Hyperactivation of the Mitogen Activated Protein Kinase (MAPK) pathway is prevalent in melanoma, principally due to mutations in the B-Raf and NRas genes. MAPK inhibitors are effective only short-term, and recurrence occurs due to functional redundancies or intertwined pathways. The remodeling of Ca^{2+} signaling is also common in melanoma cells, partly through the increased expression of T-type channels (TTCCs). Here we summarize current knowledge about the prognostic value and molecular targeting of TTCCs. Furthermore, we discuss recent evidence pointing to TTCCs as molecular switches for melanoma chemoresistance, which set the grounds for novel combined therapies against the advanced disease.

Keywords
Melanoma, T-type channels, MAPK, RAF/Ras mutants, PTEN mutants, macroautophagy, chemotherapeutic resistance

Introduction
The mitogen-activated protein kinase (MAPK) pathway plays a pivotal role in cancer progression by supporting cell proliferation, survival and migration [1]. Constitutive activation of the MAPK pathway occurs in up to 80-90% of cutaneous melanomas, mostly due to gain-of-function mutations in the B-Raf (>50%) and NRas (20-30%) genes. These cells are highly dependent on MAPK signaling and thus particularly vulnerable to MAPK inhibitors. Direct inhibition of Ras GTPases has proved unsuccessful so far, and efforts have concentrated on downstream effectors instead. However, available MAPK inhibitors targeting serine-threonine kinases BRAF or MEK, although often effective in the short-term, eventually trigger the activation of adaptive pathways that confer resistance to the treatment. One such pathway is the phosphoinositide 3-kinase (PI3K)-Akt, which can be activated by RAS and by mutations leading to the loss-of-function of phosphatase and tensin homologue (PTEN) [2], often concurrent with BRAF mutations [3]. Correspondingly, combined therapies co-targeting different components of the MAPK pathway and other survival routes are subject to intense research in order to increase treatment efficacy [4].

G1-S phase transition is dependent on Ca^{2+} influx through channels present in the plasma membrane, and subsequent activation of central Ca^{2+} regulator calmodulin (CaM). From there, Ca^{2+}-CaM facilitates cell cycle progression by binding to different cyclins/cyclin dependent kinases and by activating a plethora of kinases and/or phosphatases, in a context-dependent manner [5]. Among the many channels involved, voltage-gated T-type Ca^{2+} channels (TTCCs) play a cryptic, yet pivotal role in G1-S transition by virtue of their unique biophysical properties, which can be summarized in activation at low voltages, rapid inactivation and slow deactivation kinetics [6]. TTCCs are expressed in cancer cells and have become appealing candidates for chemotherapeutic targeting as they regulate different signaling pathways and cellular mechanisms favoring cancer cell proliferation, survival and invasiveness [7]. Because of these multilevel actions, pharmacological
block of TTCCs offers the possibility to inhibit a core pathway for cancer cells thriving, as well as putative escape routes for treatment resistance. Indeed, recent research has set the grounds for complementary targeting of TTCCs and components of the MAPK pathway.

**Prognostic value of T-type Ca$^{2+}$ channels expression in melanoma**

A pan-cancer view of TTCCs shows that their three isoforms (Ca$_{v3.1}$, Ca$_{v3.2}$ and Ca$_{v3.3}$) are expressed at variable levels according to cancer types and stages, including over- and under-expression when compared to normal tissues [7][8]. Transcriptional regulation of TTCCs is largely unknown, beyond the induction of Ca$_{v3.2}$ channels by hypoxia-inducible factors (HIFs) [9][10][11]. Of note, Cacna1g (encoding Ca$_{v3.1}$ channels) has been deemed as a tumor suppressor gene because of its aberrant methylation in 18-35% of different human primary tumors [12][13][14]. Nonetheless, the expression of transcripts for all TTCCs isoforms is increased in melanoma cells compared to melanocytes or nevus cells [7].

