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Review

The multifaceted role of TMEM16A in cancer

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ABSTRACT

The calcium-activated chloride channel TMEM16A is intimately linked to cancers. Over decades, TMEM16A over-expression and contribution to prognosis have been widely studied for multiple cancers strengthening the idea that TMEM16A could be a valuable biomarker and a promising therapeutic target. Surprisingly, from the survey of the literature, it appears that TMEM16A has been involved in multiple cancer-related functions and a large number of molecular targets of TMEM16A have been proposed. Thus, TMEM16A appears to be an ion channel with a multifaceted role in cancers.

In this review, we summarize the latest development regarding TMEM16A contribution to cancers. We will survey TMEM16A contribution in cancer prognosis, the origins of its over-expression in cancer cells, the multiple biological functions and molecular pathways regulated by TMEM16A. Then, we will consider the question regarding the molecular mechanism of TMEM16A in cancers and the possible basis for the multifaceted role of TMEM16A in cancers.

1. Introduction

Ion channels are critical for regulating ion homeostasis in any cells and thus are involved in a wide variety of physiological processes. In recent decades, a growing literature describes a contribution of ion channels in all aspects or hallmarks of cancer [1,2]. Thus, ion channels could represent an unprecedented reservoir of unused therapeutic targets. Investigating their molecular mechanisms in cancer cells is crucial to both improve our understanding of the contribution of ion channels in their cancer-related mechanisms and to better define drugs and conditions for using ion channels as therapeutic targets in cancers.

TMEM16A, also known as ANO1, DOG1, ORAOV2, or TAOS2, is a calcium-activated chloride channel (CaCC) [3–5] with physiological functions in epithelial tissues, exocrine glands, dorsal root ganglion neurons and smooth muscle [6–8]. Before its molecular identification as CaCC in 2008, TMEM16A/DOG1 was described as a biomarker for gastro-intestinal squamous cancer (GIST) [9]. Since then, a number of investigations has commented its over-expression in various cancers including Head and Neck Squamous Cancer Cells (HNSCC), Breast, Prostate, Pancreatic, Gastric, Parotid and Colorectal (CRC) cancers.

However, despite the consensus regarding its over-expression in cancers, a surprising range of the biological and molecular functions associated to TMEM16A emerged from the literature suggesting that TMEM16A has a multifaceted role in cancers. In this review, we will summarize evidence regarding TMEM16A expression in cancers and its associated biological and molecular functions. We will then review our understanding of the molecular mechanism of TMEM16A in cancers and propose hypothesis that could explain the multifaceted role observed for TMEM16A in cancers.

2. TMEM16A expression in cancer

TMEM16A is intimately associated to cancer. Historically known as DOG1 – a biomarker of Gastro Intestinal Squamous Carcinoma (GIST)

Abbreviations: 14-3-3γ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (YWHAG); α-SMA, α-smooth muscle actin; ANO1, anoctamin-1; Bcl-2, B cell lymphoma 2; CaCC, calcium activated chloride channel; CDK, cyclin-dependent kinase; COPB1, coatomer protein complex subunit beta 1; CRC, colorectal cancer; DOG1, discovered on gastrointestinal stromal tumors protein 1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptors; ERM, Ezrin-Radixin-Moesin; FADD, Fas-associated protein with death domain; GIST, gastro intestinal squamous tumors; Gli, glioma-associated oncogenes; HDAC3, histone deacetylase 3; HER2, human epidermal growth factor receptor 2; HNSCC, head and neck squamous carcinoma cells; HPV, human papilloma virus; IL-4, interleukin-4; IP_3, inositol triphosphate; IP_3R, inositol triphosphate receptor; MCL-1, myeloid cell leukemia 1; MMP, matrix metallopeptidase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLA, proximity-ligation assay; PLC, phospholipase C; SOCE, store-operated calcium entry; STAT3, signal transducer and activator of transcription 3; TGF-β, tumor growth factor-β; TNF-α, tumor necrosis factor-α; WNK, (with-no-lysine) WNK lysine deficient protein kinase; ZO-1, Zonula Occludens-1

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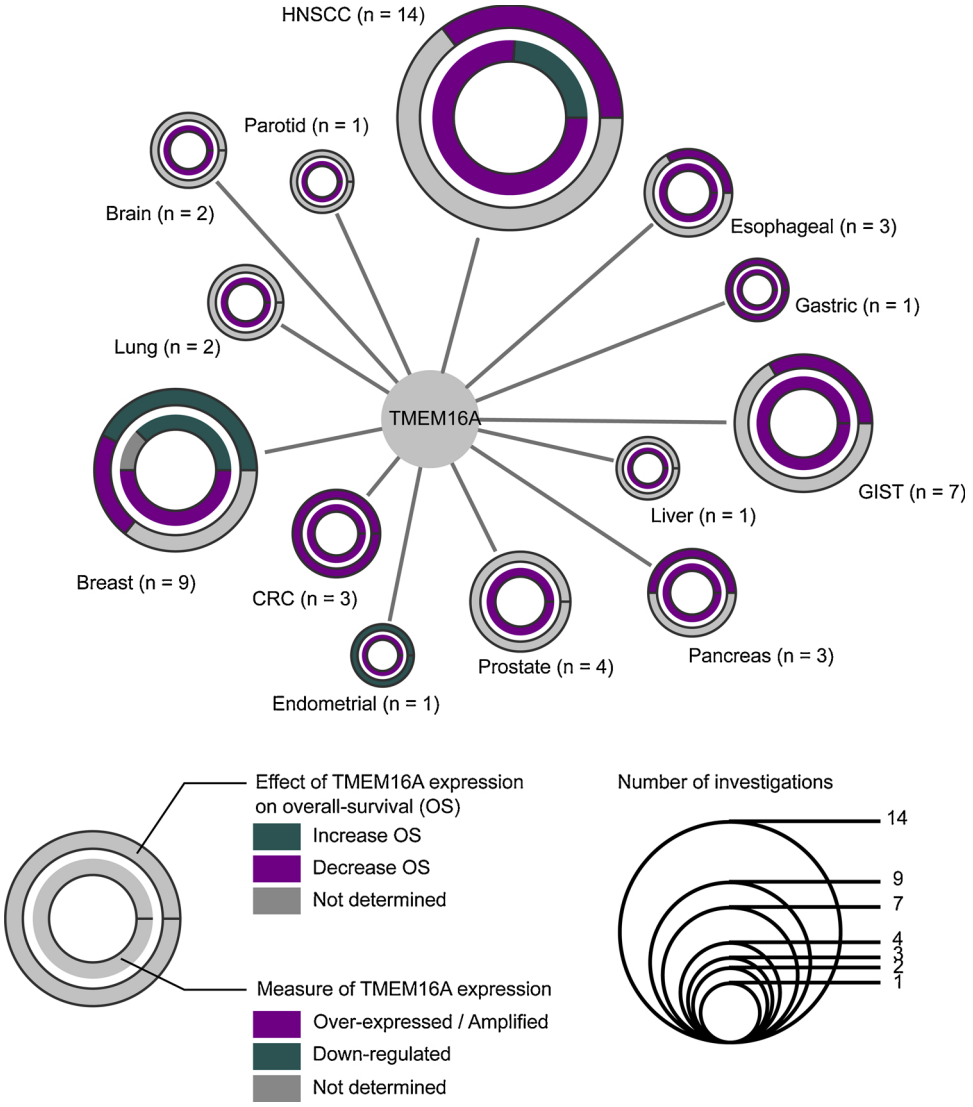

