 2000, which later turned out to match the kinase domain of the newly identified TRPM7 channel (Nadler et al., 2001; Runnels et al., 2001). Since that time, in an intensive collaboration, our labs have addressed various aspects of TRPM7 functioning and regulation, with a primary focus on actomyosin remodeling and cell adhesion, as well as PLC-signaling and Ca²⁺-signaling (Clark et al., 2006; Clark et al., 2008a; Clark et al., 2008b; Clark et al., 2008c; Langeslag et al., 2007). This introduction provides an overview of the rapidly growing literature on TRPM7 and concludes with a brief summary of the contents of this thesis.
Cellular signaling and signal transduction

Any cell, whether it is alone or organized within a tissue or organism, communicates with its environment and, like in modern society, proper communication skills are essential for development, order and survival. Communication at the cellular level, or cellular signaling, entails receiving and processing an incredibly diverse array of extracellular signals that are typically either chemical (e.g. growth factors, hormones, neurotransmitters) or physical (e.g. mechanical stress, temperature, voltage) in nature. Therefore, cells are equipped with an elaborate toolkit of receptors, ion channels, ion exchangers and pumps, enzymes, second messenger systems and structural proteins (Alberts, 2008). At the same time, cells shape and influence their environment by producing signals themselves or by counteracting extracellular inputs. For example, mechanical forces are among the most common signals that any given cell in its environment is exposed to. These originate from both the cell interior and –exterior and are typically generated by, respectively, the contractile actomyosin cytoskeleton and physical interactions with the extracellular matrix (ECM). The reciprocal interactions between cells and their environment are highly dynamic and tightly controlled to maintain functional and structural integrity of tissues. When this tissue homeostasis is disturbed, it most often is quickly restored, but when the tightly controlled balance is out of bounce and control mechanisms do not function properly, pathologies may develop. A classic example of disturbed tissue homeostasis or a disbalance in cellular signaling is cancer [see section ‘Cancer and tumor cell metastasis’].

Cellular signaling and the consequential cellular responses are determined by the receptors that respond to the initial signal. The lipid plasma membrane is impermeable to water soluble signaling molecules, and therefore most signals are passed (“transduced”) over the plasma membrane by transmembrane proteins: cell surface receptors, mechanical sensors, and ion channels. Ion channels facilitate the diffusion of ions down their electrochemical gradient over the plasma membrane and over the membranes of intracellular organelles. The resulting ion fluxes are important cellular signals, with calcium (Ca\(^{2+}\)) being arguably the most versatile and universal signaling entity [see Box 1]. The Transient Receptor Potential (TRP) channel superfamily is the latest important addition to the large family of ion channels. The discovery of TRP channels has greatly advanced our understanding of signal transduction pathways, especially sensory transduction, in a variety of fundamental cell processes [see sections ‘A remarkable cation channel family: TRP channels’ and ‘TRPM7’].

A remarkable cation channel family: TRP channels

TRP channels are identified by sequence homology and are categorized into six subfamilies that are conserved from nematodes to mammals: TRPC (‘Canonical’; members C1-7),
TRPV (‘Vanilloid’; members V1-6), TRPM (‘Melastatin’; members M1-8), TRPA (‘Ankyrin’; members A1), TRPP (‘Polycystic’; members P1-3), and TRPML (‘Mucolipin’; members ML1-3). The seventh subfamily, TRPN, has so far not been identified in mammals and is named after nompC channels (‘no mechanoreceptor potential C’) in the nematode *Caenorhabditis elegans* (see e.g. Nilius and Owsianik, 2011; for a compendium of TRP channels see http://www.iuphar-db.org/DATABASE/FamilyMenuForward?familyId=78). The TRP channel family is among the largest and most diverse families of ion channels and 28 mammalian isoforms have been identified to date. The first TRP channel was described in *Drosophila melanogaster*, a small invertebrate that becomes visually impaired when the homolog *trp* is deleted from the gene-pool (Cosens and Manning, 1969; Montell and Rubin, 1989). The family name ‘Transient Receptor Potential’ originates from the photoreceptor response in these flies that is transient rather than sustained upon prolonged illumination of photoreceptor cells in the trp mutant.

**TRP channel characteristics and function**

Although high-resolution crystal structures for full length TRP channels are not available yet (Li et al., 2011), TRP proteins are predicted to consist of six transmembrane segments with a pore-forming loop between the fifth and sixth transmembrane segment. TRP proteins assemble into tetramers to form a functional channel. The cytosolic N- and C-termini vary widely among and also within subfamilies, but contain some notable conserved domains, such as ankyrin repeats in the N-terminus (protein-protein interactions; Schindl et al., 2008; Schindl and Romanin, 2007) and the TRP domain, a 25 amino acidic segment located C-terminally to the 6th transmembrane domain (subunit tetramerization and channel gating; Garcia-Sanz et al., 2004; Garcia-Sanz et al., 2007; Phelps and Gaudet, 2007; Rohacs et al., 2005). Two TRP family members are unique among all ion channels in that they contain a protein kinase domain in the C-terminus: TRPM6 and TRPM7. Their fully functional serine/threonine kinase domains are of the α-kinase subtype and have earned these proteins the title of ‘channel-kinase’ (Clark et al., 2008a; Clark et al., 2008b; Dorovkov and Ryazanov, 2004; Middelbeek et al., 2010; Nadler et al., 2001; Runnels et al., 2001; Ryazanova et al., 2001). Another TRP family member, TRPM2 presents with a functional ADP-ribose hydrolase (Perraud et al., 2001; Perraud et al., 2003). Inherent enzymatic activity is not found in other ion channel families. In addition, TRP channels show a large diversity in ion selectivity, activation mechanisms and biological functions. Some recurring themes have nevertheless emerged in the TRP family, such as:

(i) TRP channels are non-selective channels, but almost all family-members conduct Ca$^{2+}$;

(ii) TRP channel gating is regulated by phospholipase C (PLC) and phospholipids, particularly phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P$_2$ or PIP$_2$) [see subsection ‘PIP$_2$
sensitivity and dependency of TRPM7’ in section ‘TRPM7’};

(iii) TRP channels mediate sensory transduction processes, such as vision, hearing, taste, and the perception of temperature, pain and mechanical force at both the level of the organism and the cell.

More details on TRP channel characteristics and functions are available in several excellent and comprehensive reviews (Clapham, 2003; Clapham et al., 2005; Damann et al., 2008; Minke, 2010; Montell, 2005; Nilius and Owsianik, 2011; Venkatachalam and Montell, 2007). Since this dissertation centers on the function and regulation of the Melastatin subfamily member TRPM7, however, I will focus on covering the background and specifics of this TRP channel below.

Box 1 | Ca\(^{2+}\) signaling: basics and general principles

Signals that are transduced from the cell exterior into the cytosol are typically propagated by second messengers, such as calcium ions (Ca\(^{2+}\)) (reviewed in Berridge et al., 2000). For references of generally accepted principles I refer the reader to reviews by Berridge and colleagues (2000), Taylor (2006) and Clapham (2007).

The driving force for Ca\(^{2+}\) signaling is generated by steep concentration gradients over the plasma membrane and the membranes of intracellular Ca\(^{2+}\) stores (e.g. the ER). This requires that cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is maintained at low levels (100 nM) by a tight balance of Ca\(^{2+}\) channels, -transporters and -buffering proteins. An increase in [Ca\(^{2+}\)], carries information that can be initiated by a plethora of stimuli and results from either Ca\(^{2+}\) influx from the extracellular space ([Ca\(^{2+}\])\(_e\) ~ 1 mM) or Ca\(^{2+}\) release from internal stores ([Ca\(^{2+}\])\(_{ER/SR}\) ~ 100 mM - 1 mM; Bygrave and Benedetti, 1996) or from both these routes.

Extracellular stimuli either act directly on Ca\(^{2+}\) channels in the plasma membrane or indirectly through the generation of second messengers [Figure 1]. Phospholipase C (PLC)-coupled agonists, for example, trigger the hydrolysis of phosphatidylinositol(4,5) bisphosphate (PIP\(_2\)) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (InsP\(_3\) or IP\(_3\)); all three of which are known to affect channel gating [see Chapter 4 for references]. Ca\(^{2+}\) signals are commonly amplified by Ca\(^{2+}\) release from internal stores. Ca\(^{2+}\) alone is however not sufficient to initiate this process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), but requires an additional factor, such as IP\(_3\), to activate the Ca\(^{2+}\) receptor channels in the ER membrane (e.g. IP\(_3\)R). Depletion of Ca\(^{2+}\)-stores, in turn, can evoke store-operated Ca\(^{2+}\) entry (SOCE). The molecular components that make up this major Ca\(^{2+}\) influx pathway were only recently identified by several groups: Orai1 is the Ca\(^{2+}\) channel in the plasma membrane and STIM1 the Ca\(^{2+}\)-sensor in the ER-membrane that aggregates upon a drop in ER Ca\(^{2+}\) levels and subsequently associates with Orai1 (reviewed in Taylor, 2006). The contribution of other components to SOCE-pathways is still intensively investigated, however, and especially the
significance of TRP channels is highly debated (reviewed in Berna-Erro et al., 2012; Salido et al., 2009).

In order for Ca\(^{2+}\) to function in multiple processes in the cell and to exert selective effects, the Ca\(^{2+}\) signal must be coded. Signal amplitude, timing, repetition and duration as well as subcellular localization are characteristics that determine how a Ca\(^{2+}\) signal is interpreted by the cell. In addition, the distribution of downstream Ca\(^{2+}\)-sensitive effector proteins determines how a Ca\(^{2+}\) signal is decoded. Ca\(^{2+}\) buffers can for example constrain the Ca\(^{2+}\) signal to short distances from the conducting Ca\(^{2+}\) channels. The discovery of such spatially restricted Ca\(^{2+}\) signals (high-Ca\(^{2+}\) microdomains or Ca\(^{2+}\) sparks) has highlighted a diversity of Ca\(^{2+}\) signalplexes in cells (Cheng and Lederer, 2008), of which the SNARE-complex fusion machinery in vesicle release is one of the best-described examples (Qian and Noebels, 2001; Reid et al., 1998; Schneggenburger and Neher, 2005). In addition, cell adhesions and protrusions contain numerous Ca\(^{2+}\)-sensitive proteins and Ca\(^{2+}\) channels and are regulated by Ca\(^{2+}\) sparks (Nagasawa and Kojima, 2012; Ridley et al., 2003; Tsai and Meyer, 2012). This in turn controls processes such as cell migration (Wei et al., 2009) and axonal pathfinding (Gomez et al., 2001; Gomez and Zheng, 2006; Henley and Poo, 2004; Robles et al., 2003).

