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Contents

1 Introduction

1.1 Cancer, an overview

Physiology and morphology of tissues and organs is a result of well-balanced homeostasis of living and dying cells with specific fates and functions in the body. From fertilization to a fully developed organism, cells differentiate into cell types with specific phenotypes and functions in the tissue. Differentiation and cell fate are controlled and regulated by intra- and extracellular signaling pathways and gene regulatory networks.

Cancer is still one of the most common deadly diseases worldwide. Genomic changes induce dysregulation of intracellular regulatory pathways and initiate an imbalance of cell growth and cell death. Development of cancer is a multistep process with three stages, starting with tumor initiation followed by tumor promotion and cancer progression (De Braekeleer et al. 1984, Kufe et al. 2003). Accumulation of somatic mutations inactivates tumor suppressors and activates oncogenes that encode for regulators of cell differentiation, proliferation and apoptosis. Tumor formation is initiated by genomic changes, which are mostly induced by chemical and physical mutagens. These initial mutations cause advantages for the cells, such as reduced cell death. The second step, tumor promotion, is characterised by selective clonal expansion of initiated cells. The cells start to proliferate excessively and form benign tumors. During clonal expansion and with continuous cell division rate, further accumulation of cancer-stimulating mutations induces malignant conversion of tumor cells and results in tumor progression. Malignant conversion during the tumor progression transforms tumor cells into more aggressive cells with the ability to invade the surrounding tissue and form metastasis to colonize more distant tissues and organs. One driving force of tumor progression is epithelial to mesenchymal transition (EMT). During EMT cells dissolve cell-cell contacts and turn into migratory cells (Lim & Thiery 2012). Migratory and malignant cancer cells start to spread into surrounding tissues and invade the lymphatic and hematologic system, forming metastasis in more distant organs and regions in the body.

1.2 Driver mutations in cancer

Genes that are involved in cancer development and progression encode mostly regulatory proteins and factors that are critical modulators of cell and tissue homeostasis. These genes can be subdivided into three different groups, oncogenes, tumor suppressors and care-taker genes according to their functions in the cells and their roles in cancer.

Oncogenes encode proteins that are involved in cell growth, proliferation, migration

and cell survival. Upon activation by mutation or stimulation of their expression, proto-oncogenes get hyperactivated and turn into oncogenes. Activated oncogenes stimulate signaling pathways and regulatory networks and have the potential to drive tumor progression (Lodish et al. 2000). The concept of "oncogenic addiction"describes an obvious dependency of some cancer types on one or more oncogenes (Weinstein & Joe 2008). Development of cancer mostly requires not only one oncogene, but needs activation of several other proto-oncogenes for tumor progression.

In contrast to oncogenes, tumor suppressor genes have to be deactivated or downregulated in order to stimulate a malignant conversion of tumor cells. Tumor suppressor genes encode proteins, that regulate cell cycle control, cell adhesion and apoptosis and prevent uncontrolled growth and proliferation of differentiated cells (Lodish et al. 2000). Mutation or silencing of tumor suppressor genes results in stimulation of uncontrolled tumor growth.

The third group of genes that is linked to cancer development and malignancy are the care-taker genes. The care-taker genes are important for detection of DNA damage events and involved in DNA mismatch repair and prevention of gene mutations (Deininger et al. 1990). Deletion of care-taker genes increases the probability of further mutations and stimulation of cancer promoting pathways in cancer cells.

1.3 Hallmarks of cancer

The fundamental capabilities that cancer cells acquire during tumor growth and metastasis can be summarized in the hallmarks of cancer initially proposed by Hanahan and Weinberg, specifically: sustaining proliferative signals, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death (Hanahan & Weinberg 2000) (see figure 1.1).

Normally, cell homeostasis and equilibrium of cell growth and death are regulated by cellular control mechanisms and signaling pathways. During tumor formation, cancer cells are able to sustain proliferative signaling. Mutations and altered gene expression of oncogenes stimulate cellular signaling pathways or disrupt negative feedback mechanisms and thereby support uncontrolled proliferation and cell growth.

In addition to induction and stimulation of positively acting growth and proliferation signals, cancer cells can evade growth suppressors. Upon loss of function mutations in tumor suppressors, tumor cells are able to avoid cell signaling programs that normally negatively regulate cell growth and proliferation. Evasion from growth suppressors comprises the loss of contact inhibition, a mechanism that normally causes proliferation to stop when cell-cell contacts are formed.

Controlled cell death by apoptosis requires upstream regulators and downstream effector components, both are triggered by counterbalancing pro- and anti-apoptotic proteins. The apoptosis machinery is induced by physiologic stressors that cancer cells have to tolerate during the course of tumorigenesis or as a result of anticancer therapy. During

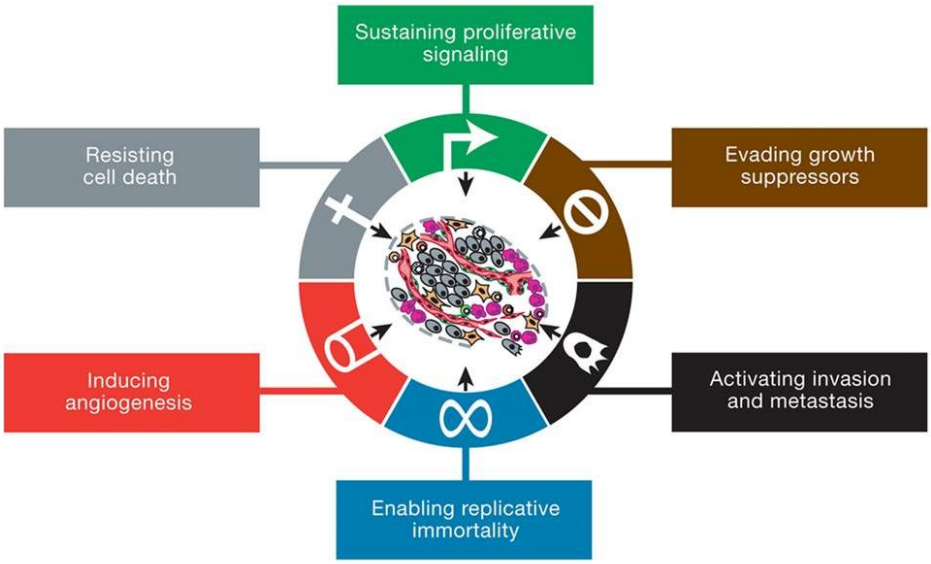

Figure 1.1: The six main hallmarks of cancer. (figure by Hanahan & Weinberg 2000)

this stress phase, cancer cells are able to repress the apoptotic machinery and develop resistances to pro-apoptotic signals. Moreover, tumor promotion can also be supported by cell death-associated autophagy and necrosis. Autophagy enables the enhanced uptake of nutrients and necrosis mediates recruitment of tumor-promoting inflammatory cells that release growth-stimulating factors and promote tumor growth and stimulation of angiogenesis.

Most cell lineages and tissue types in the body have a limited potential to divide. After a certain number of cell divisions, cells stop to divide and undergo senescence or crisis. Cells that emerge from a population in crisis circumvent the proliferation-limiting barriers, senescence and the pro-apoptotic program, and transform into further proliferating tumor cells with the capacity for replicative immortality. One of the key regulatory mechanisms in senescence and replicative immortality is the increased activity of telomerase, a nucleo-protein complex that prevents shortening of telomeres during cell replication. The telomeres are composed of multiple tandem hexanucleotide repeats that are shortened progressively in non-immortalized cells and protect the ends of the chromosomal DNAs from end-to-end fusions.

Like all other tissues in the body, tumors have to sustain sufficient nutrient and oxygen levels and the capability to dispose of accumulated metabolic waste and carbon dioxide. During embryonic development and physiological processes such as wound healing in the adult, development of vasculature involves forming of new blood vessels (vasculogenesis) and sprouting from existing blood vessels (angiogenesis). A so called angiogenic switch is activated in tumor cells and enables a tumor to sustain angiogenesis and to establish a vascular network for its own oxygen and nutrient supply. Some of these angiogenic regulator elements such as vascular endothelial growth factor (VEGF) are signaling proteins,

which bind to stimulatory or inhibitory cell-surface receptors.

The sixth hallmark of cancer cells is the ability of active invasion and metastasis, which means that affected cells spread in the surrounding tissue and invade into blood or lymphatic vessels to colonize more distant organs and parts of the body. For this processes, tumor cells frequently use the developmental regulatory program known as epithelial-mesenchymal transition (EMT). It involves dissolving cell-cell connections and transforms epithelial cells into migratory, mesenchymal cells. When the migratory cancer cells have reached the final location, EMT is reversed by mesenchymal-epithelial transition (MET) and the cancer cells start to colonize the tissue, forming metastasis.

In addition to the six initially proposed core hallmarks of cancer cells, Hanahan and Weinberg defined two emerging hallmarks characterized by deregulating cellular energetics and avoiding immune destruction, and discribed two enabling characteristics comprising genomic instability and tumor-promoting inflammation (Hanahan & Weinberg 2011).

1.4 Cutaneous melanoma

Cutaneous melanoma derives from malignant melanocytes and is still one of the most deadly cancers worldwide with very poor prognosis. Skin damage induced by sun burns and excessive exposure to the UV components in the sunlight are critical risk factors and stimulators of melanoma (Jhappan et al. 2003). Melanoma is induced and supported by frequently occurring mutations in the tumor suppressor PTEN and in proto-oncogenes like BRAF and NRAS in melanocytes in the basal epidermal layer of the skin (Chudnovsky et al. 2005; Houben et al. 2004).