Fig. 1. Expression and contribution to the overall survival of TMEM16A in cancers.
Node size is defined by the number of different articles investigating TMEM16A expression or amplification and/or TMEM16A contribution to the overall survival. The number of different articles per cancer is noted in parenthesis. Inner circle represents the distribution of results obtained from the literature per cancer types regarding TMEM16A expression. TMEM16A is either over-expressed or amplified (magenta), down-regulated (green) or not affected (grey). Outer circle represents the distribution of results obtained from the literature per cancer types regarding the contribution of a high TMEM16A expression to the overall survival (OS). A high TMEM16A expression could either reduce the OS (magenta), increase the OS (green) or have no effect or have not been determined (grey).

[9], TMEM16A has now been investigated in multiple different cancers including HNSCC [10–23], Breast [14,24–32], Brain [33,34], CRC [35–37], Esophageal [38–40], Endometrial [41], Gastric [42], GIST [10,43–47], Liver [48], Lung [49,50], Pancreatic [51–54], Parotid [55] and Prostate cancers [56–58] (Fig. 1). In the wide majority of these investigations,TMEM16A is over-expressed or amplified compared to its expression in healthy tissues (Fig. 1) suggesting that TMEM16A over-expression could represent a conserved mechanism in oncogenesis.

Interestingly, TMEM16A over-expression is not homogeneous and detailed investigations have found that TMEM16A is not over-expressed in some subtypes of various cancers such as Human Papilloma Positive (HPV)-positive HNSCC, Estrogen Receptor (ER)-positive and Human Epidermal Growth Factor Receptor 2 (HER2)-positive breast cancers and Pancreatic Neuro-Endocrine tumors [13,18,30,31,52]. Additionally, Shiwarski et al. have demonstrated that TMEM16A expression can be modulated depending on the progression of the tumor. They show that primary tumors exhibit a high level of TMEM16A whereas metastasis from lymph nodes have a low expression of TMEM16A [22]. This interesting observation has to be counterbalanced by other studies demonstrating high expression of TMEM16A in liver metastasis of CRC [35] or an amplification of the TMEM16A gene in metastasis from HNSCC [23]. It is also notable that TMEM16A over-expression in HNSCC could be predictive of the presence of distant metastasis [11].

At the clinical level, TMEM16A over-expression has been associated to multiple clinical parameters. The most recurrent one is the overall survival. In most investigations, TMEM16A over-expression is correlated with a poor prognosis [13,14,16,19–21,25,27,32,35,39,42,44,45] with the exception of endometrial cancer, HER2-positive and ER-positive breast cancers [30,31,41] (Fig. 1). In addition, TMEM16A over-expression has also been correlated to the tumor size [10,45], the presence of distant metastasis [11,23], the recurrence rate [45,49], the improvement of the clinical outcomes by the chemotherapy [32] or the clinical stage of cancer [34,41,44,56]. These findings suggest that TMEM16A represents a promising biomarker for various cancers. TMEM16A could also represent a valuable therapeutic target as its pharmacological inhibition of TMEM16A reduces tumor growth *in vivo* [59] and promotes the sensitivity of other chemotherapy [60,61] and its expression could be correlated to the improvement of clinical outcomes by chemotherapy [32].

Thus, over-expression of TMEM16A is a frequent feature observed in multiple cancers suggestive of a conserved mechanism for the promotion of carcinogenesis. However, further investigations will be required to better characterize TMEM16A expression and its contribution to clinical prognosis in specific cancer subtypes in order to improve future therapeutic strategies targeting TMEM16A.