We performed seminal research back in 2012, when we showed that cultured melanoma cells express functional voltage-gated Ca$^{2+}$ channels in their plasma membrane, including TTCCs which are barely detectable in untransformed melanocytes [15]. Of the three existing TTCCs isoforms, we also identified Ca$_{v3.1}$ as the prevalent one in highly autophagic cell lines, whereas Ca$_{v3.2}$ was prominent in the cell lines displaying a faster proliferation rate, and Ca$_{v3.3}$ was expressed in a smaller range of highly proliferative cell lines. In a follow-up histochemical study we reported that Ca$_{v3.1}$ and Ca$_{v3.2}$ immunostainings were augmented in malignant melanoma and nevi, compared to normal skin, and showed a positive association with Breslow thickness [16]. Furthermore, the Histoscore for Ca$_{v3.2}$ was higher in metastatic vs. primary melanomas, and showed a three-way correlation with proliferative (Ki-67) and hypoxia (Glut-1) markers. Chronic or acute hypoxia, known to upregulate Ca$_{v3.2}$ channels through the activation of HIF-1$\alpha$ [10][11] and HIF-2$\alpha$ [9], are critical regulators of stemness in cancer cells [17]. Moreover, a role for Ca$_{v3.2}$ in stemness maintenance was revealed in glioma stem cells [18][19]. Our study also indicated that melanoma biopsies bearing the BRAF$^{V600E}$ mutation displayed a higher immunolabeling for Ca$_{v3.1}$, which positively correlated with that of microtubule-associated protein light chain 3 (LC3), an autophagosome marker, and with loss of tumor suppressor PTEN [16]. It was previously known that BRAF$^{V600E}$-driven melanomas exhibit enhanced (macro)autophagy [20], and that autophagy inhibition improves the sensitivity of BRAF$^{V600E}$ brain cancers to BRAF inhibitor vemurafenib [21]. The trilateral links between Ca$_{v3.1}$, BRAF$^{V600E}$ mutation and autophagy were confirmed by Maiques and colleagues, who described a facilitating role of autophagy in melanoma invasivity [22]. In this work, it was also shown that Ca$_{v3.1}$ and Ca$_{v3.3}$ channel transcripts are expressed at higher levels in BRAF$^{V600E}$ compared with NRAS$^{Q61H/K/L/R}$ mutant cell lines. The reported associations of TTCCs with proliferation or autophagy markers, and with oncogenic BRAF/NRAS/PTEN mutations are indicators of bad prognosis by themselves. This was directly demonstrated in patient cohorts by *in silico* genomic analysis of The Cancer Genome Atlas (TCGA) database, which revealed that the upregulation of Ca$_{v3.1}$ or Ca$_{v3.2}$ isoforms were strongly associated to poor outcomes in Kaplan-Meier estimates [16]. Likewise, a TGCA analysis performed by Granados and colleagues demonstrated that melanoma patients with tumors displaying a higher expression of Ca$_{v3.2}$ have a worse survival, although Ca$_{v3.1}$ was neglected from this study [23]. Table 1 summarizes the reported relationships between the expression of TTCCs isoforms, associations with prognosis markers and effects of specific gene silencing.

**T-type channels as drivers of resistance to MAPK inhibitors**

The prevalence of oncogenic BRAF and RAS mutations implies the hyperactivation of pathways that favor proliferation, survival and creation of an optimal microenvironment for melanoma tumor
growth [4]. Although many patients with BRAF- or NRAS-mutant melanoma benefit initially from BRAF- or MEK- targeted therapies, resistance to the treatment or secondary malignancies develop almost inexorably. Among the known mechanisms for recurrence there is the reactivation of the MAPK pathway as a result of adaptive signaling through upstream receptor tyrosine kinases, or due to the development of secondary mutations in components of the pathway [24]. In addition, compensatory activation of PI3K [25], the unfolded protein response (UPR) [26] and autophagy [26][27] have all been shown to promote resistance to MAPK inhibitors.

The dependency of BRAF- and RAS-mutant cells on autophagy for survival within the tumor microenvironment, and their increased susceptibility to autophagy inhibitors are well-documented [21][28]. Furthermore, overexpression of oncogenic BRAF has shown to trigger autophagy and induce a senescence-like phenotype in melanoma cells [20]. The mechanisms for induction of autophagy in these cells, overwhelming the constitutive activation of anti-autophagic mammalian target of rapamycin C1 (mTORC1) by MAPK and PI3K-Akt pathways, have not been completely elucidated. In bladder cancer cells activation of the MAPK pathway can promote autophagy by ERK1/2-mediated inhibition of Beclin-1 negative regulators, thus facilitating the formation of the class III PI3K complex [29]. In addition, ERK1/2-dependent phosphorylation of Go-Interacting Protein (GaIP) has been shown to induce autophagic sequestration in colon cancer cells [30] (Figure 1).