Progression of malignant melanocytes occurs in two phases. In the first phase, called radial growth phase (RGP) malignant melanocytes spread in the upper epidermal layers, forming pagetoid spreads. The pagetoid spreading of melanoma cells during the slow RGP is limited to the epidermis. In the more rapid vertical growth phase (VGP), malignant melanocytes invade the dermis and are more prone to form metastasis (Chudnovsky et al. 2005) (see figure 1.2 and table 1.1).

Table 1.1: Clinical classification of cutaneous melanoma (Chudnovsky et al 2005).

Subtype	Frequency	Common site	Key distinguishing features
Superficial spreading melanoma	70%	Trunk of men, legs of women	RGP, 1-5 years
Nodular melanoma	10-25%	Trunk of men, legs of women	RGP, 6-18 months
Lentigo maligna melanoma	1%	Head and neck of elderly	Associated with chronic sun exposure, RGP, 3-15 years

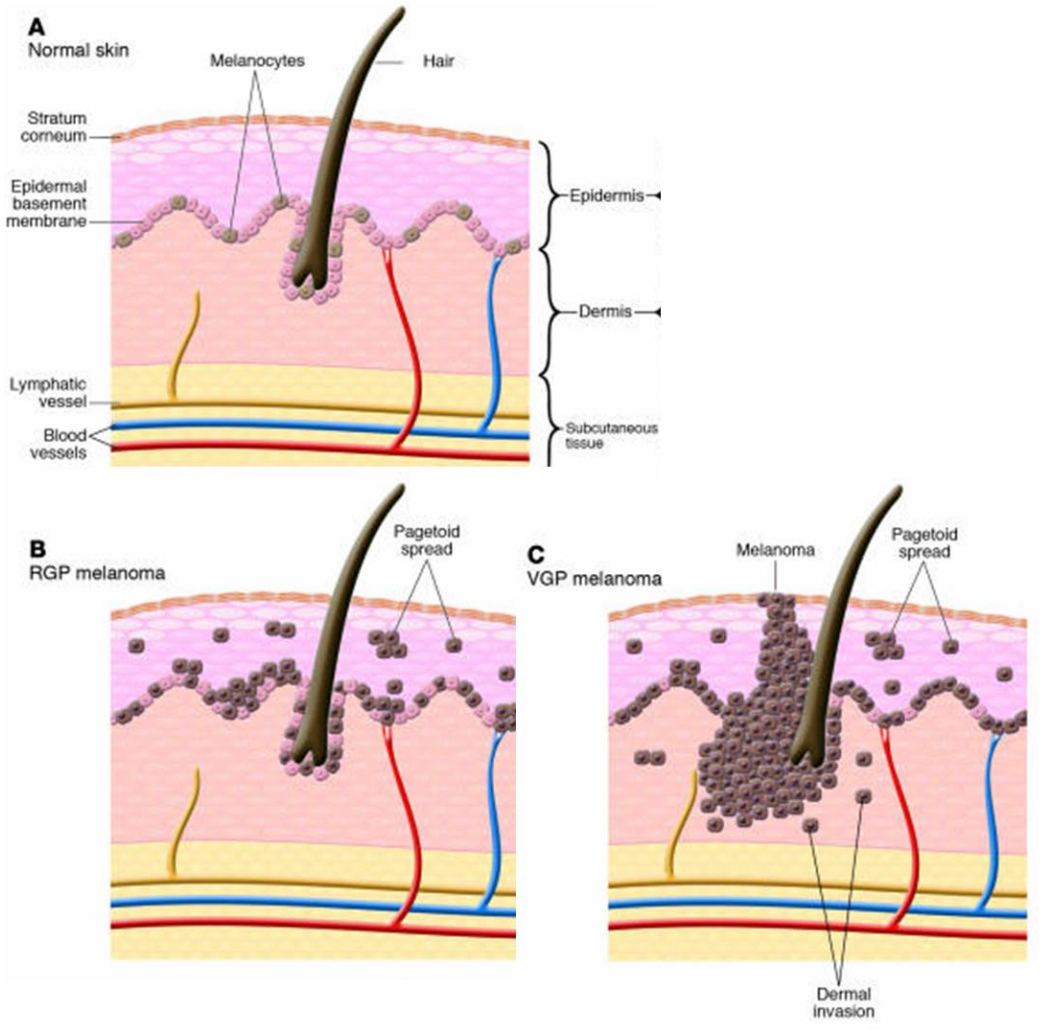

Figure 1.2: Staging of cutaneous melanoma. A. Morphology of the human skin. B. Radial growth phase (RGP). C. Vertical growth phase (VGP). (figure by Chudnovsky et al 2005)

Based on location, cell morphology and malignancy, melanomas are subdivided into different stages and subtypes. Cutaneous melanoma can be classified in lentigo maligna, lentigo maligna melanoma, superficial spreading melanoma and nodular melanoma (Chudnovsky et al. 2005; Smoller 2006) (see table 1.1). Superficial spreading melanoma is the most common melanoma type and represents 75% of all melanoma. Superficial spreading melanoma is characterized by lateral spreading of malignant melanocytes in the epidermal layer, forming pagetoid spreads during the radial growth phase. In contrast to the superficial spreading melanoma, nodular melanomas mostly do not show a radial growth phase and immediately enter a vertical growth phase. Nodular melanomas comprise very aggressive melanomas with rapid downwards growth into the dermis and high risk of metastasis (Smoller 2006). Lentigo maligna is the non-invasive phase of lentigo maligna melanoma and stays in the upper skin layers. Lentigo maligna melanomas de-

rive from the lentigo maligna stage and start to invade into the dermis. In comparison to the invasive nodular melanoma subtype, the lentigo maligna melanoma can be distinguished by a small and spindle-shaped cell morphology and characteristic spreading of cutaneous appendages in the inner skin layers. Pagetoid spread is unusual for lentigo maligna melanoma, but can appear later in melanoma progression in addition to dermal invasion (Smoller 2006).

1.5 The oncogenic BRAF V600E mutation in melanoma

Mutations of the RAS-activated kinase BRAF in the mitogen-activated protein kinase (MAPK) cascade occur in various malignant cancer types. Such mutations are especially frequent in malignant melanomas, supporting tumour promotion and progression (Brose et al. 2002). Approximately 66% of human malignant melanomas harbours an oncogenic mutation in the P-loop of the BRAF kinase (Houben et al. 2004). BRAF mutations are most frequent in invasive, superficial spreading melanoma (52–66%) and less common in lentigo malignant melanoma (14–20%) (Hertzman Johansson & Egyhazi Brage 2014). The oncogenic BRAF V600E variant is the most common BRAF mutation and harbours a single point mutation in the activation region of the kinase domain, resulting in a substitution of a glutamic acid for valine at the residue 600 in the resulting kinase protein (Brose et al. 2002). The V600E mutation prevents an interaction of the P-loop with the activation segment, which normally locks the kinase in the inactive conformation. The mutation results in a constitutively active BRAF conformation in the MAPK pathway and consequently permanent stimulation of gene regulatory signals downstream of active ERK (Wan et al. 2004; Partilas et al 2012).

1.6 Activation of the MAPK pathway by RTKs

The mitogen-dependent MAPK pathway gets initiated by the receptor tyrosine kinase (RTK)-activated G-protein RAS (Wimmer & Baccarini 2010) (see figure 1.3). Transmembrane RTKs contain an extracellular receptor domain with binding sites for specific ligands such as the growth factors EGF or FGF and an intracellular domain with protein-tyrosine kinase activity. Binding of growth factors at the extracellular receptor domain induces dimerization of two RTK proteins and upon dimerization, the cytosolic kinase domains get activated by autophosphorylation. Activated RTKs mediate recruitment and activation of the membrane-bound G-protein RAS through adaptor proteins. First, the growth factor receptor-bound protein 2 (GRB2) binds to the active RTK trough the SH2 domain, which recognises and binds to a specific phosphotyrosine residue on the activated receptor. The RTK-bound GRB2 protein recruits the guanine nucleotide exchange protein (GEF), SOS (Son of Sevenless). SOS proteins mediate the exchange of RAS-bound GDP for GTP and convert inactive GDP-bound RAS to its active, GTP-bound form. GTP-bound RAS activates the RAF kinase and initiates the MAPK cascade. The activated serine/threonine kinase RAF phosphorylates and activates the MEK and phosphorylated MEK regulates phospho-

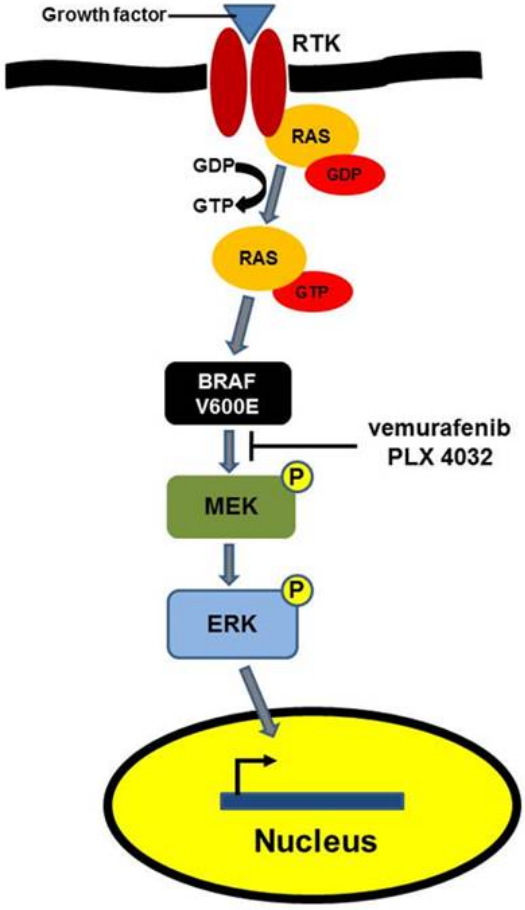

Figure 1.3: Activation of the mitogen-dependent MAPKinase pathway by receptor tyrosine kinases (RTK). Oncogenic BRAF (BRAFV600E) induces a constitutive activation of the RAF-MEK-ERK kinase cascade. Vemurafenib interferes with the phosphorylation of the MEK kinase.

rylation of the extracellular-signal regulated kinases (ERK) (see figure 1.3). Active, phosphorylated ERK (pERK) interacts with transcription factors and gene regulatory proteins in the nucleus and regulates specific gene expression. Several target genes downstream of the MAPK pathway are profoundly involved in the control of cell proliferation, cell differentiation and cell survival. In the RAF-MEK-ERK pathway three currently known RAF proteins, ARAF, BRAF and CRAF can be activated downstream of the RAS protein (Wimmer & Baccarini 2010). Beside receptor tyrosine kinases, the mitogen-dependent MAPK cascade can also be activated by G-protein-coupled receptors and integrin signals.