![Figure 1](image.png)

**Figure 1 | Major Ca\(^{2+}\) signaling pathways and components in cells.** Ca\(^{2+}\) buffer systems, Ca\(^{2+}\)-binding proteins and mitochondria; CICR, Ca\(^{2+}\)-induced Ca\(^{2+}\) release; ER, endoplasmic reticulum; IP\(_3\), inositol 1,4,5-triphosphate; IP\(_R\), IP\(_3\)-receptor channels; SERCA; sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase; SOCE, store-operated Ca\(^{2+}\) entry.
TRPM7
TRPM7 (Transient Receptor Potential cation channel, subfamily Melastatin, member 7) is an intriguing bifunctional protein that constitutes the fusion between a cation channel and a serine/threonine protein kinase domain [Figure 2]. The human TRPM7 protein is 1865 amino acids long and has a mass of 212.7 kDa (Entrez gene ID: 54822 [human] and 58800 [mouse]; http://www.uniprot.org/uniprot/Q96QT4). Up until consensus was reached on a nomenclature for TRP channels (Clapham et al., 2005; Montell et al., 2002), TRPM7 had gone by different names; melanoma alpha-kinase (Ryazanova et al., 2001), ChaK1 (Channel-Kinase1; Ryazanov, 2002), LTRPC7 (Long Transient Receptor Potential Channel 7; Nadler et al., 2001), TRP-PLIK (Transient Receptor Potential – Phospholipase C-Interacting Kinase; Runnels et al., 2001). The tissue distribution of TRPM7 is ubiquitous, but expression levels are the highest in heart, liver, bone, and adipose tissue in human samples (Fonfria et al., 2006; Nadler et al., 2001) and heart and kidney in murine samples (Runnels et al., 2001). Also, TRPM7 is constitutively expressed from early embryonic stages on, and it is essential for development as its global deletion in mice results in embryonic lethality (Jin et al., 2008; Ryazanova et al., 2010).

TRPM7 currents and the channel domain
The TRPM7 channel displays a characteristic outward-rectification current-voltage (I-V) relationship. At least, this is true in traditional whole-cell patch clamp recordings, especially when intracellular Mg$^{2+}$ is artificially lowered. The I-V-plot is linear however in perforated-patch recordings; a configuration that permits electrical coupling between the cell interior and the patch pipette while minimizing dialysis of the cytoplasm. Endogenous TRPM7 currents are small. The TRPM7 channel is constitutively open and permeates particularly the divalent cations Ca$^{2+}$ and Mg$^{2+}$ under physiological conditions (Penner and Fleig, 2007). TRPM7 currents have been extensively characterized electrophysiologically and are sensitive to inhibition by (sub)millimolar concentrations of intracellular free Mg$^{2+}$ ([Mg$^{2+}$]$_i$) and Mg$^{2+}$-nucleotides, for which the current initially was known as MagNuM (Mg$^{2+}$-nucleotide-regulated metal ion current) or MIC (Mg$^{2+}$-inhibited cation current) (Chokshi et al., 2012; Demeuse et al., 2006; Kozak and Cahalan, 2003; Nadler et al., 2001; Runnels et al., 2001; Schmitz et al., 2003). In accordance, the depletion of intracellular Mg$^{2+}$ or Mg$^{2+}$-nucleotides augments both inward and, especially, outward TRPM7 currents (Demeuse et al., 2006; Kozak and Cahalan, 2003; Langeslag et al., 2007; Nadler et al., 2001). The inhibitory action of Mg$^{2+}$ and Mg$^{2+}$-nucleotides is synergistic and does not occur only via permeation block. Available evidence from point- and deletion mutants suggests that it depends, at least in part, on divalent cation binding to different C-terminal sites, one within and one outside the kinase-domain (Demeuse et al., 2006; Schmitz et al., 2003).
The dependency of the channel function on the kinase-domain has been long debated, but the current consensus is that neither the kinase-domain nor its phosphotransferase activity are required to obtain a fully functional channel. The kinase-domain might however modulate TRPM7 gating as it appears that several α-kinase point- and deletion mutants have an altered sensitivity towards Mg$^{2+}$ and Mg$^{2+}$-nucleotides (Demeuse et al., 2006; Matsushita et al., 2005; Nadler et al., 2001; Schmitz et al., 2003).

In addition to Mg$^{2+}$ and Mg$^{2+}$-nucleotides, TRPM7 currents have been demonstrated to be sensitive to the classical signaling entities downstream of G-protein-coupled receptors (GPCRs); PLC/PIP$_2$ [see subsection ‘PIP$_2$ sensitivity and dependency of TRPM7’] and cAMP (Takezawa et al., 2004). Furthermore, TRPM7 currents show sensitivity towards pH (Jiang et al., 2005; Kozak et al., 2005) and mechanical force [see paragraph ‘Mechanotransduction’]

**Figure 2 | TRPM7 structure and domains.** A schematic overview of characteristic domains of the TRPM7 protein (amino acids refer to the human protein unless indicated otherwise). **TM**, transmembrane domain.
in subsection ‘TRPM7 in cellular physiology’]. Moreover, a number of compounds have been reported to inhibit TRPM7 currents. These include 2-aminoethyl diphenylborinate (2-APB), spermine, trivalent cations (La$^{3+}$ and Gd$^{3+}$) and the 5-lipoxygenase inhibitors NDGA, AA861 and MK886 (see e.g. Chen et al., 2010a; Langeslag et al., 2007). All of these compounds are, however, known to affect other ion channels too and therefore the search for an inhibitor specific to or selective for TRPM7 is ongoing. To date, the recently discovered compound waixenicin-A proves the most promising TRPM7-selective inhibitor, although its mode of inhibition remains to be determined (Kim et al., 2013; Zierler et al., 2011). Future follow-up studies should determine whether waixenicin-A is a suitable inhibitor for investigating the (patho-)physiological roles of TRPM7 in different processes, especially in vivo [see also Chapter 5].

In summary, TRPM7 gating has been reported to be regulated by a variety of factors, including Mg$^{2+}$ and other divalent ions, Mg$^{2+}$-nucleotides, pH, cAMP and PIP$_2$ levels. However, for each of these factors conflicting data exist and precise mechanisms of action are lacking and, therefore, remain subject of debate, stressing the importance of further investigations.

**PIP$_2$ sensitivity and dependency of TRPM7**

Phosphoinositides, and particularly PIP$_2$, are important and common regulators of many ion channels (Hilgemann et al., 2001): channel activity and localization generally depend on the presence of PIP$_2$ at the plasma membrane and TRP channels are no exception (reviewed in Hardie, 2003; Hardie, 2007; Qin, 2007; Rohacs, 2007; Rohacs, 2009; Rohacs and Nilius, 2007).

Since its initial discovery, we and others have addressed various aspects of TRPM7 regulation by PIP$_2$ in detail. Runnels and colleagues were the first to publish about the regulation of TRPM7 activity by the PLC-PIP$_2$ signaling axis (Runnels et al., 2001; Runnels et al., 2002). They had cloned full-length TRPM7 and demonstrated a clear albeit weak interaction between the TRPM7 kinase-domain and PLC$\gamma_1$ and several PLC$\beta$ isoforms that were transiently overexpressed in human embryonic kidney (HEK) cells. They also showed that TRPM7 currents could be induced by (unphysiological) depletion of intracellular Mg$^{2+}$-in whole-cell patch clamp experiments and that such currents were rapidly inhibited by activation of PLC$\beta$ and the subsequent hydrolysis of PIP$_2$. Moreover, the inhibition of TRPM7 currents by PIP$_2$ hydrolysis recovered faster when PIP$_2$ was included in the pipette, and blocking PIP$_2$ resynthesis by wortmannin delayed recovery. Subsequent studies, including our own, have obtained similar results using these conditions, putting forward the idea that PIP$_2$ is necessary to maintain TRPM7 activity (Gwanyanya et al., 2006; Kozak et al., 2005; Langeslag et al., 2007; Macianskiene et al., 2008; Oh et al., 2012; Runnels et al., 2002; Xie et al., 2011).
Whereas data obtained in our group agreed on the absolute necessity of PIP$_2$ for TRPM7 functioning at the plasma membrane, we challenged the view that PLC-mediated PIP$_2$ hydrolysis inhibits the channel (Langeslag et al., 2007). Rather, non-invasive perforated-patch recordings showed that agonists to PLC-coupled GPCRs augment TRPM7 currents at physiological [Mg$^{2+}$] (Langeslag et al., 2007). Thus, both the used electrophysiological technique and the sensitivity of TRPM7 to Mg$^{2+}$ and Mg$^{2+}$-nucleotides are crucial when considering the effect of PIP$_2$ hydrolysis on channel currents: PLC-coupled agonists indeed decreased (rather than increased) TRPM7 currents when [Mg$^{2+}$] was lowered in perforated-patches (using membrane permeable Mg$^{2+}$ chelators), resembling the effect observed with TRPM7 currents induced by Mg$^{2+}$-depletion in traditional whole-cell recordings (Langeslag et al., 2007). In sharp contrast to the complete and sustained inhibition in whole-cell recordings, the inhibition in perforated-patches was partial and transient and TRPM7 currents restored within minutes, reflecting the transient and partial depletion of PIP$_2$ in the perforated patch experiments (Langeslag et al., 2007). Xie and colleagues (2011) used a voltage-sensitive-phosphatase (Ci-VSP) system to deplete PIP$_2$ and inhibit TRPM7 in perforated-patch recordings (Xie et al., 2011). This may seem opposite to the result obtained by Langeslag and colleagues at similar [Mg$^{2+}$], but is likely to reflect the extend of PIP$_2$ degradation by the respective approaches (Langeslag et al., 2007; Xie et al., 2011). If this is true, it suggests that TRPM7 is regulated by PIP$_2$, in a biphasic manner: partial PIP$_2$ depletion potentiates TRPM7 currents until a yet undefined threshold of PIP$_2$ levels is reached that no longer supports TRPM7 activity. Such a bimodal regulatory mechanism may be envisioned if e.g. two PIP$_2$-sensing motifs were present in TRPM7, one with a high(er) and one with a low(er) affinity for PIP$_2$. The C-terminus of TRPM7 harbors several stretches of positively charged amino acids that can potentially interact with the negatively charged phosphates in PIP$_2$ (Langeslag et al., 2007; Xie et al., 2011). In analogy to TRPM8, TRPM5, TRPV5 and TRPM6, channel activity of TRPM7 was impaired by neutralizing-mutations of positively charged residues in the TRP consensus domain (for TRPM7: K1112Q, R1115Q and K1125Q) (Rohacs et al., 2005; Xie et al., 2011). The mutant TRPM6 and TRPM8 channels were shown to have an altered sensitivity towards PIP$_2$ and since these sites are conserved in the TRPM family, a similar result may be expected for TRPM7. Double– or triple-point mutant TRPM7 channels were non-functional and could therefore not be characterized (Xie et al., 2011). The smaller conductance of single-point mutant TRPM7 channels as compared to wildtype channels however was only significant for the K1112-residue. Moreover, the degree of current inhibition was marginal and not as striking as for the other TRP channels (Rohacs et al., 2005; Xie et al., 2011). This remained unexplained, but it suggests that additional factors or residues contribute to interactions between PIP$_2$ and TRPM7 and warrants further detailed investigations. Two further stretches of positive residues, which we termed P1 (aa 1147-
Taken together, it has become evident that TRPM7 requires PIP$_2$ for its activity, but the effect of PLC and PIP$_2$ hydrolysis on TRPM7 currents is complex [see also Chapter 4]. In the end what is most important is to understand the regulation of TRPM7 gating by the PLC-PIP$_2$ signaling axis under physiological conditions, with as little experimental interference as possible. In that respect, it is interesting to note that a number of cell biological investigations indicate that PLC-coupled stimuli potentiate biological processes that involve TRPM7 activity (Callera et al., 2009; Clark et al., 2006; Kim et al., 2005; Langeslag et al., 2007; Wei et al., 2009; Yogi et al., 2009).