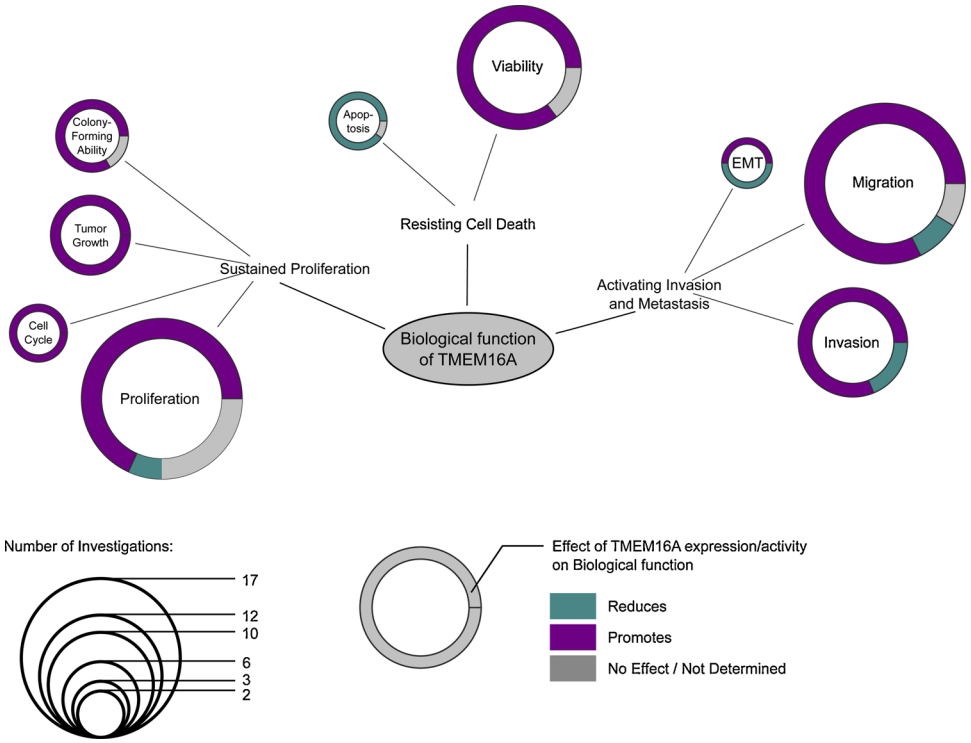

Fig. 2. Association of TMEM16A expression/activity with hallmarks of cancer.
For each hallmark of cancer as defined by Hanahan et al. [62], we associated biological functions reported in the literature to be modulated by TMEM16A expression / activity. Node size of each biological function is relative to the number of different cancer cell lines for which the contribution of TMEM16A expression/activity has been reported. The outer colored circle represents the distribution of conclusions obtained for each biological functions. TMEM16A expression/activity could either promotes (magenta), reduces (green) or have no effect (grey) on the biological function.

3. A multifaceted biological role of TMEM16A in cancer

In addition to its over-expression in multiple cancers and its contribution to several clinical features, TMEM16A has been associated to multiple biological functions in cancer cells. Hanahan and Weinberg have proposed several features or essential functions that could define a cancer cell [62]. While these functions could be seen as reducing the complexity of cancer cells, we found it interesting to overlap some of these hallmarks with different cancer functions attributed to TMEM16A in the literature.

The contribution of TMEM16A to the hallmark "Sustained Proliferation" is the most represented in the literature. Thus, molecular or pharmacological silencing of TMEM16A in cancer cells could impair proliferation [30,32,34,36,40,48,50,54,58,63–68], cell cycle [37,48,63], tumor growth *in vivo* [14,25,32,48,50,56,58], or the ability to form colony [25,50,56,63] (Fig. 2).

The contribution of TMEM16A has also been investigated with respect to the resistance to cell death of cancer cells [69]. Most of investigations across multiple cancer types have demonstrated that TMEM16A expression sustains cancer cell viability [13,22,25,30,32,37,37,56,57,61,63,64,66,70] and impair apoptosis [58,63] (Fig. 2).

The contribution of TMEM16A to the hallmark "Invasion and Metastasis" has also been extensively investigated. Most investigations conclude that TMEM16A expression promotes cancer cell migration and invasion [11,17,21,33,34,36,37,42,48,50,52,54,56,63,64,66,68,70–72] (Fig. 2). However, one report suggests a different role of TMEM16A on this hallmark [22]. In this investigation, Shiwarski et al. show that in HNSCC cancer cells, TMEM16A expression inhibits cancer cell migration and invasion while also preventing the epithelial-to-mesenchymal transition (EMT), a critical process for cancer cells to develop metastasis and secondary tumors [22]. A recent study, however found that TMEM16A expression is required for EMT, migration and invasion of gastric cancer cells [42]. Several other investigations have concluded that TMEM16A expression promotes HNSCC cancer cell migration or invasion [11,21].

Thus, it seems reasonable to conclude that TMEM16A is involved in cancer cell proliferation, apoptosis resistance, migration and invasion. However, there is heterogeneity for TMEM16A over-expression in various cancer subtypes as well as heterogeneity could be observed for the role of TMEM16A in cancer cells.

4. Multiple origins of TMEM16A over-expression in cancer

The multifaceted role of TMEM16A in cancers may reflect the origins of TMEM16A over-expression in cancer cells. Several mechanisms have been suggested to explain the over-expression of TMEM16A.

At the genomic level, amplification of the locus 11q13 is the most frequently mechanism associated to TMEM16A over-expression in cancers (Fig. 3). This amplicon has been observed in various cancers such as GIST, HNSCC, breast cancers, esophageal carcinoma and lung cancers and is correlated to an increase of the number of copies of TMEM16A gene [11,13,16,18,20,23,25,38,40,49]. Hypermethylation of the promoter region of the TMEM16A gene has been observed in HPV-positive HNSCC and in distant metastasis of HNSCC and it correlates with a low expression of TMEM16A [13,22] suggesting that hypermethylation of TMEM16A promoter could repress TMEM16A transcription. Thus, even if direct evidence of the hypomethylation of this particular genomic region in cancer has not been reported, it is conceivable that hypomethylation of TMEM16A promoter could be a putative mechanism contributing to its over-expression in cancer. In prostate cancer cells, Histone Deacetylase 3 (HDAC3) promotes the expression of TMEM16A [57] (Fig. 3). In non-cancerous cells, TMEM16A expression is induced by extracellular ligands such as Epidermal Growth Factor (EGF) or interleukin-4 (IL-4) [73,74]. Analysis of TMEM16A promoter revealed that Signal Transducer and Activator of Transcription 6 (STAT6) mediates the IL-4 induced TMEM16A expression (Fig. 3) [75]. Recently, the same group has demonstrated that TMEM16A gene transcription is regulated by Glioma-associated-oncogenes (Gli) proteins [76]. In a recent report, EGF promotes TMEM16A expression in breast cancer cells through the Epidermal Growth Factor Receptor (EGFR)-STAT3 pathway [32]. Thus, TMEM16A expression could also be modulated by soluble factors in the tumor micro-environment.