1.7 The mutant BRAF-specific inhibitor vemurafenib

Since oncogenic BRAF mutations occur very frequently in melanoma, small molecule inhibitors, such as vemurafenib and dabrafenib have been designed to specifically target

active and mutated BRAF kinase conformations (Hertzman Johansson & Egyhazi Brage 2014). The small molecule inhibitor vemurafenib (PLX4032) is a BRAF kinase inhibitor with high binding affinity to active BRAF kinase conformations and strong selectivity for V600E mutated BRAF proteins. Vemurafenib binds and blocks the ATP-binding site of active BRAF (Bollag et al. 2010; Hertzman Johansson & Egyhazi Brage 2014). Upon blocking of the BRAF ATP-binding site by vemurafenib, the mutant BRAF kinase is not able to phosphorylate MEK and the constitutively activated MAPK signaling cascade is inactivated (see figure 1.3). Vemurafenib shows a significant therapeutic effect in patients with mutant BRAF-expressing melanoma and was approved for treatment of malignant, metastatic melanoma, harboring a BRAF mutation (Chapman et al. 2011; Yang et al. 2011).

High specificity for mutated BRAF kinase conformations makes vemurafenib also a good tool for specific inhibition of the MAPK pathway in melanoma cells with BRAF V600E for performing analyses of downstream signals and associated gene expression patterns of the BRAF oncogene.

1.8 Regulation of intracellular calcium homeostasis

Intracellular calcium is an essential factor in various processes and signaling pathways within the cell metabolism, comprising cell cycle control, apoptosis, cell migration and underlying gene expression regulation (Chen et al. 2013). Intracellular calcium homeostasis involves regulation of the transmembrane calcium transport in both directions and control of intracellular calcium storage and release. Both processes require a complex system of calcium channels. Within the wide range of different calcium channel types in human cells and tissues, transmembrane plasma membrane Ca^{2+} ATPases (PMCA), intracellular inositol triphosphate receptor protein (IP3R) channels in the ER-membrane, store-operated Ca^{2+}-release-activated calcium channels (CRAC) and transient receptor potential channels (TRP) play a crucial role in the modulation of intracellular calcium concentration and calcium release. Aberrant regulation of calcium pumps and channels and disrupted calcium signaling results in altered intracellular calcium concentrations and remodeling of calcium homeostasis during tumor progression (Chen et al. 2013).

1.8.1 Plasma membrane Ca^{2+} ATPases (PMCA)

Plasma membrane Ca^{2+} ATPases (PMCA) are a subfamily of P-type ATPases, including four different isoforms and up to 20 PMCA variants encoded by four different genes. The high diversity of PMCA calcium pumps derives from alternative mRNA splicing at two different splice sites (Guerini 1998; Strehler et al. 2007). ATP-driven PMCA calcium pumps in the cell membrane actively transport intracellular calcium from the cytosol into the extracellular space. The distribution, expression and localisation of PMCA variants is cell and tissue-type specific. PMCA1 and PMCA4 members are ubiquitously expressed in almost every tissue whereas abundance of PMCA2 and PMCA3 is limited to specific tissues, such as neuronal tissue an sensory cells (Guerini 1998; Strehler et al. 2007).

The PMCA structure contains 10 transmembrane domains forming two main intracellular loops with an A-splice site, a catalytic phosphorylation site and an ATP-binding site. The intracellular C-terminal tail forms the main regulatory domain with a C-splice site, a calcium-calmodulin binding site and binding sites for protein kinases (Strehler et al. 2007; Padányi et al 2015) (see figure 1.4).

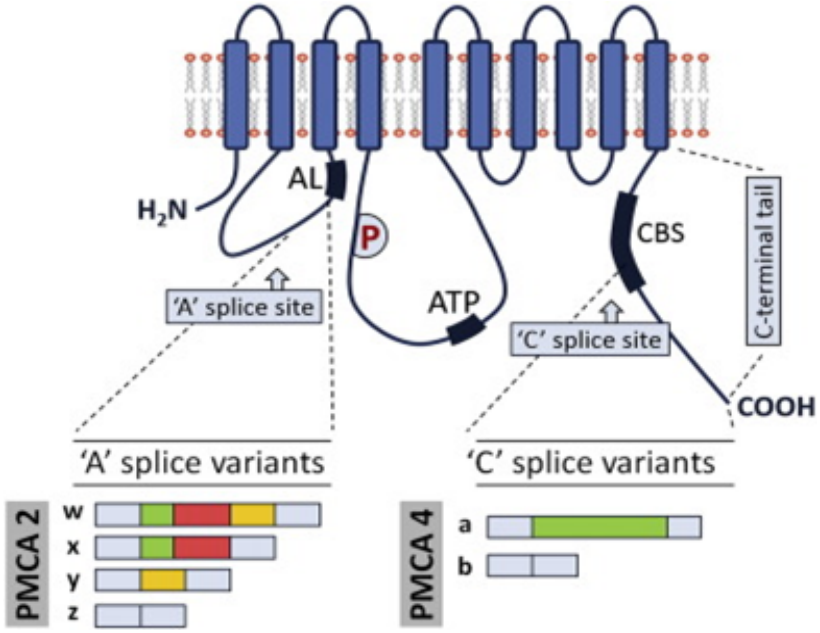

Figure 1.4: Model of plasma membrane Ca^{2+} ATPase. Catalytic phosphorylation site (P), ATP-binding site (ATP), acidic lipid (AL) binding region and calmodulin-binding sequence (CBS). A-splice variants of PMCA2 isoform and C-splice variants of PMCA4 isoform are shown. (figure by Padányi et al 2015)

In absence of the primary PMCA regulator calcium-calmodulin, the C-terminal tail binds to the two major intracellular loops and forms an autoinhibitory conformation with basal activity. The basal activity of PMCA proteins depends on the degree of interaction between the C-terminal tail and the catalytic domain and diverges within the PMCA types. In comparison to PMCA2 and PMCA3 with high basal activity, PMCA4 variants show low activity in the autoinhibited state (Padányi et al 2015). Binding of calmodulin releases the C-terminal tail from the catalytic domain and activates the PMCA pump. In addition to main modulation by calmodulin, regulation of PMCA proteins comprises binding of other PMCA interacting proteins such as calcineurine and protein kinases PKA and PKC (Strehler et al. 2007). The C-terminal region is an isoform-specific target of phosphorylation by PKA and PKC (Guerini 1998). Isoform-specific phosphorylation of PMCA proteins regulates the calcium pump activity directly by de-inhibition or indirectly by interfering with calmodulin (Brini & Carafoli 2011). Since certain PMCA calcium pumps are ubiquitously expressed and key regulators of intracellular calcium homeostasis, aberrant expression and activity of PMCA pumps play an important role in cancer.

1.8.2 Regulation of PMCA4b in cancer

PMCA4b calcium pumps are ubiquitously expressed and control maintenance of Ca^{2+}-gradients in resting cells, suggesting that PMCA4b primarily has housekeeping functions within regulation of intracellular calcium concentration (Strehler et al. 2007). Aberrant cancer cell migration in tumour metastasis was shown to be closely linked to dysregulation of intracellular calcium homeostasis (Chen et al. 2013). An upregulation of PMCA4b calcium pumps was observed in colon cancer cells and breast cancer cells upon induction of cell differentiation with histone deacetylase inhibitors (Varga et al. 2014; Aung et al. 2009; Ribiczey et al 2007). An increase of PMCA4b levels in human cancer cells upon induction of cell differentiation can be observed (Ribiczey et al 2007, Varga et al 2014). This suggests that remodeling of intracellular calcium homeostasis by PMCA4b calcium transport may be an potential new anti-cancer strategy.