The TRPM7 kinase domain
The serine/threonine protein kinase domain at the C-terminal end of TRPM7 has substantial homology to eukaryotic elongation factor-2 kinase (EEF2K) and Dictyostelium myosin heavy chain kinases (MHCK A,B and C), all of which are part of the family of atypical α-kinases (Middelbeek et al., 2010). The TRPM7 kinase-domain undergoes massive autophosphorylation (Clark et al., 2008c; Ryazanova et al., 2001) and requires Mg$^{2+}$ and ATP for its activity, but not Ca$^{2+}$ (Ryazanova et al., 2004). Autophosphorylation appears not essential for catalytic activity, but rather facilitates the recognition and subsequent phosphorylation of substrates (Clark et al., 2008c). Annexin-1 and the myosin II heavy chain (MHC-II) isoforms A,B and C are substrates of the TRPM7 kinase-domain and interactions with these substrates are regulated by Ca$^{2+}$ (Clark et al., 2006; Clark et al., 2008a; Dorovkov and Ryazanov, 2004). Annexins interact with membrane phospholipids upon Ca$^{2+}$-binding and are described to integrate Ca$^{2+}$ signaling with both membrane- and actin dynamics (Gerke et al., 2005; Hayes et al., 2004). Phosphorylation of annexin-1 by TRPM7 is stimulated by Ca$^{2+}$ and might disrupt its interaction with the membrane (Dorovkov et al., 2011). Myosin II heavy chains assemble with myosin II light chains to form the myosin II motor protein. Myosin II bundles actin filaments, creating a network known as the actomyosin cytoskeleton [see Box 2]. MHC-II phosphorylation by TRPM7 is Ca$^{2+}$-dependent and disturbs the structural integrity of myosin II proteins, which leads to relaxation of the actomyosin cytoskeleton and a concomitant decrease in cellular tension (Clark et al., 2006; Clark et al., 2008b).

Desai and colleagues (2012) recently reported a striking new feature of TRPM7: caspase-dependent cleavage of the kinase-domain, thus physically separating it from the channel-domain. This potentiated TRPM7 currents and channel-mediated apoptosis. Although the cleaved kinase-domain retained catalytically active, no biological effect independent of channel function was reported (Desai et al., 2012).
The actomyosin cytoskeleton and cellular tension

The highly dynamic actomyosin cytoskeleton is involved in the generation of mechanical forces and controls cell adhesion and migration. It comprises a network of actin filaments and myosin II motor proteins as well as many associated structural and regulatory proteins. Forces are generated by the polymerization of actin filaments and by the activation of myosin motor proteins. Myosin II dimers assemble into bipolar filaments and connect oppositely oriented actin filaments. Activated motor domains move in opposite directions along the opposing actin filaments, pulling them together, which increases contractility. Myosin II is composed of two heavy chains that include the motor domains (MHC) and associated light chains (MLC; a pair of essential– and regulatory light chains). Phosphorylation of MLC promotes myosin II filament assembly and motor activity, leading to actomyosin contraction, while MHC phosphorylation reduces myosin II filament assembly and result in actomyosin relaxation (Clark et al., 2008b; van Leeuwen et al., 1999).

GTPases of the Rho-family, including Rho and Rac, are important regulators of MLC and MHC (de)phosphorylation. Rho GTPases cycle between a GTP-bound active state and a GDP-bound inactive state and are controlled by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Rho is involved in the generation of actomyosin-based cellular tension by controlling MLC phosphorylation through the Rho-associated kinase ROCK, and it induces the assembly of focal adhesions and stress fibers. Rac is considered to antagonize Rho, reduces cellular tension and stimulates the formation of actin-rich cell protrusions known as lamellipodia (reviewed in Burridge and Wennerberg, 2004).

The actomyosin cytoskeleton is coupled to the ECM through integrin adhesion receptors. Just as the ECM affects the actomyosin cytoskeleton, myosin II-based tension also influences the physical properties of the matrix. These reciprocal interactions are to be tightly balanced in order to maintain proper cell signaling and functioning. This is exemplified by the fact that cellular tension and matrix stiffness determine cell fate, shape tissue integrity, modulate cell adhesion dynamics and drive cell migration [see Box 3], thereby contributing to cancer progression and metastasis (Goetz et al., 2011; Jaalouk and Lammerding, 2009; Paszek et al., 2005; Samuel et al., 2011).
The TRPM7 look-a-like: TRPM6
TRPM6 is TRPM7’s closest relative: it shares ~50% sequence homology with TRPM7, also conducts Mg\(^{2+}\) and contains a similar C-terminal α-kinase-domain. The kinase-domain of TRPM6 is characterized quite poorly, but was shown to phosphorylate TRPM7 (Schmitz et al., 2005) as well as MHC-II isoforms A, B and C (Clark et al., 2008a). In contrast to TRPM7, the expression of TRPM6 is limited to primarily the kidneys, the intestines and the brain. Mutations in the TRPM6 gene are associated with the autosomal-recessive disorder hypomagnesemia and secondary hypocalcemia (Chubanov et al., 2007; Schlingmann et al., 2002; Walder et al., 2002), which is characterized by low serum Mg\(^{2+}\) levels and impaired intestinal and renal Mg\(^{2+}\) (re)absorption. Later, TRPM6 was indeed demonstrated as the molecular component that mediates epithelial Mg\(^{2+}\) transport (Voets et al., 2004). Deficiency or malfunctioning of the one channel-kinase cannot be complemented by the other and therefore TRPM6 and TRPM7 are considered to be functionally non-redundant (Chubanov et al., 2007; Ryazanova et al., 2010; Schlingmann et al., 2002; Schmitz et al., 2005; Walder et al., 2002).

The Mg\(^{2+}\) versus Ca\(^{2+}\) controversy
Of the many mysteries and controversies that still surround TRPM7, the most persistent and intense discussion concerns the significance of Mg\(^{2+}\) permeation for TRPM7 functioning. Two models have arisen in the TRPM7 field; one favoring Mg\(^{2+}\) as the main permeant ion, and the other one Ca\(^{2+}\). More specifically, the proposed models describe that influx of Mg\(^{2+}\) or Ca\(^{2+}\) through the TRPM7 channel locally modulates the activity of downstream effector proteins, either directly or via the kinase-domain. Undoubtedly TRPM7 conducts Mg\(^{2+}\) as well as Ca\(^{2+}\) and both ions are indispensable for cellular function. However, while the dynamic spatiotemporal changes in Ca\(^{2+}\) levels that are driven by the large concentration gradient over the membrane are an established signal transduction element, the idea that Mg\(^{2+}\) may act similarly as a second messenger is highly debated. For instance, the driving force for Mg\(^{2+}\) is much smaller (free \([\text{Mg}^{2+}]_i \approx 0.5-1.0 \text{ mM}; \text{free } [\text{Mg}^{2+}]_e \approx 1.0-1.5 \text{ mM}).\) Although the total cellular Mg\(^{2+}\) content (= 15-20 mM) may change in response to hormonal stimuli, fluctuations in free [Mg\(^{2+}\)] are much less common, profound and dynamic, because of effective Mg\(^{2+}\)-buffering by cells (reviewed in Romani, 2011). Accordingly, spatially restricted Ca\(^{2+}\) signals are a well-accepted concept (Cheng and Lederer, 2008), whereas a similar concept for Mg\(^{2+}\) has yet to be demonstrated. Mg\(^{2+}\) though is an indispensable co-factor for the activity of kinases, transcription factors and numerous other proteins. The above, in combination with the notion that TRPM7’s inward conductance is very low at physiological membrane potentials to begin with, seemingly puts Mg\(^{2+}\) at a considerable disadvantage.
Nonetheless, TRPM7 was originally suggested to be a major influx component for Mg\textsuperscript{2+} and to regulate cellular Mg\textsuperscript{2+} homeostasis. A growth defect in TRPM7-deficient chicken DT-40 B-lymphocytes could be rescued by supplementation of supraphysiological levels of extracellular Mg\textsuperscript{2+} (Schmitz et al., 2003) or by the expression of a Mg\textsuperscript{2+}-transporter (Deason-Towne et al., 2011). In addition to that, TRPM7-deficiency lead to reduced [Mg\textsuperscript{2+}]\textsubscript{i} in DT40 as well as other cells (Abed and Moreau, 2009; Chen et al., 2012; He et al., 2005; Rybarczyk et al., 2012; Zhang et al., 2011). Low extracellular Mg\textsuperscript{2+} on the other hand reduced proliferation and migration of human osteoblasts in response to platelet-derived growth factor (PGDF), as did lowering TRPM7 expression levels by RNA interference (Abed and Moreau, 2009). Furthermore, cytoskeletal alterations and defective directional migration in TRPM7-knockdown fibroblasts (and other cell types) as well as perturbed polarized cell movements during gastrulation in *Xenopus* embryos were largely rescued by either Mg\textsuperscript{2+} supplementation or expression of the Mg\textsuperscript{2+}-transporter SLC41A2 (Liu et al., 2011; Su et al., 2011; Abed and Moreau, 2009; Callera et al., 2009; Rybarczyk et al., 2012).

Investigations using two different TRPM7-knockout mouse models generated independently by the Ryazanov– and Clapham-labs have resulted in conflicting views. Homozygous TRPM7 knockout mice were not vital due to defective embryonic development in both models (Jin et al., 2008; Jin et al., 2012; Ryazanova et al., 2010). Jin and colleagues, therefore, used targeted inducible deletion of TRPM7 in specific tissues to conclude that wildtype and knockout thymocytes and neural stem cells do not differ in the ability to mediate Mg\textsuperscript{2+} influx and have equivalent total Mg\textsuperscript{2+}-contents (Jin et al., 2008). Ryazanova and colleagues, on the other hand, found evidence for (marginal) hypomagnesaemia in heterozygous TRPM7 knock-out mice, especially when fed a Mg\textsuperscript{2+}-deficient diet. Note that these authors did not delete TRPM7, but instead used a gene-targeting vector technique to specifically disrupt its kinase encoding part, which results in TRPM7-Δkinase-mutant proteins. Consequently, the heterozygous mice would express (dysfunctional?) tetrameric TRPM7-chimera channels with any possible combination of subunits that are either wildtype or Δkinase-mutant. In addition, TRPM7 can also form heteromeric channels with TRPM6 (Li et al., 2005; Schmitz et al., 2005) to further complicate the situation.