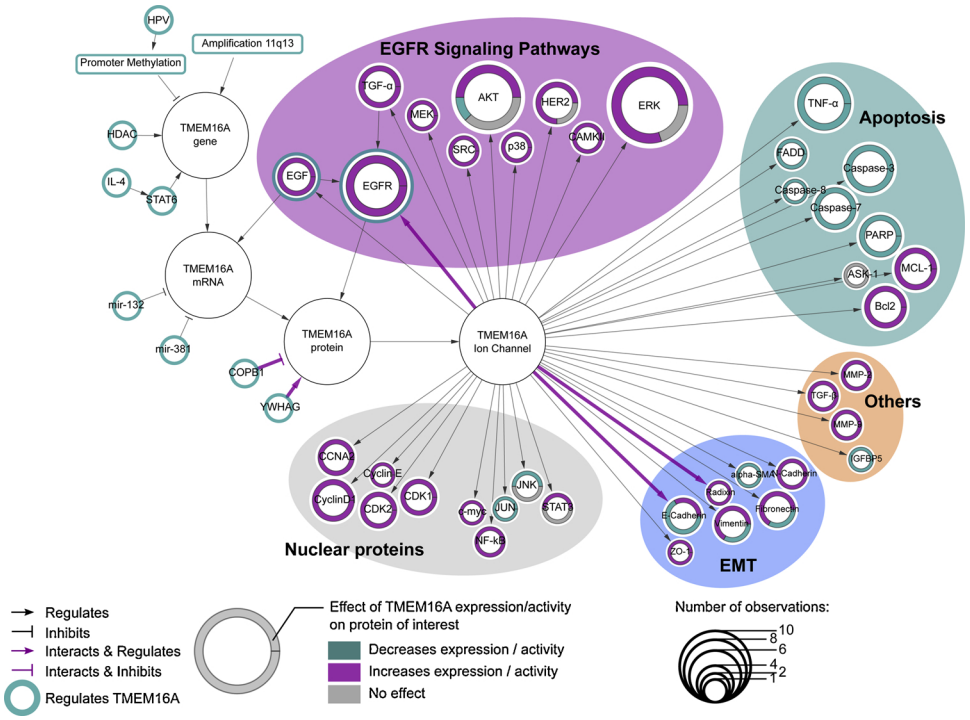

Fig. 3. Regulation of TMEM16A and its molecular targets in cancers.
Each node correspond to a protein described as either a regulator or a downstream target of TMEM16A expression/activity in cancers. Proteins represented with a thin green bordered node have been described as regulators of TMEM16A expression or activity. Proteins represented as a node with a thick colored border have been described as molecular targets of TMEM16A expression/activity. The node size of target proteins is relative to the number of different cell lines in which the contribution of TMEM16A on the expression/activity of the protein of interest has been investigated. The thick outer colored circle represents the distribution of conclusions made for each targets of TMEM16A. TMEM16A expression/activity could either promotes (magenta), inhibits (green) or has no effect (grey) on the protein expression/activity.

At the mRNA levels, miRNA-132 and 381 repress TMEM16A expression (Fig. 3). These miRNA species have been found to be down-regulated in gastric and colorectal cancers respectively [35,42] and their down-regulation correlates with a higher expression of TMEM16A expression.

TMEM16A expression is also be regulated at the post-translational level. In glioblastoma cells, TMEM16A has been found to interact with the Coatomer protein complex subunit beta 1 (COPB1) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (14-3-3γ) (Fig. 3). Such interaction could inhibit or promote the trafficking of TMEM16A to the plasma membrane [33,77]. Additionally, TMEM16A interaction with EGFR has been found to improve the stability of TMEM16A protein [61].

Thus, it appears that TMEM16A over-expression in cancer has multiple origins supporting the idea that TMEM16A over-expression is an important feature for cancer development that could be achieved through multiple ways.

5. Molecular targets of TMEM16A in cancers

Over the years, the modulation of TMEM16A expression or activity in cancer cells has been associated with the up- or down-regulation of different proteins, making it possible to create a network of protein targets of TMEM16A in cancer cells (Fig. 3). While most of these targets are probably indirectly modulated by TMEM16A, it is interesting to observe that they fall into several groups based on the biological functions they are associated with.

Alteration of TMEM16A expression affects proteins dedicated to transcription or cell division (Fig. 3). For example, cell cycle proteins such as Cyclin A2 (CCNA2), Cyclin D1, Cyclin E, Cyclin-dependent Kinases 1 and 2 (CDK1 and CDK2) are up-regulated in cancer cells with increased TMEM16A expression [34,63]. In addition, the activation of some transcription factors such as NF-κB, c-myc and STAT3 are positively correlated with TMEM16A expression whereas the activity of transcription factors JUN and JNK are negatively correlated with TMEM16A expression [32,34,58,78]. Thus, these group of targets modulated in cancer cells with increased expression of TMEM16A could contribute to the biological role of TMEM16A in cancer cell proliferation.

An other group of targets includes proteins related to apoptosis (Fig. 3). Indeed, the activity of pro-apoptotic proteins such as caspase-3, 7, 8, Fas-associated protein with death domain (FADD) and Tumor Necrosis Factor-α (TNF-α) are down-regulated while those of anti-apoptotic proteins B Cell Lymphoma 2 (Bcl-2) and Myeloid Cell Leukemia-1 (MCL-1) are up-regulated in cancer cells with increased expression of TMEM16A [25,58], indicating that TMEM16A promotes cancer cell viability and inhibits apoptosis.

At the molecular level, variety of TMEM16A functions have been implicated (Fig. 3). For example, TMEM16A could either promote or repress E-Cadherin, vimentin or fibronectin [22,42]. In addition, while in HNSCC, TMEM16A expression promotes the expression of the pro-epithelial marker Zonula Occludens-1 (ZO-1) and represses the mesenchymal marker α-Smooth Muscle Actin (α-SMA) [22], in gastric cancer cells, TMEM16A promotes the expression of the mesenchymal marker N-Cadherin [42]. It is clear that TMEM16A has an impact on several of proteins involved in EMT [22,42]. Several proteins such as radixin, Tumor Growth Factor-β (TGF-β), matrix metallopeptidases 2 and 9 (MMP2 and MMP9) associated with cancer cell migration or invasion are affected in cancer cells with increased TMEM16A expression (Fig. 3) [22,34,42].