A recent investigation in our group has revealed that inhibition of mutant BRAF in the constitutively activated MAPK pathway increases PMCA4b protein levels in malignant melanoma cells. Inhibition of mutant BRAF increased the abundance of PMCA4b proteins in the plasma membrane and enhanced intracellular calcium clearance in melanoma cells. Induced expression of PMCA4b protein by vemurafenib treatment and forced PMCA4b overexpression decreased the migration ability of melanoma cells with mutant BRAF. In addition, forced expression of PMCA4b reduced the metastatic capacity of melanoma cells in an in vivo model. Together these results suggest that PMCA4b could be an unrecognized metastasis suppressor in melanoma. (Hegedus et al, manuscript in preparation)

1.8.3 IP3R channels regulate release of ER-stored calcium

Inositol triphosphate receptor proteins (IP3R) comprise three different subtypes and form intracellular calcium channels in the membrane of the endoplasmic reticulum (ER) (Joseph et al. 1999). The IP3R channels are composed of homo- and heteromeric tetramers with four IP3R proteins (Foskett et al 2007). The six membrane spanning helical domains of each IP3R protein form the ion pore of the channel. The cytoplasmic N-terminal tail comprises the receptor domain and is subdivided into a regulatory coupling domain and an inositol-1,4,5-trisphosphate (InsP3) binding domain (Cui et al. 2004; Foskett et al 2007). Activated IP3R channels control release of intracellular ER-stored calcium into the cytoplasm. IP3R calcium channels are activated by InsP3 downstream of phospholipases PLC-β and PLC-γ (Hogan et al. 2003; Cui et al. 2004) (see figure 1.5). Upon activation of G-protein coupled receptors or tyrosine kinase receptors, the phospholipases get activated, hydrolyze the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) and release the resulting products InsP3 and diacylglycerol (DAG) (Hogan et al. 2003; Cui et al. 2004). The generated second messenger InsP3 diffuses into the cytoplasm and binds at the cytoplasmic N-terminal receptor domain of the IP3R channel. High activation of IP3R channels results in an increased release of intracellular ER-stored calcium and an accumulation of cytosolic calcium. An increase of ER-stored calcium release through the IP3R calcium channels stimulates activation of other cellular calcium channels such as store-operated CRAC channels and promotes stimulation of calcium signaling-associated

nuclear factors of activated T-cells (NFAT) transcription factors (Hogan et al. 2003) (see figure 1.5).

1.8.4 STIM proteins, calcium sensors in the ER, induce store-operated calcium entry (SOCE)

Store-operated calcium entry (SOCE) is a fundamental process in the control of calcium influx in human cells. Upon increase of cytosolic InsP3, IP3R channels are activated by InsP3 binding and ER-stored calcium diffuses into the cytosol (Chen et al. 2013; Soboloff et al. 2012). Decreased calcium in the ER stimulates the activity of stromal interaction molecule (STIM) proteins. STIM comprises two members STIM1 and STIM2, which are sensitive calcium sensors in the ER membrane. Both STIM subtypes comprise alternative splice variants (Rosado et al. 2016). STIM proteins recognize depletion of ER-stored calcium with their calcium binding domain. Upon depletion of calcium in the ER lumen STIM proteins get activated and translocate by diffusion into ER-plasma membrane junctions, where they interact with the Ca^{2+}release-activated calcium channel protein1 (ORAI) in the plasma membrane. Binding of STIM to ORAI1 proteins activates the CRAC channels (Soboloff et al. 2006; Soboloff et al. 2012). CRAC channels are composed of the channel activating element STIM and the plasma membrane channel element ORAI.

ORAI proteins comprise three different splicing variants ORAI1, 2 and 3. The ORAI protein consists of four transmembrane domains and short intracellular N- and C-terminal tails (Soboloff et al. 2006). The C-terminal coiled-coil domain indicates an important role for binding to the SOAR/CAD-domain of STIM1 and activation of the CRAC channel (Chen et al. 2013). Transmembrane domains 1 and 2 comprise the pore-forming elements (Soboloff et al. 2012; Chen et al. 2013). Interaction of the C-terminal SOAR/CAD domain of the STIM protein with the C-terminal coiled-coil domain of ORAI1 induces opening of the pore-forming four-transmembrane domain of ORAI1 and initiates activation of SOCE (Chen et al. 2013).

1.8.5 STIM protein-mediated store-operated calcium influx in cancer

STIM-mediated calcium influx through store-operated CRAC channels controls cell migration by formation of acto-myosin filaments and reorganization of the cytoskeleton (Chen et al. 2013). The assembly of contractile acto-myosin fibers during cell migration is controlled by intracellular calcium signaling. The acto-myosin contraction supports rear-end retraction and formation and disassembly of focal adhesions that are regulated by calcium-dependent signaling (Chen et al. 2013). In melanoma cells, STIM/ORA1 channel complexes mediate calcium oscillation and promote formation of invadopodium assembly during melanoma invasion (Sun et al. 2014).

1.8.6 TRP transmembrane ion channels

Transient receptor potential (TRP) channels are non-selective transmembrane ion channels and regulators of ion homeostasis in excitable and non-excitable tissue (Spassova et

al. 2004; Nilius & Owsianik 2011). TRP channels are related to the voltage- and ligand-gated potassium channel superfamily in the plasma membrane where TRP channels control Ca^{2+} and Na^{2+} entry into the cells. Thirty variants of TRP channels are subdivided into three subfamilies TRPC (Canonical), TRPV (Vanilloid) and TRPM (Melastatin), according to their homologies and functional properties (Nilius & Owsianik 2011). Each TRP protein contains a transmembrane domain with six membrane spanning helices, which can be subdivided into a sensory domain and into a pore-forming transmembrane domain, composed of 6 membrane spanning segments with a hydrophobic domain between transmembrane segment 5 and 6 (Spassova et al. 2004, Li et al 2015). Within the cell membrane TRP channels are expected to form functional transmembrane channels composed of pore-forming homo-and heteromeric tetramers with a central ion pore (Li et al 2015).

1.8.7 TRPM1 is a metastatsis suppressor in melanoma

Altered TRP ion channel expression levels and activities during progression of certain cancers has been observed (Prevarskaya et al. 2007). The TRP channel subfamily TRPM (Melastatin) plays a crucial role in regulation of melanocytic homeostasis. Regulation of melanocyte physiology and development requires functional TRPM1, TRPM2, TRPM7 and TRPM8. Among the TRPM members, decreased expression of TRPM1 is closely linked to melanoma progression. TRPM1 is ubiquitously expressed in epidermal melanocytes of benign melanocytic nevi. With increasing aggressiveness and invasiveness, the TRPM1 expression declines in malignant and metastatic melanoma cells (Prevarskaya et al. 2007). During progression of primary cutaneous melanoma, expression of TRPM1 is steadily lost during the vertical growth phase and correlates with increasing ability for metastatic invasion. Loss of TRPM1 is a commonly used indicator for melanoma classification and prognosis (Miller et al. 2004). In melanoma, TRPM1 expression is directly regulated by the melanogenesis transcription factor MITF. A MITF binding site was discovered in the promotor region of TRPM1 and indicates a regulation of TRPM1 by MITF (Miller et al. 2004).

1.9 Calcium signaling and the MAPK pathway control activation of certain transcription factors

The MAPK signaling pathway regulates expression and activation of several transcription factors that are involved in the expression regulation of calcium channels. Activation of nuclear factors of activated T-cells (NFAT) proteins is regulated by calcium signaling and can be modulated by binding of the activator protein 1 (AP1) complexes (Hogan et al. 2003). In osteoclasts, transcription of PMCA calcium pumps is stimulated by NFAT2. PMCA-mediated calcium efflux in turn prevents NFAT2 activation and controls a negative regulatory loop (Kim et al 2012). PMCA calcium pumps harbour a calcineurin binding site in the catalytic domain and are able to inhibit the calcineurin-activated NFAT pathway

(Buch et al 2005). Regulation of PMCA expression by NFAT transcription factors and interaction of PMCA proteins with the NFAT-activator calcineurin suggest a regulation of PMCA4b expression by NFAT proteins and NFAT-AP1 protein complexes in melanoma cells.

Microphthalmia-associated transcription factors (MITF) are key regulators of melanogenesis and closely linked to melanoma progression (see below chapter 1.9.3). MITF transcription factors regulate expression of genes that encode for melanogenesis regulatory proteins such as tyrosinase and tyrosinase-related protein 1 (TYRP-1) (Fang et al. 2002). In mutant BRAF expressing melanoma cells, phosphorylation of MITF proteins by ERK targets MITF proteins for proteasome-mediated degradation. Degradation of MITF proteins induces proliferation and survival of melanoma cells (Wellbrock et al. 2008).

1.9.1 NFAT, nuclear factors of activated T-cells transcription factors

Nuclear factors of activated T-cells (NFAT) are a family of calcineurin-dependent transcription factors with five different members, NFAT1-5. NFAT proteins are central signaling elements of T-cell activation. Besides regulation of immune responses, NFAT activity is also involved in proliferation, angiogenesis and apoptosis of other tissue types, such as skin, muscles and neuronal tissues (Hogan et al. 2003).

Phosphorylated NFAT proteins rest in the cytosol until they are activated by the calcium-calmodulin-dependent serine/threonine phosphatase calcineurin in response to increasing intracellular calcium (see figure 1.5). Cell-surface receptors, coupled to phospholipase C (PLC), induce accumulation of intracellular inositol-1,4,5-trisphosphate (InsP3) and stimulate release of ER-stored calcium upon InsP3 activated IP3R channels (Hogan et al. 2003; Medyouf & Ghysdael 2008). Increased calcium abundance in the cytosol induces opening of CRAC channels, resulting in an increased calcium influx into the cytosol. Activation of calcineurin by calcium-dependent calmodulin is induced upon increase of cytosolic calcium (Medyouf & Ghysdael 2008). NFAT is activated upon dephosphorylation by calcineurin and translocates to the nucleus. Activated NFATs target and regulate specific genes alone or in cooperative complexes with AP1 (Macián et al. 2001). In parallel to the activation of IP3R, the second product of PIP2 hydrolysis, DAG induces activation of the MAPK pathway and synthesis and activation of fos protein elements of the AP1 transcription regulator complex (Hogan et al. 2003) (see figure 1.5).

NFAT transcription factors are novel targets of oncogenic BRAF in metastatic melanoma, indicated by increased NFAT expression levels and activity in mutant BRAF expressing melanoma cell lines (Flockhart et al. 2009). Increased abundance of NFAT transcription factors upon mutant BRAF expression in melanoma cells suggests a crucial role of NFAT proteins in remodeling of certain cancer targets downstream of the constitutively active mutant BRAF kinase.