In contrast to the conflicting reports on Mg\textsuperscript{2+} homeostasis, it is well-documented that TRPM7 regulates basal Ca\textsuperscript{2+}-levels (Clark et al., 2006; Guilbert et al., 2009; Langeslag et al., 2007; Yang et al., 2013) as well as dynamic (receptor-mediated) Ca\textsuperscript{2+} signaling events. Anoxia-induced cell death of cortical neurons appears due to TRPM7-mediated Ca\textsuperscript{2+} overload (Aarts et al., 2003; Nunez-Villena et al., 2011). In numerous other cell types, Ca\textsuperscript{2+} signals mediated by TRPM7 facilitate cell proliferation and cell cycle progression as well as migration (Chen et al., 2010b; Hanano et al., 2004; Kuras et al., 2012; Sun et al., 2013; Wei et al., 2009). Ca\textsuperscript{2+} influx through TRPM7 also contributes to the pacemaker activity of interstitial cells of Cajal.
(Kim et al., 2005) and initiate atrial fibrosis and arrhythmia in response to transforming growth factor β1 (TGF-β1) (Du et al., 2010).

In addition to these global effects, Ca\(^{2+}\) influx through TRPM7 was proposed to exert localized effects too, because the association of TRPM7 with substrates of its kinase-domain (MHC-II and annexin-1) depended on Ca\(^{2+}\) (Clark et al., 2006; Dorovkov and Ryazanov, 2004). The validity of this ‘TRPM7 local Ca\(^{2+}\) model’ was recently supported by the finding of TRPM7-mediated microdomains of Ca\(^{2+}\) influx that were proposed to guide the turning of migrating fibroblasts towards growth factor cues (Wei et al., 2009) [see also Chapter 5].

Where does this now leave us? Certainly, the permeability of TRPM7 for both Mg\(^{2+}\) and Ca\(^{2+}\) are associated with several processes. On top of that, Mg\(^{2+}\) and Ca\(^{2+}\) fluxes affect each other, making it almost impossible to effectively separate the two. Recent reports question the importance of Mg\(^{2+}\) permeation. In part because of the discovery of several new Mg\(^{2+}\) transporters, such as SLC41A1/2, MagT1, Mrs2 and TRPM6 (Romani, 2011). Additionally, conditional and inducible TRPM7 knockout mouse models are viable and do not present with gross disturbances in Mg\(^{2+}\) homeostasis (Jin et al., 2008; Jin et al., 2012; Sah et al., 2013a), and it is hard to envision that fast and dynamic signaling events evoked in response to receptor-stimulation may result from Mg\(^{2+}\) changes. In contrast, local Ca\(^{2+}\) alterations mediated by TRPM7 are now well-established (Wei et al., 2009) [see also Chapter 5]. Finally, it is important to realize that TRPM7 likely also affects cells in ways independent of ion influx altogether (Sah et al., 2013a; Sah et al., 2013b) [see also Chapters 3 and 5].

**TRPM7 in cellular physiology**

The multifunctional character of TRPM7, as a channel and a kinase, render it a protein capable of integrating multiple signaling pathways. In accordance with this notion, TRPM7 has been proposed to function in several fundamental physiological processes.

(i) **Mg\(^{2+}\) homeostasis**: TRPM7 was the first identified ion channel with a significant permeability to Mg\(^{2+}\) and has ever since been put forward as an essential determinant of cellular Mg\(^{2+}\) homeostasis. This view has however also been challenged [for a comprehensive discussion on this matter see subsection ‘The Mg\(^{2+}\) versus Ca\(^{2+}\) controversy’].

(ii) **Proliferation and survival**: There is a vast body of evidence that has identified TRPM7 as an important regulator of cell proliferation and survival. Induced deletion of the TRPM7 gene in DT-40 B-lymphocytes causes growth arrest and subsequent cell death, which was attributed to disrupted Mg\(^{2+}\) homeostasis (Deason-Towne et al., 2011; Nadler et al., 2001; Sahni et al., 2010; Schmitz et al., 2003). Human retinoblastoma cells and human microvascular endothelial cells (HMECs) deficient in TRPM7 arrested in different phases of the cell cycle, which was related to either influx of Ca\(^{2+}\) or Mg\(^{2+}\) through TRPM7 (Baldoli...
and Maier, 2012; Hanano et al., 2004). This suggests that TRPM7 function is differentially controlled during different phases of the cell cycle and indeed TRPM7 was found to be upregulated specifically in the G1 phase in mast cells (RBL-2H3) (Tani et al., 2007). In prostate cancer cells TRPM7 mediated Ca²⁺-dependent proliferation (Sun et al., 2013) whereas TRPM7 silencing in MCF-7 breast cancer cells interfered with proliferation, and TRPM7 expression in grade III tumor samples was associated with the proliferative marker Ki67 and tumor size (Guilbert et al., 2009). Numerous other cell types and animal models were found to require TRPM7 for proliferation (Abed and Moreau, 2007; Du et al., 2010; Jiang et al., 2007; Yee et al., 2011), growth (Elizondo et al., 2005) and survival (Jin et al., 2008; Ryazanova et al., 2010). On the other hand, increased expression of TRPM7 can also promote neuronal cell death, while TRPM7 silencing can promote proliferation of human umbilical vein endothelial cells (HUVECs) (Inoue and Xiong, 2009; Nunez-Villena et al., 2011). Additionally, in contrast to Guilbert and colleagues (2009), we did not observe proliferation differences between TRPM7-silenced and control breast cancer cell lines (MCF-7 and MDA-MB-231), nor did TRMP7 expression correlate with proliferation indicators in primary tumor samples (Middelbeek et al., 2012, see Chapter 2). Furthermore, tissue-specific deletion of the TRPM7 gene recently demonstrated that certain cell types remained viable and grew normally, and therefore do not require TRPM7 for proliferation (Jin et al., 2012; Sah et al., 2013b). Thus, the role of TRPM7 in proliferation depends on cell type and possible compensatory mechanisms. In most of these studies, cation fluxes have been implicated. Further research is required to gain better insight into the responsible mechanism(s) as well as on how TRPM7 itself is controlled during the cell cycle.

(iii) Embryonic development: Three non-conditional TRPM7 knockout mouse lines, generated by different methods, were not viable and died before embryonic day 7.5 (E7.5) (Jin et al., 2008), but conditional deletion of TRPM7 at later stages (E14.5 to adult) did not appear to result in gross developmental defects (Jin et al., 2012). Tissue-specific and timed TRPM7 deletion have illustrated that the development of organs and tissues depends on TRPM7 expression in a spatiotemporally complex manner. The brain, for example, appears to develop normally after TRPM7 deletion at E10.5 whereas kidneys deficient in TRPM7 from E11.5 were morphologically and histologically distinct from wildtype kidneys (Jin et al., 2012). TRPM7 expression in the heart is essential up to E9 (early cardiogenesis) whereas it appears dispensable from E12.5 (late cardiogenesis) (Sah et al., 2013a). Finally, deletion of TRPM7 in thymocytes attenuated their differentiation into mature T-lymphocytes (Jin et al., 2008). As a result thymic architecture was abnormal, which was attributed to the selective loss of medullary cells due to aberrant growth factor expression patterns and subsequent expression and activity of the transcription factor STAT3. Taken together, these results
emphasize that TRPM7’s role in embryonic development may differ depending on during which stage of embryogenesis TRPM7 was deleted and in which progenitor cells.

Several zebrafish mutants (touchtone/nutriaj124e1 / j124e2 / b508 / b722, sweetbreadp75fm / p82mf and touchdownts310c / b508 / mi174) with similar phenotypes were found to correspond to the TRPM7 gene and indeed morpholino-mediated TRPM7 knockdown phenocopied the TRPM7 mutants (Elizondo et al., 2005; Low et al., 2011; Yee et al., 2011). The severity and specifics of phenotypic traits may depend on the specific mutant TRPM7 allele and include; (i) hypopigmentation of the skin due to perturbed development of melanophores; (ii) impaired touch-evoked escape behaviors; and (iii) proliferation-associated defects in development of the skeleton and exocrine pancreas (Elizondo et al., 2005; Low et al., 2011; Yee et al., 2011). Like melanophores in zebrafish, pigment cells derived from the neural crest in mice also require TRPM7 during development (Jin et al., 2012). Why this cell type seems particularly sensitive to TRPM7 expression is, however, not understood.

The early development of Xenopus embryos appears to be critically determined by TRPM7 expression in the dorsal marginal zones, because both TRPM7 upregulation by injection of RNA or its downregulation by morpholinos were shown to severely disrupt gastrulation (Liu et al., 2011).

Taken together, knock-out approaches in different animal models (Elizondo et al., 2005; Jin et al., 2008; Jin et al., 2012; Liu et al., 2011; Ryazanova et al., 2010; Sah et al., 2013a; Sah et al., 2013b; Yee et al., 2011) have illustrated that TRPM7 is expressed from early embryonic stages on and is essential for proper development.

(iv) Adhesion and migration: Cell adhesion and migration critically depend on actomyosin remodeling [see Box 3]. Work from the Frank van Leeuwen-lab, in a continuing collaboration with our group, has established that TRPM7 associates with the actomyosin cytoskeleton via its kinase-domain and controls actomyosin contractility and cell adhesion architecture (Clark et al., 2006; Clark et al., 2008a; Clark et al., 2008b). Overexpression of TRPM7-HA up to 2-3 fold of endogenous levels in mouse neuroblastoma cells (N1E-115) caused increased cell spreading, indicative of decreased cytoskeletal tension (actomyosin relaxation). In accordance with earlier observations by van Leeuwen and colleagues (1999), N1E-115 cell spreading was the result of reduced actomyosin-based cellular tension, which we showed to be (partially) mediated by the TRPM7 α-kinase (Clark et al., 2006; Clark et al., 2008b; van Leeuwen et al., 1999). Furthermore, the reduced cellular tension was accompanied by increased cell adhesion and the formation of specialized cell adhesions known as invadosomes. Finally, TRPM7 was found to localize to invadosomes and activation of TRPM7 by the PLC-coupled receptor agonist bradykinin augmented their formation [see also Chapters 3 and 5]. In accordance with the observations in N1E-115 cells, we and others demonstrated
that shRNA-mediated TRPM7 knockdown in breast cancer cell lines (MDA-MB-231 and MCF-7) and fibroblasts increased cellular tension, accompanied by reorganizations of the actomyosin cytoskeleton (Middelbeek et al., 2012; Su et al., 2011). TRPM7 overexpression in HEK-293 cells on the other hand decreased adhesion by controlling the protease m-calpain through increases of ROS and nitric oxide and subsequent activation of p38 MAP kinase and c-Jun N-terminal kinase (Su et al., 2006; Su et al., 2010). The interactions between TRPM7 and the actomyosin cytoskeleton may be reciprocal as pharmacologically-induced rearrangements of the actomyosin cytoskeleton were demonstrated to regulate TRPM7 currents (Jeong et al., 2006). Further study is required however to fully establish this concept [see also Chapter 3].