Epidermal growth factor receptor (EGFR) is a tyrosine kinase that has been extensively investigated in various cancers [79–83]. In cancer cells, most recurrent molecular targets associated with TMEM16A are also associated with EGFR signaling pathways (Fig. 3). Initially, Britschgi et al. report that TMEM16A expression is able to regulate EGFR constitutive phosphorylation and associated signaling pathways such as SRC, Akt, ERK and CamKII in HNSCC and breast cancer cells [25]. They also demonstrated that TMEM16A promotes autocrine secretion of the EGFR ligands EGF and TGF-α [25]. Then, while elucidating the interactome of TMEM16A in HNSCC cancer cells, Bill et al. found interaction of TMEM16A and EGFR independent of either EGFR or TMEM16A activities [61]. They also reported that EGFR and TMEM16A show mutual regulation of their expression levels promoting their stability [61]. Interestingly, the pharmacological inhibition of TMEM16A enhanced the sensitivity to EGFR-based therapeutic strategies in HNSCC cancer cells strengthening the relationship between EGFR and TMEM16A [60,61].

In breast cancer, EGF-induced EGFR-STAT3 signaling pathway promotes TMEM16A expression [32]. Recently, we found that TMEM16A is required for promoting EGF-induced EGFR signaling in a pancreatic cancer cell line [52]. Interestingly, while confirming the close relationship between TMEM16A and EGFR, we also found that Akt and ERK signaling are not affected by TMEM16A expression suggesting the involvement of a different EGFR-related signaling pathway [52]. Moreover, TMEM16A expression has a profound effect on the phosphoproteome of pancreatic cancer cells and impairs the EGFR-related signaling pathways [52]. TMEM16A can also modulate the expression of HER2, an other member of human epidermal growth factor receptor (HER), thereby enlarging the role of TMEM16A in signaling involving tyrosine kinases receptors [78,84]. Thus, at the molecular level, TMEM16A also has multifaceted roles on a variety of molecular targets that converge to the multiple oncogenic function regulated by TMEM16A.

6. Understanding the mechanism of TMEM16A in cancers

The multifaceted molecular and cellular roles of TMEM16A in cancer cells raise the question regarding the molecular mechanism for TMEM16A.

As TMEM16A is an ion channel, it is tempting to speculate that TMEM16A exerts its pro-oncogenic function through chloride transport. However, TMEM16A-related chloride conduction may not be mandatory for its pro-oncogenic effects [13,54,63], as indicated by the difference observed between the use pharmacological blockers of TMEM16A and the molecular silencing of TMEM16A protein expression. Thus, TMEM16A blockers T16Ainh-A01 or CaCCinh-A01 does not reproduce the effect observed using siRNA on the migration of the pancreatic cancer cell line BxPC-3 [54]. Similarly, the use of T16Ainh-A01 does not reproduce the effect observed with the silencing of TMEM16A expression on the viability of the colorectal cancer cell line HCT-116 [63]. Conversely, the reduction of HNSCC cell viability by the use of CaCCinh-A01 is not observed when silencing TMEM16A expression [13]. However, other investigations observed that TMEM16A blockers inhibit cancer cell proliferation and/or migration to the same extent as the molecular silencing of TMEM16A [11,25,33,37,58–60,64]. Moreover, over-expression of non-conductive TMEM16A mutants (R621E, K668E and K610A) failed to reproduce the increase of proliferation observed with wild-type TMEM16A [14,25,32].

Taken together, these studies suggest that both the protein expression and the ion conduction of TMEM16A may contribute to its pro-oncogenic functions, consistent with the idea of a very multifaceted role of TMEM16A in cancers.

6.1. TMEM16A-interacting partners

Independent or synergistic to its ion channel function, TMEM16A protein may modulate cancer cell function by interacting with other proteins as described for other ion channels [85–91]. In an attempt to identify TMEM16A-interacting partners, two independent investigations have been conducted to determine the TMEM16A interactome [61,92].

TMEM16A has been found to interact with the Ezrin-Radixin-Moesin (ERM) network in HEK cells over-expressing TMEM16A (Fig. 4) [92]. The ERM network is an intermediate in the connection between the actin cytoskeleton and the plasma membrane. This network may regulate cancer cell migration, invasion or adhesion, raising the possibility that the TMEM16A interaction with components of ERM network could modulate cancer cell migration [93,94]. TMEM16A interaction with radixin was observed by co-immunoprecipitation in HNSCC cell lines and has been found to be mediated by the phosphorylation of S970 in the TMEM16A C-terminal domain [22]. Interestingly, while the over-expression of TMEM16A inhibits EMT and promotes proliferation in T24 cell line, over-expressing TMEM16A S970A (a mutant not interacting with radixin) fails to inhibit EMT but still promotes T24 proliferation [22]. This suggest that TMEM16A interaction with the ERM network could contribute to one aspect of its biological functions in cancer cells. The relationship between TMEM16A and other components of the ERM network (ezrin and moesin) has not been elucidated yet in the context of cancer.

In HNSCC cancer cells, characterization of the TMEM16A interactome has revealed that TMEM16A interacts with EGFR (Fig. 4) [61], supporting the notion that TMEM16A is strongly associated to EGFR signaling pathways in cancer cells [25,52,61]. The TMEM16A/EGFR interaction was also observed by proximity-ligation assay (PLA) in pancreatic cancer cells (Fig. 4) [52]. Mutagenesis experiments revealed that TMEM16A/EGFR interaction is independent of TMEM16A ion channel or EGFR kinase activities and is mediated by the juxta-membrane domain of EGFR. While the direct binding site of EGFR on TMEM16A has not been identified, the C-terminal domain of TMEM16A has been found to be non-essential suggesting a different binding site than that for radixin [61]. EGFR interaction with TMEM16A is associated to proliferation of HNSCC and pancreatic cancer cell migration [52,61]. Compiling studies of TMEM16A interaction with EGFR and radixin, we can thus associate TMEM16A protein with two different partners with distinct and unique binding sites for the modulation of at least two different cancer functions, indication that the multifaceted role of TMEM16A in cancer may be due to multiple interactions on multiple locations on the TMEM16A protein.