The above-reported upregulation of TTCCs in BRAFV600E mutant cells adds a piece to the puzzle of melanoma progression which gets even more complicated upon analysis of tumor resistance to MAPK inhibitors. Following the trail of research performed in glioblastoma (GBM), which unveiled a positive association between the expression of Ca3.1 and chemotherapeutic resistance to alkylating agent temozolomide [31], Barceló and colleagues have recently reported the increased expression of Ca3.1 channel transcripts in vemurafenib-resistant BRAFV600E-mutant, compared to vemurafenib-sensitive BRAFV600E melanoma cells [27]. In addition, consultation of the Gene Expression Omnibus repository database revealed higher levels of Ca3.1 transcripts in melanomas from patients after treatment with BRAF inhibitors. Like temozolomide-resistant GBM cells, vemurafenib-resistant melanoma cells displayed increased autophagosomal-associated LC3-phosphatidylethanolamine conjugate (LC3-II) levels, indicating enhanced basal autophagy with respect to their vemurafenib-sensitive counterparts. Moreover, the authors found that PTEN-deficient vemurafenib-resistant cells expressed the highest levels of Ca3.1 mRNA. The circle of evidence was closed by PTEN gene knockdown in PTEN-competent cells, resulting in augmented expression of Ca3.1 and conversely, overexpression of PTEN in PTEN-deficient cells resulting in reduced Ca3.1 transcript levels. However, the analysis of Ca3.2 transcripts in these conditions was not performed.

Another recent article reported that TTCC expression was upregulated in partially reprogrammed (by transduction of genes encoding pluripotency factors) BRAF and NRAS mutant-melanoma cells, acquiring features of stemness, slower proliferation and enhanced invasivity [23]. De-differentiated cells increased the expression of transcripts for Ca3.2 (particularly the NRAS mutants), in agreement with its known role in stemness, although the analysis of Ca3.1 transcripts was omitted. However and in contrast to the above-mentioned work, when melanoma cells were challenged with vemurafenib, both Ca3.1 and Ca3.2 isoforms were upregulated. Crucially, de-differentiated BRAF or NRAS mutant cells also became resistant to MAPK inhibitors such as vemurafenib (targeting BRAF) and trametinib (targeting downstream MEK1/2). These results are summarized in Table 2.

**Targeting of T-type channels in vitro and in vivo**

Many of the existing compounds with a proven ability to block TTCCs, originally developed against cardiovascular disorders or neurological conditions, display antagonism against different types of Ca2+, Na+, and K+ channels and metabotropic receptors [32][33]. Tetralol-derivative mibefradil and
diphenylbutylpiperidine-derivative pimozide are amongst the most widely used pharmacological blockers of TTCCs in cancer research, both in vitro and in preclinical models. These compounds are cytotoxic at about 5 to 10-fold supramaximal concentrations, suggesting that at least part of these effects are off-target [34]. In our pioneering works we used both compounds at 10 µM because we estimated half maximal effective concentrations (EC_{50}) on cell viability between 5-8 µM, depending on melanoma cell lines [47]. We found that exposure to mibebradil or pimozide at 10 µM exerted a dual effect by inducing cell cycle arrest and caspase-dependent apoptosis in a wide range of melanoma cells [35]. Apoptosis was preceded by endoplasmic reticulum stress and activation of the UPR. In addition, concomitant increased levels of LC3-II and of p62, a protein that acts as cargo receptor and becomes an autophagy substrate [36], as well as the accumulation of polyubiquitin aggregates, indicated a defective autophagy. Gene knockdown of TTCCs by siRNA transfection partially mimicked the effects of pharmacological blockers on UPR and autophagy markers, and induced apoptotic cell death.

Because reportedly vemurafenib-resistant BRAF^{V600E} cells display increased expression of Ca_{3.1}, it could be expected that their viability is more dependent on these channels and thus more sensitive to TTCC blockers, compared to non-adapted BRAF^{V600E} cells. However, Barceló and colleagues did not observe significant differences in the EC_{50} of mibebradil on both cell types. In addition, the PTEN status did not affect the sensitivity of melanoma cells to mibebradil, in spite of decreasing (when overexpressed) or decreasing (when knocked down) Ca_{3.1} transcript levels manifold [27]. In contrast, Granados and colleagues described enhanced sensitivity to mibebradil or its derivative NNC-55-0396 (but not to diphenylpiperazine-derivative lomerizine) for de-differentiated BRAF^{V600E} and RAS mutant melanoma cell lines, irrespective of their PTEN status (Table 2). Furthermore, mibebradil, NNC-55-0396 or knockdown of Ca_{3.2} induced apoptosis, differentiation and restored the sensitivity of de-differentiated BRAF^{V600E} vemurafenib-resistant cells to MAPK inhibition.