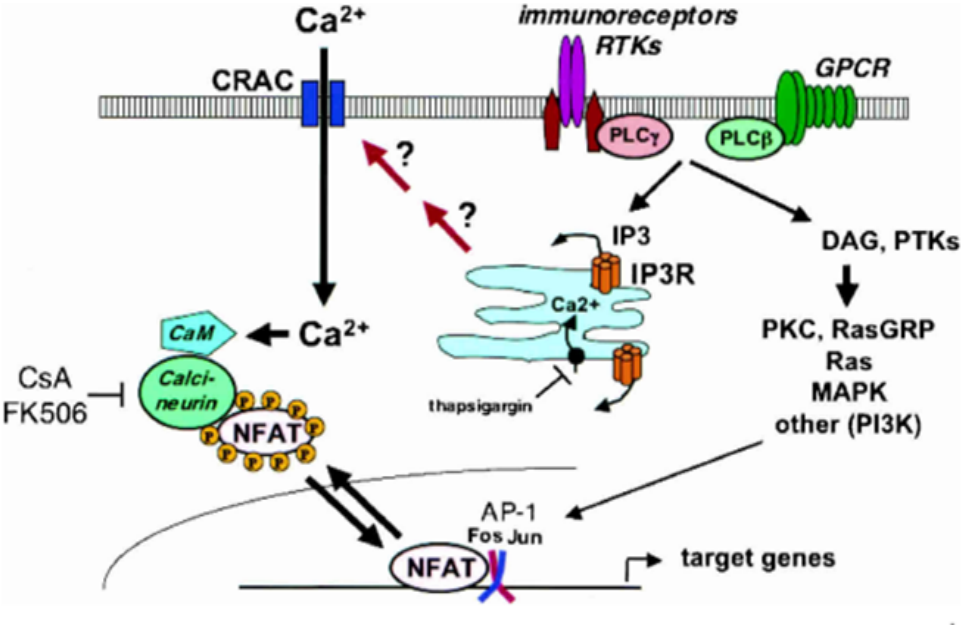

Figure 1.5: Regulation of nuclear factors of activated T-cells (NFAT) transcription factors by release of ER-stored calcium and stimulation of calcineurin: Receptor tyrosine kinases (RTK) and G-protein coupled receptors (GPCR) activate phospholipases PLC-β and PLC-γ, which hydrolyse the membrane lipid phosphatidylinositol-4,5-bisphosphate and release inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to the IP3R channels and induces ER-stored calcium release. Increase of calcium in the cytosol induces the activation of calineurin by calcium-dependent calmodulin (CaM). DAG stimulates the MAPK pathway and regulation of AP1 complexes. (figure by Hogan et al. 2015)

1.9.2 The AP1 transcription regulator complex

The activator protein 1 (AP1) is a transcription regulator complex composed of homo- and heterodimers of fos (c-fos, fosB, Fra1, Fra2) and jun (c-jun, junB, junD) basic region-leucine zipper (bZIP) DNA binding proteins (Karin 1995). The basic region-leucine zipper DNA binding motif allows jun-jun homodimerization and heterodimerization of jun proteins with fos proteins (Shaulian & Karin 2001). Jun protein formed AP1 complexes recognize TRE elements (phorbol 12-O-tetradecanoate-13-acetate (TPA) response elements) and target TPA-stimulated genes (Schoenthal et al 1992; Shaulian & Karin 2001). AP1 transcription regulator complexes activate or repress expression of several target genes involved in regulation of proliferation, cell growth, cell survival and cell death (Shaulian & Karin 2001). Synthesis and activation of AP1 complexes is stimulated by growth factors, proinflammatory cytokines, T-cell activators, neurotransmitters and UV irradiation (Karin 1995). Growth factor response induces activation of the MAPK cascade and stimulation of AP1 complexes by active ERK and c-jun N-terminal kinase (JNK). Upon activation of the MAPK the phosphorylated ERK kinase supports the synthesis of c-fos and formation of AP1 complexes with c-jun. Fos proteins do not form homodimers, but fos proteins, such as c-fos and FRA1 dimerize with jun proteins and form AP1 complexes, which are more

stable than AP1 complexes with jun proteins alone (Karin 1995).

Within the diverse group of jun proteins, c-jun regulates the expression and function of cell cycle regulators such as cyclin D1 and represses the transcription factor p53 and the cyclin-dependent kinase inhibitors p16 and p21 (Schreiber et al 1999; Shaulian & Karin 2001). C-jun activity is regulated through the MAPK cascade by c-jun N-terminal kinase (JNK), a member of the MAPK family. JNK phosphorylates the N-terminus of the c-jun protein and stimulates the activation of c-jun complexes (Karin 1995; Shaulian & Karin 2001).

Regulation of the cell cycle control factors is antagonized by the jun protein junB, which upregulates tumor suppressors. Since c-jun regulates proliferation and cell cycle control through repression of tumor suppressors and stimulation of cyclin D1 transcription, c-jun was discovered to act as an oncogene in cancer upon mutation or altered expression and activation (Schreiber et al. 1999). Furthermore inhibition of the mutant BRAF kinase in melanoma cells induces down regulation of the fos protein Fra1 (Obenauf et al. 2015). Dysregulation of AP1 elements and remodeling of AP1 regulating pathways such as the MAPK cascade contribute to repression of tumor suppressors and stimulation of oncogenes during cancer development and result in promotion of uncontrolled proliferation and cell cycle progression.

1.9.3 MITF transcription factors, master regulators of melanogenesis

The microphthalmia-associated transcription factor (MITF) family consists of five members, encoded by a single MITF gene (Hou et al. 2000; Hartman & Czyz 2014). The MITF gene expresses the MITF variants MITF-A, MITF-B, MITF-C, MITF-H and MITF-M by different usage of alternative promotors (Hartman & Czyz 2014). All MITF isoforms harbor a basic helix-loop-helix-leucine zipper DNA-binding domain and differe in their N-terminal domain (Hou et al. 2000).

MITF transcription factors are master regulators of gene expression in cell-type specific differentiation and cell cycle control in melanocytes, osteoclasts, mast cells and during development of the retinal pigment epithelium. MITF-M transactivates expression of the differentiation mediators tyrosinase and tyrosinase-related protein 1 (TYRP-1) exclusively in melanocytes and induces melanogenesis (Hou et al. 2000; Fang et al. 2002). Mutations in the MITF gene and dysfunction of the MITF-M protein prevent appropriate binding to the M-Box promotor sequence of specific target genes resulting in a reduction or loss of melanocytes (Fang et al. 2002). Knockout of MITF in mice results in loss of pigmentation and confirms the fundamental role of MITF transcription factors in normal development of melanocytes and retinal pigment cells (Dynek et al. 2008).

Since MITF is an essential regulator of cell differentiation programs in melanogenesis, oncogenic activity and dysregulated expression of the MITF gene were observed in malignant melanoma. Oncogenic BRAF stimulates upregulation of MITF expression through upregulation of the POU domain transcription factor (BRN2) (Wellbrock et al. 2008). In melanoma cells BRN2 expression is strongly upregulated by the activated MAPK signaling

pathway (Goodall et al. 2004; Wellbrock et al. 2008). BRN2 targets the MITF promotor and increases MITF transcription. In contrast, hyper-activated ERK kinases phosphorylate MITF and target MITF proteins for degradation in melanoma with oncogenic BRAF. Degradation of MITF upon mutant BRAF expression stimulates cell cycle control elements such as cyclin dependent kinases and supports proliferation and survival in melanoma cells (Wellbrock et al. 2008).

1.10 Histon acetylation and deacetylation are dynamic modulators of gene expression

Histone modifications are key regulators of chromatin packing (Bannister & Kouzarides 2011). Especially histone acetylation and deacetylation are crucial regulatory processes in chromatin remodeling and epigenetic regulation of global gene expression. Imbalanced histone acetylation status has been recognized to play an important role in remodeling of gene expression and activation of transcription factors during development and progression of cancer (Andreoli et al. 2013; Ropero & Esteller 2007).

1.10.1 DNA packaging in eukaryotic cells

Eukaryotic cells use specific protein complexes for packaging of DNA into a more compact form called chromatin. This DNA-protein complex formation allows compacting of DNA with a length of around 3 billion base pairs into 23 chromosomes. The DNA is wrapped two times around a histone protein octamer (nucleosome), formed of two H2A-H2B histone dimers and a tetramer of H3 and H4 histone proteins. An additional histone type, H1, stabilizes the DNA loops around nucleosomes and connects two neighboring nucleosomes for a further compaction of the chromatin (Annunziato et al. 2008). Histone proteins comprise a globular histone core and a N-terminal tail, protruding from the protein core. Histone modifications modulate chromatin packaging and transition between tightly packed heterochromatin and a more relaxed euchromatin. These modifications comprise acetylation, methylation and ubiquitylation on the N-terminal histone tails (Bannister & Kouzarides 2011).

1.10.2 Histone acetylation

Histon acetylation is associated with formation of euchromatin, a chromatin state characterised by DNA relaxation and de-silencing of gene expression. Histone acetylation marks are set on the lysine residue of the N-terminal histone tails by histone acetylases (HAT). HAT transfers an acetyl group to the NH^{3+} residue of the lysine, using acetyl-CoA as a acetyl group donor (Bannister & Kouzarides 2011). Binding of acetyl residues neutralises the positive charge of the lysine and destabilises the electrostatic interaction between DNA and the histone proteins (Finley & Copeland 2014). Reduced binding of the DNA to the nucleosome complex makes the gene more accessible for gene expression regulatory

proteins. HATs are enriched in active genes and are co-activators of gene expression. Increased histone acetylation by HAT proteins results in de-silencing of genes and enables binding of RNA polymerase II (Wang et al. 2010).