In addition of the ERM network, Perez et al. found that TMEM16A interacts with the SNARE protein complex (including Syntaxin-4, Syntaxin-7, VAMP3 and STXBP3) (Fig. 4) [92]. While these proteins might be associated with TMEM16A biogenesis and trafficking, the interaction of TMEM16A with this particular protein complex could hypothetically be associated with the TMEM16A-dependent EGF and TGFα secretion observed in breast and HNSCC cancer cells [25]. So far, no experiments have been performed to elucidate the relationship between the vesicle trafficking network and TMEM16A.

Survey of the literature reveals that additional proteins interact with TMEM16A. In cancer cells, COPB1 and 14-3-3γ have been found to interact with TMEM16A (Fig. 4) and to inhibit or promote TMEM16A trafficking at the plasma membrane respectively [33,77]. 14-3-3γ interacts with the residue T9 of TMEM16A [33], thus providing additional evidence of multiple protein binding sites on TMEM16A.

In non-cancer cells, TRPC6, TRPV4, CFTR, LRRC8A, CLCA1, CAMK2G and inositol triphosphate receptor 1 (IP_3R1) interact with TMEM16A and regulate its activity (Fig. 4) [8,95–102].

It is interesting to note that from TMEM16A interactomes defined by a proteomic approach, only TMEM16A itself and SLC3A2 have been found in both studies [61,92]. This absence of overlap between both proteomics approach could be attributed to a difference in the techniques, the methodology, or the biological model. Moreover, from the list of TMEM16A-interacting partners obtained by the proteomics approach, only radixin and EGFR have also been observed as TMEM16A-interacting partners in other investigations conducted in cancer cells [22,52], suggesting that great effort will be required to validate all of these putative partners of TMEM16A and to characterize their interaction and function in cancer cells.

6.2. TMEM16A ion conduction

As discussed above, the pharmacological inhibition of TMEM16A using various molecules inhibits several cancer cell functions such as proliferation, migration or apoptosis resistance [13,25,33,37,52,54,58–60,63–66,68,70,72,103]. This suggests that TMEM16A channel activity contributes to the multifaceted role of TMEM16A in cancer. How might TMEM16A-mediated chloride current mediate pro-oncogenic functions?