The proposed role of TRPM7 as a regulator of the actomyosin cytoskeleton and cell adhesion(s) (Clark et al., 2006) has been supported by the observation that TRPM7 contributes to migration of several cell types. TRPM7 expression is positively correlated with migration in most cell lines examined so far, including neuroblastoma cells [Chapter 3], breast cancer cells (Middelbeek et al., 2012, see Chapter 2) and other tumor cell lines [Table 1], as well as non-tumor cell lines, such as vascular smooth muscle cells, osteoblasts, fibroblasts and T-lymphocytes (Abed and Moreau, 2009; Baldoli and Maier, 2012; Callera et al., 2009; Kuras et al., 2012; Liu et al., 2011; Su et al., 2011; Wei et al., 2009). In contrast, TRPM7 downregulation increased the migration of HEK-293 cells (Su et al., 2006) and recently also human umbilical vein endothelial cells (Baldoli et al., 2013). The mechanism by which TRPM7 would regulate migration, however, is still poorly understood. Some insight was provided by an elegant study of Wei and colleagues (2009), who proposed that microdomains of high Ca\(^{2+}\) at the leading lamella of WI-38 human embryonic lung fibroblasts steer directional migration in response to growth factors or mechanical stimuli. Following TRPM7 knockdown or its pharmacological inhibition, these so-called Ca\(^{2+}\) flickers disappeared, accompanied by a loss of turning of migrating fibroblasts (Wei et al., 2009). In addition, TRPM7 activation by bradykinin caused Ca\(^{2+}\) influx and migration of nasopharyngeal tumor cells (Chen et al., 2010b).

As for the responsible mechanism, we recently demonstrated that TRPM7 affected the migration of breast cancer cells through its regulatory control over myosin II-based cellular tension (Middelbeek et al., 2012, see Chapter 2). The regulation of actomyosin contractility by TRPM7 is mediated through Ca\(^{2+}\)-dependent association between myosin IIA and the TRPM7 kinase-domain (Clark et al., 2006; Clark et al., 2008b). While these observations suggest that TRPM7-mediated Ca\(^{2+}\) influx regulates cell migration, other studies have challenged this view and argue for a prominent role of Mg\(^{2+}\) influx (Abed and Moreau, 2009; Callera et al., 2009; Rybarczyk et al., 2012; Su et al., 2011).

Where the understanding of how TRPM7 regulates migration in vitro is already
poorly defined, its contribution to \textit{in vivo} migratory processes is virtually unknown. The strong requirement for TRPM7 expression during distinct stages of early development and organogenesis, times in which several migration programs are initiated, has in most models so far been attributed to cell survival and proliferation whereas migration has not been addressed \textit{[see previous paragraph ‘Embryonic development’].} However, TRPM7 has been described to affect polarized cell movements during gastrulation in the \textit{Xenopus} embryo (Liu et al., 2011). Cells gain migratory properties during gastrulation because they undergo epithelial-to-mesenchymal transition (EMT). EMT is characterized by the loss of cell polarity and the breakdown of cell-cell adhesions, effectively transforming epithelial cells into cells with mesenchymal traits. Besides the importance of EMT for development, its significance for wound healing and initiation of metastasis has also been widely recognized. Since TRPM7 was found to participate in the regulation of EMT induction in breast cancer cell lines (Davis et al., 2013) and to promote breast cancer metastasis in mouse xenograft experiments (Middelbeek et al., 2012, \textit{see Chapter 2}), this suggests that TRPM7 may play a more general role in migratory processes \textit{in vivo}. The continuing progress that is being made in the field of (intravital) microscopy, combined with the possibility to generate inducible and tissue-specific knockouts in different animal models, will contribute to elucidation of the role of TRPM7 in migration processes \textit{in vivo}.

\textbf{(v) Mechanotransduction:} Mechosensitive or stretch-activated ion channels are triggered by mechanical deformation or stretch of the membrane or cytoskeleton (Sachs, 2010). Several members of the TRP channel family, including TRPM7, have emerged as potential mechosensors (reviewed in Christensen and Corey, 2007; Kuipers et al., 2012; Lin and Corey, 2005; Sharif-Naeini et al., 2008; Yin and Kuebler, 2010). Proposed models that describe the activation of TRPM7 and its functioning in cell processes therefore often include a mechanical component. However, the direct evidence that TRPM7 itself is sensitive to mechanical stimuli is limited and observations are partially conflicting.

Initially, TRPM7-GFP was found to accumulate at the plasma membrane in HEK-293 cells in response to fluid flow (shear force), concomitant with an increase in TRPM7 currents (Oancea et al., 2006). In two consecutive studies, Numata and colleagues (2007) demonstrated that single-channel– and whole-cell TRPM7 currents were augmented by suction via the patch pipette and osmotic cell swelling, respectively (Numata et al., 2007a; Numata et al., 2007b). siRNA targeted against TRPM7 blocked these effects in HeLa and HEK-293 cells. Conditions that do not allow for efficient exocytosis or vesicular trafficking were ineffective, however, indicating that TRPM7 may be directly activated by mechanical stimuli, rather than being incorporated in the plasma membrane as suggested by Oancea and colleagues (Numata et al., 2007a; Numata et al., 2007b; Oancea et al., 2006). Bessac
and Fleig (2007) on the other hand, provided a different explanation by showing in an
elegant set of experiments that TRPM7 was responsive to osmotic gradients and that
the potentiation of TRPM7 currents was directly related to the [Mg^{2+}]. In line with these
observations, whole-cell TRPM7 currents were not affected by cell swelling because the
intracellular Mg^{2+} concentration is clamped by the pipette, which suggests that TRPM7 is not
sensitive to mechanical stretch of the membrane (Bessac and Fleig, 2007). Taken together,
these electrophysiological studies do not unambiguously isolate TRPM7 as a cellular
mechanosensor.

Using Ca^{2+}-imaging to look for intracellular Ca^{2+} signals and channels that regulate
directional cell migration, Wei and colleagues (2009) found high-Ca^{2+} microdomains in
the leading lamella of migrating cells that appeared mediated by TRPM7 (Wei et al.,
2009). Importantly, in addition to growth factors, these so called Ca^{2+} flickers could also
be evoked by mechanical stimuli, including shear stress induced by fluid flow, pulling on a
flexible substrate (cell stretching) and suction (~40 mm Hg) applied through a patch-pipette.
Pharmacological treatments that are considered to affect local membrane tension also
correlated with Ca^{2+} flicker generation (Wei et al., 2009). Ca^{2+} flickers partly overlapped with
focal adhesions, leading to the proposal that Ca^{2+} flickers are evoked by mechanical traction
forces at focal adhesions in migrating cells (Wei et al., 2009). In Chapter 5, we addressed this
hypothesis for cell adhesions in neuroblastoma cells.

The characteristic touch-evoked escape response in zebrafish requires functional
TRPM7 at distinct periods during development (Elizondo et al., 2005; Low et al., 2011).
TRPM7 did not however act as a mechanosensor in the sensory neurons that respond to the
mechanical stimulus. Rather it was suggested that TRPM7 participates in the electrochemical
communication with downstream neurons in the ‘touch-evoked escape neural circuit’ (Low
et al., 2011).

Recapitulating, despite some promising initial observations much work is still required
to establish a general role for TRPM7 in cellular mechanotransduction. It is important to
note that an ion channel is considered a mechanoreceptor when it is directly activated
by mechanical forces (due to either changes in membrane tension or mechanical forces
conveyed through structural elements that are tethered to the channel), while it is not when
it merely responds to mechanically induced signals generated in the cell by other processes
(reviewed by Christensen and Corey (2007); Sharif-Naeni and colleagues (2008); Yin and
Kuebler (2010)). The available evidence suggests that TRPM7 is not a primary mechanosensor
itself but instead acts as a downstream effector protein in a mechanotransduction pathway.
Future work should however proof this concept.
Box 3 | Cell-matrix adhesions and modes of migration

Cell adhesions

Integrin adhesion receptors mediate physical interactions between cells and their environment and couple the actomyosin cytoskeleton through a number of accessory proteins to ECM components. They assemble in cell-matrix adhesions (or cell adhesions), which represent a focal point for cells to communicate with their environment. Cell adhesions act as mechanosensors, for example, and transduce mechanical cues that originate from the ECM, which in turn regulate cell adhesion formation and turnover.

Focal adhesions are considered to mature from focal complexes, which are the first detectable cell adhesions of the (migrating) cell that appear as dot-like structures at the leading lamellipodium. Focal adhesion assembly is initiated by integrin-ECM interactions that trigger integrin activation and the subsequent recruitment of adaptor proteins, such as vinculin, talin and kindlin. Ongoing recruitment of other adaptors and signaling proteins, such as focal adhesion kinase, results in the further maturation of focal adhesions, which ultimately localize as elongated structures at the end of actin-filament bundles named stress fibers [Figure 3A]. The size of focal adhesions is proportional to the level of actomyosin-based tension (Balaban et al., 2001).

Invadosomes are specialized cell adhesions that share several features with focal adhesions; they are dynamic, mechanosensitive, actin-rich complexes [Figure 3B] (Collin et

![Figure 3 | Cell-matrix adhesions. (A) Vinculin (green) highlights elongated focal adhesions that are located at the end of stress fibers in MDA-MB-231 breast cancer cells (red, actin). (B) Vinculin (green) is located in a ring around the actin-dense core (red) of invadosomes in mouse neuroblastoma cells that overexpress TRPM7 (N1E-115/TRPM7). (C) Invadosomes (red, actin) have degraded the underlying matrix (green), as indicated by small black holes in an otherwise confluent layer of gelatin (green, Gelatin-Oregon Green 488). N1E-115/TRPM7 were seeded on coverslips coated with Gelatin-Oregon Green 488 and matrix degradation was assessed after 24 hours. F-actin in A-C was visualized by Alexa-568 phalloidin. Dashed square in A and B indicates zoomed region. Scalebar = 10 µm.](image-url)
al., 2008; Linder et al., 2011; van den Dries et al., 2012). Their distinguishing feature is, however, the ability to degrade ECM-components to permit cell invasion \[Figure 3C\] (Linder, 2007; Linder et al., 2011; Murphy and Courtneidge, 2011). Invadosome is an umbrella term for podosomes and invadopodia, two closely related protrusive cell adhesions. Although these structures are very similar in molecular composition, functional and morphological differences have been proposed, for example; podosomes are considered less stable than invadopodia (turnover rates of several minutes versus hours, respectively) and protrude less into the surrounding matrix. Invadopodia have recently been defined as specific features of invasive tumor cells, whereas podosomes are found in normal cell types that are capable of passing tissue boundaries, such as cells of the monocytic lineage, endothelial cells and smooth muscle cells (Murphy and Courtneidge, 2011). Invadosomes consist of an actin-dense core that is surrounded by a ring of contractile and accessory proteins and they contain many actin-binding and regulatory proteins including vinculin, $\alpha$-actinin, myosin II and the Arp2/3 complex (Block et al., 2008; Wernimont et al., 2008). Their formation, dissolution, architecture and function is influenced by actomyosin-based tension and the stiffness of the underlying matrix (Alexander et al., 2008; Burgstaller and Gimona, 2004; Clark et al., 2006; van Helden et al., 2008) as well as by soluble factors, such as growth factors and reactive oxygen species (Diaz et al., 2009; Eckert et al., 2011; Varon et al., 2006; Yamaguchi et al., 2005). The relevance of invadosome formation \textit{in vivo} has long been debated, but has been convincingly demonstrated in recent years with the discovery of podosomes in VSMCs of isolated aortas (Quintavalle et al., 2010), invasive structures in \textit{C. elegans} anchor cells that invade epidermal basement membranes (Ziel et al., 2009) and invadopodia in metastatic breast cancer cells that are essential for protease-dependent invasion, intravasation and formation of lung metastases (Gligorijevic et al., 2012).