The effect of mibebradil on vemurafenib-resistant melanoma was further analyzed in vivo. Barceló and colleagues orally treated immunodeficient SCID xenografted mice with vemurafenib, mibebradil or both for 2 weeks, on a daily basis [27]. Co-administration of mibebradil delayed the growth of vemurafenib-sensitive and resistant cell line-derived xenografts, when compared to either untreated mice or mice treated with only vemurafenib. Similarly, Granados and colleagues orally administered vemurafenib and/or mibebradil to immunodeficient NSG xenografted mice: both drugs synergized in two sequential applications for up to 20 days, to slow tumor growth and extend the survival of xenografted mice in Kaplan-Meier estimates [23].

The role of TTCCs in melanoma motility and invasivity has also been under scrutiny. Both parameters were reduced by mibebradil, as well as by the macroautophagy inhibitor chloroquine, specifically in BRAF^{V600E} but not in NRAS^{Q61L} melanoma cells [22]. In addition, gene knockdown of Autophagy-Related Gene 5 (Atg5, key for phagophore extension) reduced migration of both cell lines in wound-healing assays, and silencing of Ca_{3.1} or Ca_{3.2} (the latter more strongly) reduced the invasion capacity of BRAF^{V600E} mutants in transwell assays. The expression of Snail1, a zinc-finger transcription factor involved in migration, paralleled the autophagic status and was reduced by both TTCC pharmacological blockers and Ca_{3.1}/Ca_{3.2} gene knockdown. These results contribute to conflicting evidence about the involvement of autophagy in metastasis. In early stages, autophagy may oppose metastasis by limiting necrosis and inflammation. However, when metastasis is initiated, autophagy may promote detachment from the extracellular matrix (ECM) and survival to anoikis [37][38][39]. Nonetheless, the influence of autophagy on the Epithelial-Mesenchymal Transition (EMT), the process by which epithelial cancer cells lose adhesion to the ECM, de-differentiate and migrate to a distant location, can be double-sided. For example, in GBM cells autophagy inhibition by gene silencing of Beclin-1, Atg5, or Atg7 increased the migration and invasion capacities [40]. These
disparate conclusions indicate that the links between autophagy and EMT may occur at different levels and vary according to cancer types and underlying genetics.

Concluding remarks
Altogether, data obtained in the last decade suggest that TTCC pharmacological blockers might be used to inhibit signaling pathways used in melanoma progression and invasion. Moreover, two recent reports indicate that TTCC targeting might prevent, overcome or delay melanoma resistance to MAPK inhibitors. Of note, the relationship between BRAF\(^{V600E}\) and TTCCs appears to be bidirectional: (1) the expression of TTCCs is increased in BRAF\(^{V600E}\) mutant cells, and further increased in mutant cells resistant to MAPK inhibitors. Hence, the MAPK pathway controls the transcription of genes encoding TTCCs. (2) Gene silencing or pharmacological block of TTCCs restore the sensitivity to MAPK inhibitors. Hence, TTCCs seem to activate the MAPK pathway downstream of BRAF or, at least, to make melanoma cells dependent on this pathway (Figure 1). The molecular mechanisms behind this two-way positive feedback are unknown at present.

In addition, the expression of Ca\(_{v}3.1\) was found to correlate with loss of PTEN. PTEN acts as a tumor suppressor by inhibiting the PI3K-Akt pathway as well as other PI3K-independent actions [41]. PTEN loss-of-function occurs in 30-60% of melanomas, and has been suggested to cooperate with BRAF\(^{V600E}\) mutations in the genesis of metastatic melanoma [42]. Importantly, both preclinical and clinical studies have shown that PTEN deficiency impairs BRAF-targeted treatments [41][43]. Thus, combinatorial targeting of MAPK and PI3K-Akt pathways is a prospective approach to overcome resistance and cross-resistance mechanisms [44]. Although no direct interactions have been reported between TTCCs and components of the PI3K-Akt pathway, TTCC pharmacological block or gene silencing are known to dephosphorylate prosurvival Akt in GBM [45][19] and ovarian [46] cancers. Altogether, the activity of TTCCs would allow to circumvent BRAF inhibition by dual downstream activation of the MAPK and the mTORC2-Akt pathways. In turn, activation of these pathways would feed back on TTCC expression (Figure 1). The positive correlation between the expression of Ca\(_{v}3.1\) and autophagy, and the reported effects of TTCC pharmacological block/silencing on UPR and autophagy, are also part of the jigsaw for adaptation of melanoma cells to MAPK inhibitors.