Ion fluxes are essential to maintain osmolarity and intracellular

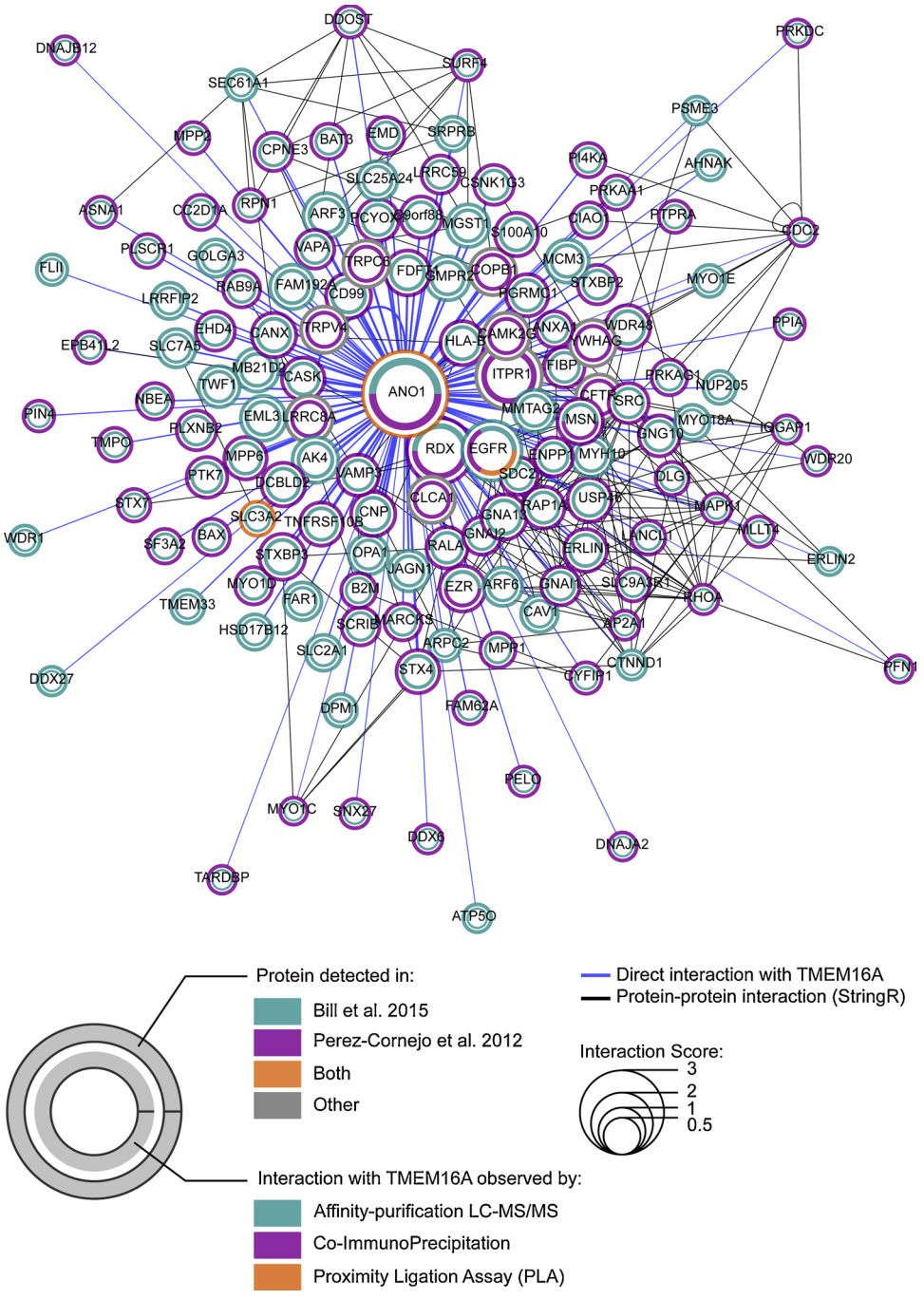

Fig. 4. TMEM16A-interacting partners.
Each TMEM16A interacting partner found in the literature has been represented as a single node connected to TMEM16A by a blue edge. The node size and edge width are relative to the interaction score calculated as the relative ascending rank order obtained in each of interactome published [61,92] + 1 for each data demonstrating a direct interaction between TMEM16A and the partner of interest by either co-immunoprecipitation (co-IP) or proximity-ligation-assay (PLA). Network obtained has been enriched for high confident protein-protein interaction (PPI) (confidency = 0.8, additional interactors = 0) using StringApp (version 1.4.2) integrated in Cytoscape 3.7.0. Then, the network layout have been obtained by using the integrated prefuse-direct force layout algorithm. The outer circle of each node represents the detection of TMEM16A-interacting protein in the interactome published by Bill et al. (green), the one published by Perez et al. (magenta), both (orange) or neither of them (grey). The inner circle represent the method used to demonstrate the interaction between TMEM16A and the protein of interest (proteomic approaches = green, co-IP = magenta or PLA = orange).

water content and ion composition in every single cell. In the context of cancer, ion fluxes and associated changes in the intracellular water content regulate cell volume and thus are critical for morphological changes occurring during cell division, migration, invasion, EMT or for preventing cell death [104,105]. Thus, osmoregulation represents a mechanism that associates ion fluxes and different cancer cell functions and could, at least in part, explain the multifaceted role of TMEM16A. In agreement with a contribution of TMEM16A to cancer cell osmoregulation, several reports have observed that both molecular silencing and TMEM16A inhibition are able to affect cancer cell volume or morphology [11,22,71].

Lysine deficient kinases (WNK) are considered to be intracellular chloride sensors [106,107] and regulate cell volume, proliferation and EMT [108,109]. In cancer cells, WNK kinases regulate PI3K-Akt, TGF-β and NF-kB signaling and autophagy [110,111]. In pancreatic cancer cells, we have found that both TMEM16A expression and EGFR activation modulate the phosphorylation of WNK1 kinases (Crottes et al. Unpublished data). This suggest that TMEM16A-related chloride current may mediate a part of its pro-oncogenic effects through WNK kinases signaling. Further experiments are required to validate this molecular mechanism.

Recently, our group observed that TMEM16A-related chloride currents are critical for the distribution and clustering of Phosphatidylinositol 4,5-bisphosphate (PIP_2) at the plasma membrane (PM) [112,113]. In cancer cells, lipid distribution in microdomains at the PM is critical for cancer cell proliferation and migration [80]. It is also a critical determinant for the interaction of ion channels with various receptors such as EGFR at the surface of cancer cells [80]. PIP_2 is the precursor of inositol triphosphate (IP_3) obtained by the cleavage of PIP_2 by phospholipase C (PLC) (which can be activated by EGFR). IP_3

is a well characterized second messenger that activates IP_3R which initiates intracellular calcium (Ca^{2+}) release known to be a regulator of TMEM16A activation (discussed below) [98].

Our recent study linked TMEM16A activity, EGFR and IP_3-dependent intracellular Ca^{2+} release to pancreatic cancer cell migration [52]. Combined with the known interaction of TMEM16A with IP_3R [97,98], these results allowed us to propose the following mechanism in which TMEM16A-related chloride currents promote PIP_2 clustering at the PM in close vicinity to the EGFR/TMEM16A/IP_3R macro-complex. This restricted spatial localization of all three molecules will promote the release of IP_3 from the PM and subsequently intracellular Ca^{2+} release in response to EGFR activation. The spatial localization and the dynamics of these interactions need to be further investigated, to test this hypothesis as the basis for unifying the ion conduction and physical interaction of TMEM16A into one molecular mechanism to define the multifaceted role of TMEM16A in cancer cells.

Similar to the multiple roles observed for TMEM16A protein interactions in cancer cells, TMEM16A-mediated ion conduction may have multiple roles in cancer cells by modulating osmoregulation, WNK kinases activities or lipids distribution. Thus, both the TMEM16A physical interaction and its biophysical properties contribute to the multifaceted role observed for TMEM16A in cancer.

6.3. Regulation of TMEM16A chloride transport

As we discussed above, TMEM16A protein could be modulated at the transcriptional, translational or post-transcriptional levels in cancer cells. TMEM16A ion conduction could also be modulated by multiples mechanisms.

Since its identification as a CaCC [4,5,73], several regulatory mechanisms of TMEM16A chloride channel have been identified [114]. TMEM16A ion conduction can be affected by direct binding of calcium [115–118], membrane potential [115,119,120], lipid environment [112,113,121,122], phosphorylation of serine residues, [99,100,122], the presence of protons or temperature alteration [123,124]. As discussed previously, its interaction with other ion channels and kinases also modulates its ion conduction [8,95–98,101,102].

Taken individually, each of these regulatory mechanisms of TMEM16A is relevant in cancer. For instance, membrane potential changes in tumors cells has been observed since the late 1950's [125]. Lipid environment is essential for the proper function of ion channels in cancer cells [80]. As in non-cancerous tissues, finely tuned regulation of Ca^{2+} homeostasis is critical for cancer cells [126]. Local extracellular acidification is regulated by ion channels to promote extracellular matrix digestion and cancer cell invasion [127,128].

However, although pharmacological approaches have emphasized the importance of TMEM16A-dependent chloride transport for cancer-related functions (as discussed above), the investigation of the molecular mechanisms regulating TMEM16A chloride currents in cancer cells remains largely unknown.