HAT proteins can be subdivided into two subfamilies, Type A and Type B. The nuclear Type A members are divided into three subfamilies: GNAT, p300/CB, and MYST (Hodawadekar & Marmorstein 2007). Type B members are frequently found in the cytoplasm and acetylate newly synthesized histones, H3 and H4, for their appropriate incorporation into chromatin (Andreoli et al. 2013). Histone acetylation is reversed by deacetylation.

1.10.3 Histone deacetylations

Deacetylation via histone deacetylases (HDAC) supports reconstruction of the tight binding of the DNA around the histone core and remodeling of heterochromatin (Ropero & Esteller 2007). HDACs remove the acetyl groups from the histone tails and restore the electrostatic affinity of the negatively charged DNA and the positively charged histone complex. In contrast to HAT proteins, HDAC proteins are co-repressors of gene transcription by regulating histone deacetylation and chromatin condensation (Wang et al. 2010).

Histone deacetylases are subdivided into four classes and grouped according to their homologies and cofactor activation (de Ruijter et al. 2003; New et al. 2012). ClassI HDACs 1, 2, 3 and 8 are related to the yeast transcription regulator RPD3 and are expressed in almost every tissue. ClassII HDAC4, 5, 6, 7, 9 and 10 share similar domains with the yeast deacetylase Hda1 and have a more tissue-specific expression pattern. Most of the HDACs are localized in the nucleus according to their function as chromatin modulator. While ClassII HDACs are able to shuttle between the nucleus and the cytoplasm, almost all classI HDAC members lack a nuclear export signal (NES) and are limited to the nucleus (de Ruijter et al. 2003). HDACs of classIII comprise seven members of sirtuins, SIR1-7, with homologies to the silent information regulator 2 (Sir2)-related protein family. HDAC11 is the only ClassIV HDAC. According to their cofactor specificity, members of classIII HDAC proteins are activated by NAD^+ binding, while activation of classI, II and IV HDAC members requires Zn^{2+} binding (de Ruijter et al. 2003).

HAT and HDAC activity is not limited to control of acetylation marks on histone N-terminal residues and involves also acetylation and deacetylation of non-histone proteins (Singh et al. 2010). Gene expression regulation by HAT and HDAC comprises both, chromatin remodeling and acetylation of non-histone proteins that are involved in transcriptional regulation, for example transcription factors like p53, STAT proteins, E2F, MyoD and NF-κB or nuclear receptors (Ropero & Esteller 2007; Singh et al. 2010). Acetylation affects protein stability, DNA binding affinity, transcriptional activation and protein interaction of non-histone proteins (Spange et al. 2009).

1.10.4 HDAC1 and HDAC2

Ubiquitously expressed HDAC1 and HDAC2 belong to HDAC protein classI and have high similarity with each other with 82% amino acid identity suggesting similar functions

(Brunmeir et al. 2009; de Ruijter et al. 2003). HDAC1 and HDAC2 form homo- or heterodimers and get incorporated into several co-repressor complexes such as Sin3, NuRD (nucleosome remodeling and deacetylating) and Co-REST complexes. The N-terminal HDAC association domain (HAD) allows dimerization, protein-protein interaction and direct interaction with DNA binding proteins (Brunmeir et al. 2009). Activation of HDAC1 and HDAC2 requires dimerization. The catalytic domain that comprises the largest part of the HDAC protein and harbours the active site controls the deacetylation activity of the HDAC protein. The tubular pocket of the active site mediates removal of acetyl groups using a charge relay system. The charge-relay system consists of histidines (His 131 and His 132), aspartic acids (Asp 166 and Asp 173) and tyrosine (Tyr 297) arrangements and requires binding of HDAC cofactor Zn^{2+} at the bottom of the tubular pocket (Finnin et al. 1999; de Ruijter et al. 2003). Like most classI HDAC proteins, HDAC1 and HDAC2 lack a nuclear export signal (NES) and are restricted to the nucleus (Kim & Bae 2011; de Ruijter et al. 2003). HDAC1 and HDAC2 complexes are highly involved in chromatin compaction and gene transcription regulation of target genes upon chromatin remodeling and direct interaction with transcription factors (Ropero & Esteller 2007). Hyperacetylation upon inhibition of HDAC proteins, such as nuclear HDAC1 and HDAC2, reverses gene repression and stimulates re-expression of silenced genes (Kraemer et al. 2003).

1.10.5 HDAC inhibitors demonstrate therapeutic effects in human cancer

In contrast to what was previously believed, cancer development is not only induced by accumulation of somatic mutations and genomic changes, but involves epigenetic changes as well (Ropero & Esteller 2007; Andreoli et al. 2013). Since histone acetylation and deactylation epigenetically regulate global gene expression, an imbalanced activity of HAT and HDAC is linked to epigenetic control of oncogenes and tumor suppressors in cancer. Hyperacetylation upon inhibition of HDAC reverses transcription activity of repressed genes and induces re-expression of genes involved in cell differentiation (Kraemer et al. 2003). Moreover, epigenetic regulation of gene expression comprises interaction of HDAC proteins with non-histone proteins, such as the transcription factor p53 (Singh et al. 2010; Kim & Bae 2011). Treatment with the HDAC inhibitors suberoylanilide hydroxamic acid (SAHA) or trichostatin A (TSA) leads to induction of cell differentiation in breast and colon cancer cells (Ribiczey 2008; Varga et al. 2014). According to their biochemical structure and different specificity to HDAC classI, II and IV, HDAC inhibitors are classified in hydroxamic acids, short-chain fatty acids, cyclic tetrapeptides and benzamides (Kim & Bae 2011; Wagner et al. 2013). The appropriate pharmacophore of the inhibitor molecule binds the histone deacetylase at the tubular pocket of the active site and chelates the HDAC activating co-factor Zn^{2+}. Chelating of the HDAC-activating co-factor Zn^{2+} by the HDAC inhibitor blocks the activation of the HDAC (Finnin et al. 1999).

Treatment of colon and breast cancer cells with HDAC inhibitors induced an upregulation of the PMCA4b calcium pump associated with induction of cell differentiation (Ribiczey 2008; Varga et al. 2014). Epigenetic regulation of the ubiquitously expressed PMCA4b calcium pump by HDAC suggests that HDAC inhibition could play a role in

PMCA4b expression not only in colon and breast carcinoma, but also in malignant melanoma.

1.10.6 ClassI HDAC protein-specific inhibitor valproic acid

While SAHA is a pan-inhibitor of class I and class II HDAC proteins, valproic acid, a member of the aliphatic acid class of HDAC inhibitors, specifically inhibits classI HDAC proteins (Dokmanovic et al. 2007; Kraemer et al. 2003). Valproic acid was previously used in anti-epileptic treatment, but was also recognised to induce inhibition of HDAC1 and HDAC2 and activation of cell differentiation programs in carcinoma cells (Kraemer et al. 2003). Valproic acid treatment selectively inhibits classI HDAC proteins and induces proteasomal-degradation of HDAC2 proteins in cancer cells (Kraemer et al. 2003). Since valproic acid primarily inhibits classI HDAC members localized in the nucleus, valproic acid can be used to specifically inhibit HDAC1 and HDAC2 proteins and analyse gene expression activity of epigenetically controlled genes upon chromatin remodeling.

1.11 Aim

BRAF is one of the most frequently mutated genes in melanoma. Inhibition of the mutated BRAF kinase with vemurafenib shows therapeutic benefits in treatment of metastatic melanoma with mutated BRAF. Development of drug resistance and limited therapeutic response in treatment of melanoma with BRAF inhibitors require investigation of additional molecular targets for metastatic melanoma. Intracellular calcium signaling and maintenance of intracellular calcium homeostasis by a network of calcium pumps and channels are key regulators of a balanced cell metabolism. Dysregulation of intracellular calcium signaling is a driving force in tumor progression and metastasis.

The aim of this thesis was to investigate the regulation of the PMCA4b calcium pump and other calcium channels, and of calcium signaling-associated transcription factors in wildtype and mutant BRAF expressing cancer cells. The following analysis were performed:

- Since PMCA4b protein expression was upregulated by inhibition of mutant BRAF and since forced expression of PMCA4b inhibited migration of melanoma cells, regulation of PMCA4b transcript expression was analyzed upon mutant BRAF inhibition with vemurafenib in melanoma cells .
- Regulation of other calcium channels with a crucial role in remodeling of intracellular calcium homeostasis was investigated upon treatment with vemurafenib.
- Regulation of transcription factors, associated with calcium signaling and melanoma development was analyzed upon treatment with vemurafenib.
- Since the investigated calcium channels and transcription factors are also known to be under epigenetic control, regulatory effects of the HDAC inhibitor valproic acid alone and in combination with vemurafenib were analyzed.

- To analyze regulation of PMCA4b transcript expression by wildtype and mutant BRAF in an otherwise isogenic background, melanoma cells were transiently transfected with wildtype and mutant BRAF.
- To analyze regulation of PMCA4b upon stable long-term expression of mutant or wildtype BRAF, retroviruses expressing the different BRAF variants were generated and melanoma cells were transduced with these retroviruses.
- To investigate regulation of PMCA4b expression by wildtype and mutant BRAF in colon cancer, colon carcinoma cells were transduced with wildtype and mutant BRAF expressing retroviruses.