Cell adhesion dynamics and cell migration
Cell migration critically depends on actomyosin remodeling and the generation of mechanical forces. Actin polymerization at the leading edge drives the formation of filopodia and lamellipodia, that are stabilized by cell adhesions. Traction forces at these cell adhesions provide the means for the cell to pull itself forward. Net forward movement requires cell contraction and disassembly of adhesions in the trailing edge of the cell \[Figure 4\] (Ridley et al., 2003).

The above simplified model describes migration in fibroblasts, but other cell types can achieve movement in different ways, often depending on the tissue context (e.g. 2D versus 3D, matrix stiffness and composition), and cells are capable of switching between migration modes depending on the circumstances (Friedl and Wolf, 2010). The migration of individual cells is classified as either mesenchymal-type or amoeboid-type. The mesenchymal mode
of migration, as detailed in Figure 4, is developed by cell types that adhere strongly to the substrate and have highly organized cytoskeletons, such as fibroblasts (Friedl et al., 1998). Amoeboid migration, in contrast, does not involve strong interactions with the ECM. Fast moving cells, such as immune cells, propel forward because of cell body deformations mediated by actin protrusion and myosin II-based tension, and this amoeboid movement is largely integrin-independent as well as protease-independent (Lammermann and Sixt, 2009). In vivo many cells migrate collectively in groups, interconnected by cell-cell contacts.

![Figure 4 | Mesenchymal migration. Typical example of a (randomly) migrating breast cancer cell (MDA-MB-231).](image)

1. A MDA-MB-231 cell adheres to the underlying matrix through focal adhesions that are visualized by Vinculin-GFP and migrates directionally from the right side (trailing edge) towards the left side (leading edge).
2. The rear of the cell is immobile due to strong adhesion to the underlying matrix. In contrast, the leading edge is highly dynamic: actin polymerization drives the extension of the ‘old’ lamellipodium as well as the formation of a new protrusion, both of which require formation of de novo focal adhesions for stabilization.
3. As the cell moves forward by pulling on focal adhesions in the leading edge, the cell stretches and the focal adhesions in the trailing back experience high mechanical (pulling) forces, because of which they increase in size.
4. The cell is changing the direction of migration and therefore retracts the ‘old’ lamellipodium and extents the new protrusion that formed at step 2. Retraction of the ‘old’ lamellipodium requires the disassembly of focal adhesions and extension of the new lamellipodium is driven by actin polymerization.
5. The cell has turned direction, but is still attached at the rear.
6. The last steps of this mesenchymal mode of migration involves disassembly of focal adhesions at the trailing edge and contraction of the cell body. Time interval between frames is 5-10 frames.
Pathological conditions associated with TRPM7

Biological dysfunctioning of TRPM7 has been associated with a number of pathological conditions, including cancer progression, cardiovascular diseases and neurodegenerative diseases.

(i) **Amyotrophic lateral sclerosis - parkinsonism dementia complex (ALS/PDC):** The TRPM7 gene variant T1482I was reportedly associated with ALS/PDC (5 out of 22 Guamanian patients clinically diagnosed with ALS/PDC versus 0 of 23 control patients), but a subsequent study failed to observe this association in a larger patient cohort (Hara et al., 2010; Hermosura et al., 2005).

(ii) **Familial Alzheimer’s disease (FAD):** The (indirect) evidence for a role of TRPM7 in the pathogenesis of FAD is based on observations that presenilin mutations that are associated with FAD cause an imbalance in PIP_2 metabolism. The lowered PIP_2 levels, in turn, suppressed the activity of TRPM7 and subsequent Ca^{2+} influx, as assessed in human embryonic kidney (HEK)-293 cells and Chinese hamster ovarian (CHO) cells (Landman et al., 2006; Oh et al., 2012). Whether TRPM7 activity is affected in more physiological models of FAD, and if so, whether restoring TRPM7 activity protects from the development of this neurodegenerative disorder has not been addressed.

(iii) **Cardiovascular disease:** Mg^{2+} influx through TRPM7 has been implicated in several processes in the vasculature that are associated with hypertension (Touyz, 2008; Yogi et al.,...
The vasoconstrictor agents angiotensin II and aldosterone regulate Mg$^{2+}$-dependent proliferation of vascular smooth muscle cells (VSMCs) through induction of TRPM7 expression (He et al., 2005). In addition, TRPM7 was downregulated in VSMCs of spontaneous hypertensive rats and angiotensin II failed to increase its expression (Touyz et al., 2006). Furthermore, mice with low intracellular Mg$^{2+}$ levels presented with increased blood pressure, impaired endothelial function, abnormal vascular structure and increased expression of vascular inflammation markers and TRPM7 (Paravicini et al., 2009). The pro-inflammatory agent bradykinin was indeed shown to induce TRPM7 expression in VSMCs and bradykinin-mediated upregulation of inflammation markers could be blocked by the TRPM7 inhibitor 2-APB (Yogi et al., 2009). Bradykinin-induced VSMC migration was also demonstrated to require TRPM7-mediated Mg$^{2+}$ signaling (Callera et al., 2009).

TRPM7 has also been proposed to contribute to heart function. Firstly, TRPM7 was upregulated and TRPM7 current density was increased in atrial fibroblasts of patients that suffer from the arrhythmia, atrial fibrillation (Du et al., 2010). Differentiation of fibroblasts into myofibroblasts was increased in these patients as compared to controls and could be decreased by shRNA-mediated knockdown of TRPM7. Moreover, TRPM7-shRNA treatment blocked the induction of myofibroblast differentiation by transforming growth factor β1 (TGF-β1) (Du et al., 2010). Secondly, TRPM7 was recently shown to affect cardiac automaticity in the embryonic myocardium and sinoatrial node, which is important in the control of heart rate. TRPM7 did so via transcriptional regulation of Hcn4 expression, while it did not likely contribute to diastolic Ca$^{2+}$ influx (Sah et al., 2013b). Finally, cardiac-specific TRPM7 deletion in developing mice (between E9 and E13) may lead to cardiac dysfunction, such as heart block and ventricular arrhythmias (Sah et al., 2013a).

(iv) Ischemic stroke: Several lines of evidence argue that TRPM7 contributes to the pathogenesis of ischemic stroke (reviewed in Bae and Sun, 2011). Exposure to prolonged oxygen glucose deprivation (OGD) induces formation of reactive oxygen species (ROS) and Ca$^{2+}$ overload in cortical neuron cultures, and this effect is reduced upon TRPM7 knockdown (Aarts et al., 2003). In addition, the characteristic current induced by OGD, I$_{OGD}$, has all the hallmarks of TRPM7 (I$_{MIC/MagNuM}$). Thus TRPM7 contributes significantly to OGD. Follow-up studies demonstrated that hypoxia and ROS (H$_2$O$_2$) activate TRPM7 channels, increasing neuronal injury in primary cortical neurons due to either Ca$^{2+}$ overload or possibly influx of Mg$^{2+}$ or Zn$^{2+}$ (Coombes et al., 2011; Inoue et al., 2010; Zhang et al., 2011).

Importantly, in vivo models of ischemic stroke have substantiated the in vitro results. TRPM7 becomes upregulated in hippocampal neurons in mice challenged with ischemic insults and high TRPM7 expression levels are maintained during subsequent reperfusion. Conversely, TRPM7 expression levels were reduced after application of neuronal growth
factor (NGF), which is considered a neuroprotective factor during ischemia (Jiang et al., 2008) or after application of neuroprotective Ginsenoside-Rd (Zhang et al., 2012). Suppression of TRPM7 in CA1 hippocampal neurons by *in vivo* injections of shRNA also prevented ischemia-associated deficits (Sun et al., 2009). On the other hand, TRPM7 gene variants (single nucleotide polymorphisms or SNPs) in humans were not associated with risk of ischemic stroke in a prospective case-control study (Romero et al., 2009).

(v) **Cancer:** There is compelling evidence that TRPM7 is an important player in cancer progression. TRPM7 plays pivotal roles in cancer aggressiveness, particularly in cell proliferation, survival, adhesion and migration. Moreover, several studies have reported that TRPM7 expression is increased in primary tumor samples. The potential role of TRPM7 in pathophysiological processes that underlie cancer is summarized in Table 1 and addressed in greater detail in the next section. I particularly concentrate on TRPM7's control of actomyosin remodeling, cell adhesion and Ca\(^{2+}\) signaling, because that has been the focal point of this thesis [see Chapters 2, 3 and 5].

**Cancer and tumor cell metastasis: The TRPM7 connection.**
Cancers are clinically very heterogeneous and even the different cancer subtypes show large heterogeneity [see Box 4], a fact with important consequences for the success and efficacy of treatment. Recent research efforts therefore concentrate on the identification of molecular factors that may explain cancer heterogeneity and subsequent classification of patients in different treatment groups.

**Tumorigenesis and metastasis**
The malignant transformation of normal cells has been characterized by general principles, regardless of the cancer type (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). First, a tumor forms due to accumulation of mutations that ultimately disturb the intricate balance of proliferation and cell death (apoptosis), leading to its uncontrolled and limitless growth. Whereas TRPM7 has been shown to be required for cell proliferation and cell survival in diverse cell types it does not appear that TRPM7 generally provides a growth advantage to cells or protect them from apoptosis [see paragraph ‘Proliferation and survival’ in subsection ‘TRPM7 in cellular physiology’]. Like any other tissue or organ, further tumor growth relies on the supply of oxygen and nutrients and the disposal of metabolic waste material by the cardiovascular system. Growing tumors, therefore, induce and sustain neovascularization through the secretion of pro-angiogenic factors that act on endothelial cells. A tumor is considered malignant when the tumor cells are transformed and have lost the differentiated phenotype of the tissue of origin.
Figure 5 | Metastatic dissemination (of carcinomas) step-by-step. (A) (1) Cells exist in the context of a 3D environment that comprises other cells and ECM. The reciprocal interactions between cells and their environment are tightly balanced and coordinated to reach a state of tissue homeostasis. (1 → 2) Any (combination) of a diverse set of defined – and yet unknown factors can induce the malignant transformation of a cell. This includes aberrant signaling due to dysregulated interactions between cells and their environment. (2) A transformed cell grows out to become a tumor after it has acquired specific traits that increase its growth potential and enable it to resist anti-growth signals and apoptosis. While the primary tumor affects its surroundings, disturbing tissue homeostasis, this tumor microenvironment itself also affects the tumor. An altered composition of the ECM, increased ECM stiffness and immune cell infiltration are for example known determinants of cancer progression and correlates of tumor aggressiveness. (2 → 3) Tumor cells in the primary tumor acquire metastatic traits, such as anchorage-independence, loss of cell-cell contacts and the ability to migrate. (3) These metastatic tumor cells are highly invasive and complete a complex metastatic cascade (B) to establish secondary tumors in a distant tissue. (B) Cancer progression or metastasis involves a number of steps that occur in two major phases; dissociation and colonization. (continued on next page)
From this point on, further progression of tumors proceeds via several well-defined steps that together constitute the metastatic cascade [Figure 5]. Metastasis involves dissociation of tumor cells from the primary tumor, migration through stroma and across tissue boundaries (invasion) towards the vasculature and entry into the circulation (intravasation). Subsequently, tumor cells are to survive in the bloodstream, escape from the vasculature (extravasation), and finally, adapt to a new environment to colonize distant sites (Chaffer and Weinberg, 2011). Metastatic spread is the hallmark of malignant cancer and the main cause of cancer-related death (Chaffer and Weinberg, 2011). However, many of the individual steps in the metastatic cascade are only poorly understood at a mechanistic and cellular level. Genetic and molecular insight into what factors determine the metastatic potential of tumor cells might, therefore, improve the identification of patients at high risk. To this end, we have explored the functional consequences of differential TRPM7 expression in neuroblastoma and breast cancer cells, with a specific focus on the acquisition of metastatic features as discussed in Chapters 2 and 3 [see also Box 4 for an overview of the clinical features of breast cancer and neuroblastoma].