Importantly, one cannot entirely rely on extrapolation of regulatory mechanisms of TMEM16A observed in non-cancerous cells to the context of cancer. For example, while the activation of TMEM16A-mediated chloride current occurs downstream of either IP_3R-dependent intracellular Ca^{2+} release or extracellular Ca^{2+} fluxes mediated by TRPC6, TRPV4 or ORAI1 in non-cancerous cells [8,98,129–131], TMEM16A is upstream of the calcium signaling and controls both intracellular Ca^{2+} release and store-operated Ca^{2+} entry induced by EGFR activation in pancreatic cancer cells [52].

Investigating the different known activators of TMEM16A in the context of cancer will provide valuable inputs to improve our understanding of the function and regulation of TMEM16A in cancer cells.

7. Elucidating the multifaceted functions of TMEM16A in cancers

We have discussed above how the multifaceted role of TMEM16A in cancers could be explained by either protein-protein interaction, ion conduction or both. However, it is interesting to speculate whether, in a single cancer cell, TMEM16A could contribute to all of these functions simultaneously or asynchronously. The resolution of this question has not yet been achieved as several reports indicate that TMEM16A was unable to concomitantly regulate both proliferation and migration in the same cancer cells [11,21,22,42,54] while other reports demonstrate that TMEM16A could regulate both proliferation and migration in the same cancer cells [34,48,50,56,63,68].

7.1. A cell-specific mechanism

In their recent review, Wang et al. proposed that multifaceted functions of TMEM16A could be explained by a cell-specific role of TMEM16A that depends on the cellular environment [132]. Thus, the TMEM16A oncogenic function is dependent on the cancer cell type under investigation and its protein expression profile. While it is an interesting hypothesis that could explain why TMEM16A could promotes proliferation, migration or cell death depending on the cancer cell types, this hypothesis may not account for the multiple function that TMEM16A could exert in a single cancer cell.

7.2. TMEM16A and the plasticity of cancer cells

Composition of cellular membranes is highly heterogeneous. In any cellular membrane, micro-domains such as lipid rafts may have different lipid and protein compositions. These micro-domains are involved in intracellular signaling and inter-organelle communications.

In cancer cells, ion channels may exert intriguing functions on lipid rafts, thus promoting the formation and the stability of macro-complexes *via* their interaction with various receptors, other ion channels and signaling molecules [80,86,91]. By doing so, ion channels contribute to sustained downstream signaling pathways associated to these receptors or proteins. Disturbing ion channel function or expression or lipid composition will abrogate the formation of such macro-complexes and inhibit the associated downstream signaling pathways. These ion channels-dependent complexes could form in response to the sensing of extracellular cues such as extracellular matrix or growth factors. Thus, ion channels could promote cancer cell adaptive response by the formation of such macro-complexes [86,90,133].

As discussed above, TMEM16A promotes PIP_2 clustering at the plasma membrane [112] and interacts with various receptors (EGFR), signaling molecules (the ERM network) or other ion channels expressed in different sub-cellular compartments (IP_3R) [22,52,61,92,98]. Thus, we hypothesize that TMEM16A may contribute to the formation of such macro-complexes associated to lipid rafts (enriched in PIP_2) to promote the adaptive response of cancer cells to various stimuli.

Thus, either by being involved in signaling confined to multiple lipid rafts at different sub-cellular locations or by contributing to adaptive response to extra- or intra-cellular cues, TMEM16A may regulate various signaling pathways and biological functions in a single cancer cell thus leading to the multifaceted role of TMEM16A in cancer cells.

Supporting this hypothesis, our recent investigation in pancreatic cancer cells observed that TMEM16A interacts with EGFR and sustains ligand-dependent EGFR activation and signaling pathways by initiating intracellular Ca^{2+} release and store-operated calcium entry (SOCE) [52]. This study provides an example of a TMEM16A-related macro-complexes (TMEM16A/EGFR) involved in the sensing of extracellular growth factor (EGF) and involving the intracellular inter-organelle communication (Intracellular Ca^{2+} release + SOCE). Further experiments will be required to define each aspect of this particular molecular mechanism of TMEM16A and determine if by this mechanism, TMEM16A can indeed regulate multiple oncogenic functions in the same cancer cell.

8. Perspectives and future directions

In this review, we summarize the multifaceted role of TMEM16A in cancer. TMEM16A over-expression is a common feature of multiple cancers suggesting that TMEM16A is a promising biomarker.

However, a survey of the literature suggests that the role of TMEM16A could not be attributed to a single function or molecular mechanism. Whether by the origins of TMEM16A over-expression, signaling pathways, proteins or cancer cell functions regulated by TMEM16A, all of these converge toward a complex and multifaceted role of TMEM16A. Aggregating evidence from the literature suggests that both TMEM16A protein and TMEM16A-related ion conduction contribute to its cancer-related functions. TMEM16A could thus be engaged in a large variety of different signaling pathways by either its ability to interact with multiple proteins or its ion conduction that regulates ion homeostasis in cancer cells thereby contributing to critical functions in cancer cells such as osmoregulation, lipid distribution or calcium homeostasis.

To explain the multifaceted role observed for TMEM16A in cancers, Wang et al. recently proposed that TMEM16A could be associated to a cell-type specific mechanism [132]. However, this hypothesis suggests that TMEM16A modulates a single function in a unique cancer cell line.

In this review, we propose a complementary hypothesis that may explain the multifaceted role of TMEM16A in cancer. Our hypothesis proposes that TMEM16A contributes to the formation of macro-complexes of proteins in specialized micro-domains such as lipid rafts. Such specialized macro-complexes will promote the adaptive response to various stimuli thus associating TMEM16A to the regulation of multiple functions in cancer cells.

This hypothesis is partially supported by current evidence, it will require further investigation to characterize each aspect of the molecular mechanism of TMEM16A to elucidate its multifaceted role in cancer.

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Declaration of Competing Interest

No conflict of interest to declare.

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