2 Materials and Methods

2.1 Cell culture

Cells were cultured in T25 or T75 cell culture flasks with appropriate cell culture medium in a humidified atmosphere at 37°C and 5% CO_2. MEWO (BRAF wildtype), A2058 and A375 (both BRAF V600E mutant) melanoma cell lines were maintained in DMEM medium containing 10% fetal calf serum (FCS). The Caco2 colon cancer cells were cultivated in RPMI medium containing 20% FCS (see table 2.1). HEK293 human embryonic kidney cells were used for generation of pseudotyped retroviruses and maintained in MEME medium containing 10% FCS. Cells were passaged 1-2 times a week in a dilution ratio of 1:10 or 1:20 depending on cell confluence.

Table 2.1: Cell lines used

Cell lines	**Morphology**	**Standard growth medium**	**Source**
MEWO	fibroblastoid	DMEM, 10% FCS	ATCC
A2058	epithelial	DMEM, 10% FCS	ATCC
A375	epithelial	DMEM, 10% FCS	ATCC
A375-GFP	epithelial	DMEM, 10% FCS	Luca Hegedus, Medical University of Vienna
A375-PMCA4b-I	epithelial*	DMEM, 10% FCS	Luca Hegedus, Medical University of Vienna
A375-PMCA4b-II	epithelial*	DMEM, 10% FCS	Luca Hegedus, Medical University of Vienna
Caco2	epithelial	RPMI, 20% FCS	ATCC
HEK293	epithelial	MEME, 10% FCS	ATCC

* In contrast to the A375 parental line with a slender shape and more outgrowth, the PMCA4b-expressing cells are more round-shaped, with a typical front-to-rear polarity

PMCA4b overexpressing A375 melanoma cells and GFP expressing A375 cells were kindly provided by Luca Hegedus and the cells were kept in DMEM medium containing 10% FCS (see table 2.1).

2.2 Treatment of melanoma cells with vemurafenib, valproic acid and a combination of both compounds

MEWO, A375 and A2058 melanoma cells were treated with the mutant BRAF specific inhibitor vemurafenib to inhibit the constitutively activated MAPK pathway and the HDAC

inhibitor valproic acid. Since all three cell lines express wildtype NRAS, MEWO cells were treated as BRAF wildtype control. Vemurafenib and valproic acid were dissolved in sterile DMSO. One-2.5x 10^5 cells were seeded into a 6-well plate and incubated for 24h under normal culture conditions. After 24h the cells were washed with PBS and incubated with either vemurafenib (0.5μM), valproic acid (2mM) or a combination of both drugs, diluted in 2ml DMEM medium with 10% FCS. The final DMSO concentration in the experiments did not exceed 0.01%. For each cell line and time point an untreated control was incubated with DMEM medium only and a DMSO control was incubated with sterile DMSO (less then 0.01%) diluted in DMEM medium. Cells were treated for 8h-72h and afterwards transferred into Trizol reagent for RNA isolation.

2.3 RNA isolation with Trizol reagent

The cells were placed on ice to stop the cell metabolism and washed with cold PBS. After washing, 1ml Trizol reagent (see table 2.2) was added to each well. The cells were washed or scrapped into the Trizol and transferred into centrifugation tubes. The cells were mixed by vortexing and lysed in the Trizol reagent for 5min at room temperature. Two hundred μl chloroform were added and mixed with the cell lysate. The mixture was centrifuged for 15min with 12200g at $4°$C. The upper, aqueous phase was transferred to a new tube and mixed with 500μl isopropanol. The RNA was precipitated with isopropanol for 1h at $-20°$C and centrifuged for 5min with 12200g at $4°$C. The RNA pellet was washed with 1ml 75% ethanol, air-dried and the RNA resolved in 15μl RNAse-free water. The RNA concentration was determined with a NanoDrop 1000 spectrophotometer (Peqlab) and the RNA quality was checked by gel electrophoresis. Two μg of isolated RNA were diluted in 10μl RNAse-free water and mixed with 3μl vistra green loading dye. The samples were loaded on a 1.5% agarose gel and separated for 30-45min at 120V.

Table 2.2: Trizol reagent

guanidine thiocyanate, final conc. 0.9M
ammonium thiocyanate, final conc. 0.45M
(ammonium rhodanide)
sodium acetate pH 4.8, final conc. 0.12M
glycerine, final conc. 6%
phenol saturated with water, final conc. 39%
RNAse-free water

2.4 Gel electrophoresis

Gel electrophoresis was used for visualization and purification of DNA or RNA samples. For a gel with 1-2% agarose, appropriate amounts of agarose were dissolved in 50 or 100ml TBE buffer (see table 2.3). The gel was placed into an electrophoresis tank filled with TBE buffer. Ten-50μl of sample were mixed with 3-8μl vistra green loading dye (see table 2.4) and loaded onto the gel. Additionally, a 1kb or 100bp DNA ladder was loaded to

determine the size of the DNA fragments. The gel was run 5min at 60V and then 30-40min at 120V. The gels were scanned with a Typhoon Trio FluorImager (GE Healthcare) to detect the fluorescence of the DNA or RNA-bound vistra green and to visualize the fragments in the gel.

Table 2.3: TBE buffer

10.8g/l Trizma base (Sigma)
5.5g/l boric acid (Sigma)
4ml/l 0.5M EDTA, pH = 8.0
dH_2O to 1l

Table 2.4: Vistra green loading dye

666μl 6x loading dye (Fermentas)
700μl dH_2O
500μl 80% glycerol
133μl 0.5M EDTA
1μl vistra-green 10000x (Amersham)

2.5 Reverse transcription of RNA to cDNA

For quantitative real time PCR, the isolated mRNA was reverse transcribed into cDNA with Revert Aid reverse transcriptase (Thermo Fisher Scientific). First, 1μg of RNA was diluted in 6.5μl RNAse-free water and incubated at $70°$C for 10min. Two μl of M-MLV RT buffer, 0.5μl random hexamer primers, 0.5μl Revert Aid reverse transcriptase and 0.25μl Ribolock RNAse inhibitor were mixed with the RNA (see table 2.5). The reaction mix was incubated for 1h at $42°$C and then the reverse transcriptase was inactivated at $70°$C for 10min. The obtained cDNA was diluted with 10μl RNAse-free water and stored at $-20°$C.

Table 2.5: RT-mix for cDNA (1μg RNA)

4μl 5x M-MLV RT buffer
1μl dNTPs (10mM)
1μl Reverd Aid reverse transcriptase (200U/μl)
0.5μl Ribolock RNase inhibitor (40U/μl)
1μl random hexamer primers (0.5μg/μl)

2.6 Taqman quantitative real time PCR

For semi-quantitative analysis of the PMCA4b transcript levels in the treated melanoma cells, Taqman quantitative real time PCR was performed in a Maxima Probe/ROX master mix (Thermo Fisher Scientific) with PMCA4b specific TaqMan probes (Applied Biosytems, see table 2.7). One μl of cDNA was mixed with 6.25μl Maxima Probe/ROX master mix, 0.625μl TaqMan probe and diluted with 4.625μl nuclease-free water in a total reaction volume of 12μl. Each sample was measured in duplicates in a MicroAmp Fast Optical

96-well reaction plate (Applied Biosytems). The prepared plate was placed into an ABI Prism 7000 SDS Thermocycler (Applied Biosystems) and the PCR program was started (see table 2.6). The fluorescence signals of the PCR reactions were measured after every cycle. Additionally, GAPDH (glycerinaldehyd-3-phosphat-dehydrogenas) was measured for each sample and used for normalization (see table 2.7).

Table 2.6: Quantitative real time PCR settings

	Temperature [°C]	Time [min]	Repetition
Stage1	50	02:00	1x
Stage2	95	10:00	1x
Stage3	95	00:15	40x
	60	01:00	

Table 2.7: Taqman probes used for expression analysis

Target	Assay ID
GAPDH	Hs99999905_m1 Applied Biosystems
PMCA4b	Hs00608066_m1 Applied Biosytems

2.7 SYBR Green quantitative real time PCR

For semi-quantitative analysis of transcript levels of transgenic BRAF and of additional calcium channels and transcription factors in the treated melanoma cells, SYBR Green quantitative real time PCR was performed in a Maxima SYBR/ROX master mix (Thermo Fisher Scientific) with specific primers. The primers were designed with the Clone Manager program using the mRNA sequences of the NCBI gene sequence database and the primer design tool. Primer settings were set to an optimal length between 19bps and 20bps, a CG range between 40 and 70% and an annealing temperatures between 59 and 65°C (list of primers see table 2.8). The optimal PCR product length was set between 90 and 130bps.

For the PCR reactions 1μl of cDNA was added to a final 12μl reaction volume containing 0.5μl forward and 0.5μl reverse primer for the gene of interest and 6μl of Maxima SYBR/ROX master mix (Thermo Fisher Scientific) diluted with 4μl of dH_2O. The PCR procedure was performed as already described for the Taqman qPCR analysis (see figure 2.6), but in contrast to the Taqman detection system, fluorescence was measured with the SYBR Green system. Again the housekeeping gene GAPDH was additionally measured for each sample and used for normalization.