In conclusion, TTCCs are valuable prognostic markers and promising molecular targets in melanoma. However, further research is required to elucidate the coupling of TTCC-mediated Ca\(^{2+}\) entry with adaptive signaling pathways and cellular processes used in melanoma progression, invasion and chemoresistance, in which macroautophagy appears to play a leading role. In addition, the pharmacological targeting of TTCCs needs to be further outlined. The different TTCC isoforms perform different functions in cancer physiology, and currently available compounds are not only unable to distinguish between them, but at cytotoxic concentrations exert off-target effects which need to be carefully dissected. On that account, the delivery of efficacious therapies targeting TTCCs in melanoma is likely to parallel the timeline for development of highly selective inhibitors.

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CONFLICT OF INTEREST
The authors state no conflict of interest.
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Figure Legends:

Figure 1:
Double-sided interaction of TTCCs with MAPK and mTORC2-Akt pathways define two feedback loops (red arrows). The expression of TTCCs is increased in cells with overactive MAPK (BRAFV600E and RAS mutants) and PI3K/mTORC2-Akt (RAS mutants/PTEN mutants) pathways. Conversely, block/gene silencing of TTCCs restore the sensitivity of resistant cells to MAPK inhibitors, and inhibit the mTORC2 pathway. Both these pathways are known to favor cell growth at the transcriptional level and inhibit autophagy initiation by mTORC1 activation. Contrarywise, ERK1/2 may induce macroautophagy by facilitating Beclin-1-mediated nucleation of the phagophore and by GaIP-mediated autophagic sequestration. Arrow tips indicate whether the modulation is positive (pointed) or negative (round). RTK stands for Receptor Tyrosine Kinase.
Table 1: Correlation between TTCCs expression and prognostic markers, and effects of gene silencing in melanoma cells
N.A.: not addressed

<table>
<thead>
<tr>
<th>TTCCs isoform</th>
<th>Positive associations</th>
<th>Prognostic</th>
<th>Effects of gene silencing</th>
<th>References</th>
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<tr>
<td>( \text{Ca}_3.1 )</td>
<td>Breslow thickness, Autophagy (LC3-II), EMT (Snail1), ( \text{BRAF}^{V600E} ), PTEN deletion</td>
<td>Negative</td>
<td>Cell cycle arrest, Unfolded protein response, Defective autophagy, Apoptosis, Reduced migration/invasion, Increased sensitivity to vemurafenib</td>
<td>[15, 16, 27, 35]</td>
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<td>( \text{Ca}_3.2 )</td>
<td>Breslow thickness, Metastasis, Stemness (Sox2, SSEA1), Proliferation (Ki-67), Hypoxia (Glut-1)</td>
<td>Negative</td>
<td>Cell cycle arrest, Unfolded protein response, Defective autophagy, Apoptosis, Differentiation, Increased sensitivity to vemurafenib and trametinib</td>
<td>[15, 16, 23, 27, 35]</td>
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<tr>
<td>( \text{Ca}_3.3 )</td>
<td>Metastasis, ( \text{BRAF}^{V600E} )</td>
<td>N.A.</td>
<td>N.A.</td>
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Table 2: Mutational status of melanoma cells relative to cell phenotype, TTCC expression and sensitivity to TTCC inhibition. N.A.: not addressed

<table>
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<tr>
<th>Mutational status</th>
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<th>Phenotype</th>
<th>Relative expression of TTCC isoforms</th>
<th>Sensitivity to pharmacological block or gene silencing</th>
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<td>Δ LC3-II</td>
<td>Δ Autophagy</td>
<td>Δ Ca,3.1</td>
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<td>[16, 22, 27]</td>
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<td></td>
<td>Δ Snail1</td>
<td>Δ Invasivity</td>
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<td>Δ Ca,3.3</td>
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<td>Vem.-resistant <strong>BRAF^{V600E} (vs sensitive)</strong></td>
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<td>Δ Autophagy</td>
<td>Δ Ca,3.1</td>
<td>Similar</td>
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<td>N.A.</td>
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