**Tumor cell adhesion and migration in metastasis**

For a tumor cell to successfully metastasize and form a secondary tumor it needs to acquire specific metastatic traits, most notably loss of cell-cell adhesion, anchorage-independent growth and migration. As mentioned before, EMT-like processes are considered important drivers of such malignant transformation (Kalluri and Weinberg, 2009). In non-epithelial cancers trademarks of EMT have been observed too, such as up- and downregulation of EMT-associated transcription factors, partly mimicking EMT and therefore termed EMT-like processes (Kahlert et al., 2013). EMT is evidenced by drastic reprogramming of the cell's transcriptional profile, altering the expression of many markers such as Snail, Twist and E-cadherin (Peinado et al., 2007), as well as by phenotypic and morphological changes. EMT induction can proceed via a variety of factors and mechanisms, including (Ca\(^{2+}\)-dependent) growth factor signaling (Davis et al., 2013; Graham et al., 2008; Lo et al., 2007; Yilmaz and

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**Figure 5 | continued.**

(3 - Tumor microenvironment) Dissociation involves the escape from the primary tumor, which requires the loss of cell-cell contacts, passage of tissue boundaries (basement membrane), migration through the stroma and entry into the circulation (local invasion and intravasation). Next, anchorage-independence allows tumor cells to survive transportation in the circulation. (3 - Distant tissue) Colonization begins with the adhesion of circulating tumor cells to the vessel wall after which they exit the circulation and migrate into the surrounding tissue of a distant organ (extravasation). Finally, only tumor cells that are able to adapt to the new environment and that can initiate proliferation survive and may establish a secondary tumor at a distant site.
Table 1 | Overview of TRPM7 in tumor cell migration and tumor progression.

<table>
<thead>
<tr>
<th>Model system</th>
<th>Assayed by</th>
<th>Effect / Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast adenocarcinoma</td>
<td>MCF-7 knockdown</td>
<td>Proliferation (possibly Ca(^{2+}))</td>
<td>Guilbert, 2009</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231; MCF-7 stable (shRNA) knockdown</td>
<td>Migration (actomyosin contractility)</td>
<td>Middelbeek, 2012 (see Chapter 2)</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231; MDA-MB-435S; MCF-7 knockdown and overexpression</td>
<td>Migration (kinase-dependent and channel-independent)</td>
<td>Guilbert, 2013</td>
</tr>
<tr>
<td>Animal model</td>
<td>Experimental metastasis (tail-vein injections) stable (shRNA) knockdown</td>
<td>MDA MB-231 cells with TRPM7 knockdown establish fewer metastases than control cells</td>
<td>Middelbeek, 2012 (see Chapter 2)</td>
</tr>
<tr>
<td>Tissue</td>
<td>Adenocarcinoma and matched noncancerous control tissue IHC (26 paired samples) and Semiquantitative PCR (6 samples)</td>
<td>Stronger staining and higher mRNA expression in tumor sections (correlation with grade III tumors with a higher Ki67 score or tumor size)</td>
<td>Guilbert, 2009</td>
</tr>
<tr>
<td></td>
<td>Ductal adenocarcinoma tissue and adjacent non-tumoral tissue IHC and RT-PCR (59 samples)</td>
<td>TRPM7 overexpressed in tumor samples versus control and association with proliferative parameters (grade, Ki67 index and tumor size)</td>
<td>Dhennin-Duthille, 2011</td>
</tr>
<tr>
<td></td>
<td>Expression analysis in two independent patient cohorts (368 and 144 patient samples) Microarray and RT-qPCR (6 samples)</td>
<td>Negative correlation with recurrence-free survival and distant metastasis-free survival. No correlation with proliferative markers and tumor size.</td>
<td>Middelbeek, 2012 (see Chapter 2)</td>
</tr>
<tr>
<td></td>
<td>ER- invasive ductal carcinoma versus non-invasive areas IHC (8 paired samples)</td>
<td>Stronger staining in tumor areas.</td>
<td>Guilbert, 2013</td>
</tr>
<tr>
<td>Colorectal adenoma</td>
<td>Case-control study 688 adenoma cases, 210 hyperplastic polyp cases and 1306 polyp-free controls Genotyping (linkage analysis for the Thr1482Ile polymorphism)</td>
<td>The 1482Ile allele is associated with higher risk for development of adenoma or hyperplastic polyps (especially in combination with high Ca(^{2+}):Mg(^{2+}) diets).</td>
<td>Dai, 2007</td>
</tr>
<tr>
<td>Gastric adenocarcinoma</td>
<td>Cell line(s) DT-40 lymphocytes (chicken) knockdown and pharmacological inhibition</td>
<td>Survival and apoptosis (Mg(^{2+})).</td>
<td>Kim, 2008, 2011</td>
</tr>
<tr>
<td>Haematopoietic cancer</td>
<td>Cell line(s) AGS knockout</td>
<td>Proliferation/quiescence (Mg(^{2+})).</td>
<td>Schmitz, 2003</td>
</tr>
<tr>
<td></td>
<td>RBL-I (rat); Jurkat T-cell lymphoma pharmacological inhibition by waixenicin A</td>
<td>Proliferation (G0/G1 arrest).</td>
<td>Sahni, 2010</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Model system #1</th>
<th>Assayed by</th>
<th>Effect / Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head and neck cancer</td>
<td>Cell line(s) Squamous cell carcinoma (FaDu; SCC25)</td>
<td>knockdown and pharmacological inhibition</td>
<td>Proliferation (Ca²⁺).</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma (5-8F; 6-10B)</td>
<td>knockdown and overexpression</td>
<td>Migration (Ca²⁺). Higher TRPM7 expression in a metastatic cell line compared to a non-metastatic control.</td>
<td>Chen, 2010</td>
</tr>
<tr>
<td>Lung adeno carcinoma</td>
<td>Cell line(s) A549</td>
<td>knockdown and pharmacological inhibition</td>
<td>EGF promotes migration through upregulation of TRPM7.</td>
</tr>
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<td>Neuro blastoma</td>
<td>Cell line(s) N1E-115 (mouse)</td>
<td>stable overexpression</td>
<td>Adhesion and migration.</td>
</tr>
<tr>
<td>Animal model Experimental metastasis (tail-vein injections)</td>
<td>stable overexpression</td>
<td>TRPM7 overexpressing N1E-115 cells establish more metastases than control cells.</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Tissue Expression analysis in two independent patient cohorts</td>
<td>microarray (88 and 251 patient samples)</td>
<td>TRPM7 expression does not correlate with patient survival (or other clinical parameters).</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Pancreatic adeno carcinoma</td>
<td>Cell line(s) Bx-PC3; PANC-1; MIA-Pa-Ca2; PL45; CAPAN-1; HPAF-II; Panc 02.03</td>
<td>knockdown and RT-qPCR</td>
<td>Proliferation (Mg²⁺) Increased expression in 5 out of 7 tumor cell lines.</td>
</tr>
<tr>
<td></td>
<td>Bx-PC3</td>
<td>knockdown</td>
<td>Migration (Mg²⁺).</td>
</tr>
<tr>
<td></td>
<td>Bx-PC3; PANC-1</td>
<td>knockdown</td>
<td>Senescence (Mg²⁺).</td>
</tr>
<tr>
<td>Animal model Zebrafish</td>
<td>swd(p75m) and TRPM7(j124e1) mutants</td>
<td>Proliferation (Mg²⁺).</td>
<td>Yee, 2011</td>
</tr>
<tr>
<td>Tissue Healthy exocrine pancreatic tissue versus adenocarcinoma tissue</td>
<td>IHC (18 samples) RT-qPCR (6 samples)</td>
<td>Upregulation in tumor samples. Intensity of TRPM7 staining correlates with tumor grade and is inversely associated with overall survival.</td>
<td>Rybarczyk, 2012</td>
</tr>
<tr>
<td>Control ductal epithelial tissue versus adenocarcinoma tissue</td>
<td>IHC (5 samples)</td>
<td>Stronger staining in tumor samples or areas.</td>
<td>Yee, 2011</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>Cell line(s) DU-145; PC3 and control RWPE cells</td>
<td>knockdown and overexpression</td>
<td>Proliferation (Ca²⁺).</td>
</tr>
<tr>
<td>Tissue Control and tumor samples</td>
<td>RT-qPCR (4 samples)</td>
<td>Increased expression in tumor samples.</td>
<td>Sun, 2013</td>
</tr>
<tr>
<td>Retino blastoma</td>
<td>Cell line(s) Y79</td>
<td>knockdown</td>
<td>Proliferation (Ca²⁺).</td>
</tr>
</tbody>
</table>

#1 All cell lines are of human origin unless indicated otherwise.
Christofori, 2009). TRPM7 has recently been identified in a targeted siRNA-based screen as a powerful facilitator of Ca\(^{2+}\)-dependent EMT induced by epidermal growth factor (EGF) in breast cancer cells (Davis et al., 2013). However, the involvement of TRPM7 in this process needs further study as TRPM7 knockdown did only affect part of the many EMT markers that are commonly activated downstream of the EGF receptor (Davis et al., 2013).

In addition to the loss of cell-cell contacts, metastatic transformation involves dynamic regulation of integrin-mediated cell-matrix adhesions. Changes in the integrin expression profile of tumor cells, such as induction of \(\alpha\nu\beta3\), enhance their ability to migrate and to form metastases and correlates with tumor aggressiveness in several human cancers (Felding-Habermann et al., 2001; Pecheur et al., 2002; Sloan et al., 2006; Moschos et al., 2007). In addition, anchorage-independent growth (or anoikis-resistance) enables disseminating tumor cells to survive independent from cell-matrix interactions in otherwise lethal environments, such as the blood circulation or the lymphatic system (Guadamillas et al., 2011; Kim et al., 2012). The acquisition of migratory potential is another characteristic feature of disseminating tumor cells. As detailed in Box 3, cell migration demands the modulation of cell-cell and cell-matrix interactions, adjustments of cell shape and control of actomyosin-based cellular tension to generate and withstand mechanical (traction) forces. These processes are driven by continuous and tightly controlled reorganizations of the actomyosin cytoskeleton. Members of the family of Rho GTPases, especially Rho, Rac and Cdc42, are key regulators of actomyosin remodeling and cellular tension [see Box 2 and 3]. The importance of properly controlled actomyosin remodeling, cellular tension and migration for cellular physiology and tissue homeostasis is emphasized by the fact that deregulated Rho GTPase signaling is associated with tumorigenesis and metastasis of several human cancers (reviewed in Ellenbroek and Collard, 2007). TRPM7, the new player in this game, affects cellular tension, cell-matrix interactions and migration [see paragraph ‘Adhesion and migration’ in subsection ‘TRPM7 in cellular physiology’ and see Chapters 2, 3 and 5]. Consequently, altered TRPM7 activity or expression levels may increase the metastatic potential of tumor cells, leading to higher risk of metastasis formation and associated worse patient prognosis [see Table 1 and Chapters 2 and 3]. It is, however, currently largely unknown what aspects of TRPM7-mediated signaling potentiate the metastatic potential of tumor cells, and secondly, during which steps of the metastatic cascade this is activated or required.

Ca\(^{2+}\) signaling in cancer
Most studies on TRPM7 have concentrated on the function of TRPM7 as an ion channel, with particular focus on the activation and gating mechanisms and its permeability to Ca\(^{2+}\) and Mg\(^{2+}\). Considering the prominent regulatory role of Ca\(^{2+}\) signaling in a variety of cancer-
relevant processes, such as proliferation, apoptosis, angiogenesis and migration [see Box 1 and 3], it should come as no surprise that other Ca\textsuperscript{2+} signaling proteins, beside TRPM7, have been associated with cancer too. These include TRPM8 and TRPV6, store-operated Ca\textsuperscript{2+} channels (Orai1) and Ca\textsuperscript{2+} transporters (PMCA, SERCA and SPCA), as recently reviewed elsewhere (Chen et al., 2013; Fiorio et al., 2012; Lee et al., 2011; Monteith et al., 2012; Prevarskaya et al., 2011). Our understanding of how dysregulation of Ca\textsuperscript{2+} signaling may increase the metastatic potential of tumor cells is improving slowly but steadily. Many questions remain however. To name a few: What Ca\textsuperscript{2+} transporter or channel plays what role during what stage of the process? Which Ca\textsuperscript{2+} channel is involved in which cancer? Are there common principles for functioning of these Ca\textsuperscript{2+} channels in tumorigenesis and metastasis? Which channels with altered expression in cancer could represent biomarkers or pharmaceutical targets? And, lastly, in the light of the increasing attention for Mg\textsuperscript{2+}; What role does Mg\textsuperscript{2+} permeation play in these processes? (see e.g. Castiglioni and Maier, 2011; Wolf and Trapani, 2012).

**Box 4 | Breast cancer and neuroblastoma**

To provide some clinical and pathological background for Chapters 2 and 3, breast cancer and neuroblastoma are here briefly introduced.

**Breast cancer**

Breast cancers are among the most frequent cancers (Ferlay et al., 2010) and commonly originate from epithelial cells that line the milk ducts (ductal carcinoma) or lobules (lobular carcinoma). The main metastatic sites are lung, liver and bone (Weigelt et al., 2005). It is a heterogeneous disease, both in etiology and responsiveness to treatment, and efforts to optimize the treatment of individual patients have focused on the optimal (sub)classification of breast cancers. This has led to the development of a set of classification tools that are currently combined to diagnose breast cancer and to decide on the most appropriate therapy. Light microscopy of primary tumor tissue sections is used to assess tumor grade; loss of the differentiated appearance of normal breast tissue. Tumor grade ranges from low grade (G1: well differentiated), intermediate grade (G2: moderately differentiated) and high grade (G3+4: poorly differentiated or undifferentiated). Assessment of tumor stage according to the TNM system is based on the size of the primary tumor (T) and its spread to nearby lymph nodes (N) and to more distant organs (M). In recent years, the clinical and pathological factors described above have been supplemented with gene expression profiling. This allows for the molecular characterization of tumor samples and luminal (A and B), basal-like and HER2-enriched are the best described, and most common, subtypes identified. The
majority of tumors classified as basal-like are defined as triple-negative, which refers to the lack of expression of the estrogen receptor (ER), the progesterone receptor (PR) as well as the human epidermal growth factor receptor (HER2/neu) (Schnitt, 2010). Receptor status is an important clinical feature and together with molecular markers categorizes patients into therapeutic groups. Consequently, receptor status is a potential confounding factor that needs to be taken in consideration in statistical analyses that test the prognostic value of candidate genes and expression signatures for tumor progression. Breast cancer cell lines that are commonly used in the lab also differ in hormone receptor expression patterns and therefore represent different breast cancer subtypes or therapeutic groups.

**Neuroblastoma**

Neuroblastoma is the most common solid tumor of childhood and originates from precursor cells derived from the neural crest. Primary tumors form in tissues of the peripheral sympathetic nervous system, most commonly in the adrenal glands. The main metastatic sites of neuroblastoma are the liver, bone and bone marrow (DuBois et al., 1999). Patients that present with localized tumors typically have a good prognosis for recovery after surgical removal (and chemotherapy) and in some cases the tumor will regress spontaneously to a benign phenotype (Hero et al., 2008). Neuroblastoma is a very heterogeneous cancer though and the majority of patients present with widespread metastasis at diagnosis, categorized as high-risk disease, and have a poor prognosis, despite aggressive therapy regiments. Neuroblastomas are classified at diagnosis according to the new International Neuroblastoma Risk Group Staging System (INRGSS) that is based on tumor imaging by CT and MRI: Stage L1 and L2 tumors are localized tumors without or with image-defined risk factors, respectively. Stages M or MS are defined as (distant) metastatic disease, with for Stage MS the additional characteristics that metastasis is restricted to skin, liver or bone marrow in patients younger than 18 months (Monclair et al., 2009). Patient prognosis and treatment are in turn based on tumor stage, age at diagnosis, tumor grade, DNA ploidy and *MYCN* gene amplifications, resulting in four pre-treatment risk groups: very low, low, intermediate, and high risk. *MYCN* is considered the golden standard prognostic marker in neuroblastoma, yet it only accurately predicts patient outcome in a subset of patients and therefore current and future investigations are focused on the genetic profiling of tumors in an attempt to identify new prognostic markers and metastatic or high-risk signatures (Asgharzadeh et al., 2006).
Thesis outline

While TRPM7 has emerged as an exciting new component of the molecular toolkit that regulates cell adhesion, the specific function of TRPM7 is poorly defined [see paragraph ‘Adhesion and migration’ in subsection ‘TRPM7 in cellular physiology’]. TRPM7 may depend on both conductive properties as well as its enzymatic activity to control adhesion. We have investigated the effect of global and localized Ca\textsuperscript{2+} influx through TRPM7 on the actomyosin cytoskeleton, cellular tension and cell adhesion. Given that Ca\textsuperscript{2+} signaling and cellular tension have been clearly linked to metastasis and aggressiveness of tumor cells, we formulated the hypothesis that TRPM7 makes a decisive contribution to metastasis.

In Chapters 2 and 3 we used a multidisciplinary approach and investigated the effect of differential TRPM7 expression levels for the invasive potential of tumor cells and metastasis. In Chapter 2 we analyzed the mRNA expression profiles of primary breast carcinoma tumors and the associated patient survival plots and pathological parameters from two independent breast cancer patient cohorts and found that higher TRPM7 expression levels correlated with metastasis. Subsequent biochemical– and cell biological assays revealed that the motility of otherwise highly invasive mammary carcinoma cell lines was greatly diminished by lowering TRPM7 expression levels, as was their ability to develop into metastases in mouse xenograft experiments. Our studies into neuroblastoma provide further support for a regulatory role of TRPM7 in cancer progression and metastasis, as described in Chapter 3. Mild overexpression of TRPM7 in a mouse neuroblastoma cell line (N1E-115) greatly improved the cell adhesive– and migratory properties of these otherwise poorly adhesive and moderately motile cells. In vivo, these findings were reflected in an enhanced metastatic potential and the resulting pronounced formation of metastases. In addition, we demonstrated that TRPM7 is part of a cytoskeletal complex whereof the vast majority of components are known regulators of cellular tension and cell adhesion dynamics. Finally, expression levels of several of these ‘TRPM7 interactors’ associated with patient survival in two independent neuroblastoma patient cohorts.

In Chapters 4 and 5 we employed fluorescence microscopy and biophysical techniques to study the signaling pathways upstream and downstream of TRPM7 activation, with a focus on Ca\textsuperscript{2+} permeation. Chapter 4 describes the characterization of TRPM7 gating in response to G\textsubscript{q}\textsubscript{11}-protein-coupled receptor stimulation. We present evidence for a prominent and essential regulatory role of PLC\textsubscript{δ1} and PIP\textsubscript{2} (hydrolysis) to initiate and maintain opening of the TRPM7 channel. Moreover, our findings illustrate that TRPM7 activity regulates PIP\textsubscript{2} levels and we showed that TRPM7 opening is governed by a unique feedforward activation loop. In Chapter 5 we examined the role of TRPM7-mediated Ca\textsuperscript{2+} influx in cell adhesion as formulated in our working model [Figure 6]. We developed TIRF-based Ca\textsuperscript{2+}-imaging methodology and image-analysis routines and used that to demonstrate that TRPM7
can generate highly repetitive Ca\(^{2+}\) events that are localized to distinct microdomains in mouse neuroblastoma cells (N1E-115). The appearance of these Ca\(^{2+}\) sparks, however, did neither correlate with the spatial distribution of invadosomes nor modulate their dynamics noticeably. Importantly, the results in Chapter 5 require us to reconsider the idea that Ca\(^{2+}\) influx through the TRPM7 channel is a dominant factor in regulating TRPM7-mediated actomyosin remodeling and cell adhesion dynamics.

In Chapter 6, the key thoughts about TRPM7 that arise from our studies are summarized and discussed in relation to the available literature.

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**Figure 6 | Working model of TRPM7s functioning in cell adhesion and actomyosin remodeling.** TRPM7 activation can proceed via a number of proposed mechanisms, including PLC-mediated PIP\(_2\) hydrolysis and mechanical force. Upon local activation, TRPM7 sets up a microdomain of high Ca\(^{2+}\) levels and low PIP\(_2\) levels (1) which sustains TRPM7 activation (2) and triggers both the (de)activation of Ca\(^{2+}\)- and PIP\(_2\)-sensitive downstream effector proteins (3) as well as the association of the TRPM7 kinase-domain with its substrates, such as myosin II heavy chain isoforms (4). This ultimately results in the reorganization of the actomyosin cytoskeleton and tuning of cellular tension, which in turn affect cell adhesion architecture and –dynamics (5). While we here specifically describe localized TRPM7-mediated signaling, as could occur at cell adhesions, cell-wide TRPM7 activation would proceed via the same model in which the spatial constraint of TRPM7-mediated signals is lost.
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