Table 2.8: Primers used for SYBR Green expression analysis

Oligo Name	Sequence (5'-3')	Annealing temperatur °C
IP3R1 forward	TTG GGC CTG GTT GAT GAT CG	64
IP3R1 reverse	TTT GGG CAG AGT AGC GGT TC	64
IP3R2 forward	AGA AGA ATG CCA TGC GTG TG	63
IP3R2 reverse	ACC CTC GCT TCT CAG TTT CC	63
IP3R3 forward	CCT AAG AAG TTC CGT GAC TG	59
IP3R3 reverse	TCC TTG TCC TGC TTA GTC TG	60
MITF forward	AGA CAT GCG CTG GAA CAA GG	65
MITF reverse	GAG CAA CAA ATG CCG GTT GG	64
NFAT1 forward	ACG AGC TTG ACT TCT CCA TC	61
NFAT1 reverse	ATG GCT TGA GGC CAT AGT CC	63
NFAT2 forward	CCA GTC CCT TCC AAG TTT CC	62
NFAT2 reverse	GTT CTT CCT CCG CTG ACT TC	62
NFAT3 forward	GGT GGA GTC TGA GCT AAA TG	59
NFAT3 reverse	TAC AGG CTC CAG GGA TCT TC	62
NFAT4 forward	GGA TTA CCG TCT CAC TCT TC	59
NFAT4 reverse	GGG TTT GGG ACC ACC TAA TG	62
ORAI1 forward	TGG ACG CTG ACC ACG ACT AC	66
ORAI1 reversev	CCT CGA TGT TGG GCA GGA TG	65
RYR2 forward	ATG TAT CTG TGC TGC CTG TC	61
RYR2 reverse	CTT CTG ATC GCT GCT TAG AG	59
STIM1 forward	GAT GGA CGA TGA TGC CAA TG	61
STIM1 reverse	GAA GGT GCT GTG TTT CAC TG	61
STIM2 forward	AAC GAC ACT TCC CAG GAT AG	60
STIM2 reverse	ACC ACA TCC AAT GCC TTG AG	62
TRPM1 forward	GTG TCA GCA CAG GTG TTA TC	60
TRPM1 reverse	TCC TTT CCA ACC AGG TCT TC	61
GAPDH for SYBR	AGCTCACTGGCATGGCCTTC	62
GAPDH rev SYBR	ACGCCTGCTTCACCACCTTC	62
BRAF forward	CCACAGAGACCTCAAGAGTA	59
BRAF reverse	GACTTCTGGTGCCATCCACAAA	65
c-fos forward	CTGTGGCTTCCCTTGATCTG	62
c-fos reverse	TGAGGAGAGGCAGGGTGAAG	65
c-jun forward	CTGCATGGACCTAACATTCG	60
c-jun reverse	CCCGCTTTGTGTTCTTAAGG	61
FRA1 forward	ACACCCTCCCTAACTCCTTTC	62
FRA1 reverse	TGCTGCTACTCTTGCGATG	62

2.8 Cloning of wildtype and mutant BRAF expressing retroviral vector plasmid

2.8.1 Preparation of the plasmid vector

For creating BRAF wildtype or mutant BRAF expressing retroviruses, the open reading frames of wildtype and mutant BRAF were first subcloned into a retroviral expression plasmid. The used retroviral expression vector pQCXIP (Clonetech) contains a retroviral LTR (long terminal repeat) region, an IRES (internal ribosome entry site) sequence and resistance genes for ampicillin (for bacterial selection) and for puromycin (for antibiotic se-

lection in mammalian cells). First, 4μg of the plasmid DNA were digested with fast digest sticky end restriction enzymes using 2μl EcoRI and 2μl XhoI (both from Thermo Fisher Scientific, 1 fast digest units/μl each) in a total reaction volume of 50μl with fast digest reaction buffer (Thermo Fisher Scientific). After incubating for 3h at 37°C, the enzymes were heat inactivated and 1μl alkaline calf intestinal phosphatase (Thermo Fisher Scientific, 1unit/μl) was added to the reaction mix. The linearized plasmid vector was incubated for 45min at 37°C with the alkaline phosphatase to dephosphorylate the DNA ends and to prevent re-ligation of the plasmid backbone during the following insert ligation. The vector product was mixed with 8μl vistra green loading dye and run on a 1.5% agarose gel. The appropriate vector DNA band was purified from the agarose gel with Promega Wizard DNA purification kit, diluted in dH_2O and stored at −20°C.

After ligation of the first insert fragment (see insert ligation procedure), 4μg of the resulting plasmid backbone were digested again with 2μl EcoRI fast digest sticky end restriction enzyme in a total reaction volume of 50μl with fast digest reaction buffer. After digesting the plasmids at 37°C for 3h, the DNA ends were again dephosphorylated with alkaline phosphatase. The digested product was separated on an 1.5% agarose gel and the appropriate DNA fragment purified from the gel with Promega Wizard DNA purification kit. The DNA was diluted in dH_2O and used as plasmid backbone for ligation of the second, remaining BRAF fragment.

2.8.2 Preparation of the BRAF insert

For production of BRAF DNA inserts, template plasmids with inserted open reading frames of either wildtype or mutant BRAF were used. The BRAF inserts were divided into two shorter fragments and ligated in two separate steps, because this matched better with the available restiction enzyme sites. First a shorter fragment containing the N-terminal part of BRAF was cut out directly from 4μg of the template plasmid with fast digest sticky end restriction enzymes using 2μl EcoRI and 2μl XhoI (both from Thermo Fisher Scientific, 1 fast digest units/μl each) in a total reaction volume of 50μl with fast digest reaction buffer (Thermo Fisher Scientific). The template plasmid and the enzymes were mixed with the reaction buffer and incubated for 3h at 37°C. The second and longer part of the BRAF inserts was amplified with Q5 High Fidelity polymerase (New England Biolabs) PCR reaction in a Q5 reaction buffer (New England Biolabs), using 300ng of the BRAF expressing template plasmids and 0.5μM of specific primers in a final reaction volume of 50μl (see table 2.9). The primers were designed to contain EcoRI restriction sites (see table 2.10) to create EcoRI DNA overhangs for later ligation. The PCR was initiated with a denaturation phase at 98°C for 3min, followed by 33 cycles with denaturation at 96°C for 50sec, annealing at 62°C for 50sec and elongation at 72°C for 6min. A final elongation phase was set to 72°C for 9min.

Table 2.9: PCR mix with Q5 High Fidelty polymerase 50μl

10μl 5x Q5 reaction buffer
6.25μl dNTPs, final conc. 1.25mM
1.25μl BRAF EcoRI forward primer, final conc. 0.5μM
1.25μl BRAF EcoRI reverse primer, final conc. 0.5μM
0.5μl Q5 High Fidelity polymerase (0.02U/μl)
0.5-1.4μl template plasmid (300ng)
29-30μl dH_2O

The PCR-amplified insert was digested with EcoRI restriction enzyme to create sticky ends. Two μl of EcoRI restriction enzyme were added to the PCR product and incubated at $37°$C for 2h. The digested PCR product was separated on a 1.5% agarose gel and the appropriate insert band purified from the gel with Promega Wizard DNA purification kit. The insert DNA was diluted in dH_2O and stored at $-20°$C.

Table 2.10: PCR primer

Oligo Name	**Sequence**	**Annealing temperature** $C°$
BRAF rev EcoRI	TTAGCGAATTCAGCTGGG	64
BRAF for endo EcoRI	CAGTCCGAGACAGTGTAA	59

2.8.3 T4 ligase overnight ligation and transformation into competent JM109 E.coli bacteria

For ligation of the BRAF inserts into the prepared retroviral expression plasmid backbone, 2μl of T4 DNA ligase (Thermo Fisher Scientific) were mixed with 50ng plasmid and 32ng insert (1:3 ratio) in a final reaction volume of 20μl with T4 DNA ligase buffer. Additionally, a ligation control reaction without an insert was prepared to check the degree of re-ligation of the plasmid backbone. The ligation mixes were incubated overnight at $16°$C and on the next day the T4 ligase was inactivated for 10min at $75°$C. For transformation of the generated plasmids into competent bacteria, 5μl ligation reaction mix were mixed with 50μl of competent JM109 E. coli bacteria and placed on ice for 20min. The transformation was induced by a 1.5min heat shock at $42°$C. After shaking in 950μl SOC-medium (see table 2.11) for 1h at $37°$C, the bacteria were harvested and spread on agar plates containing LB-medium (see table 2.12) and ampicillin for selection of bacterial colonies containing the ampicillin resistance gene of the transformed plasmid. The plates were incubated at $37°$C overnight and then stored at $4°$C before picking colonies for overnight cultures in LB-medium.

Table 2.11: SOC-medium (=SOB Medium containing 20mM glucose)

20g tryptone (Sigma-Aldrich)
5g yeast extract
0.5g NaCl
10ml KCl (250mM)
5ml $MgCl_2$ (2M)
dH_2O to a total of 1l
20ml glucose solution (1M)

Table 2.12: Agar plates with ampicillin for E.coli bacteria

20g LB-broth (Sigma-Aldrich)
15g agar (Sigma-Aldrich)
dH_2O to a total of 500ml
500μl ampicillin, final conc. 0.1mg/ml

Table 2.13: LB-medium (Luria-Bertoni medium), pH = 7

10g tryptone (Sigma-Aldrich)
5g yeast extract
10g NaCl
dH_2O

2.8.4 Plasmid isolation

For a 'quick and dirty' control DNA preparation, individual bacterial colonies were picked and cultivated in 3ml LB-medium containing 100μg/ml ampicillin at 37°C on a shaker overnight. One or 1.5ml of the overnight cultures were used for plasmid isolation. First, bacteria were pelleted and re-suspended in 700μl STET-buffer (see table 2.14). Thirteen μl lysozym (10mg/ml) were added to lyse the bacterial cell walls before the reaction was stopped at 100°C. Bacterial cell wall remnants, attached chromosomal DNA and denatured protein were pelleted by centrifugation and removed. The supernatant with the plasmid DNA was transferred into a fresh tube and mixed with 1μl RNAseA (10mg/ml). The plasmid DNA was precipitated for 20min in 700μl isopropanol at −20°C. After precipitation, the DNA was harvested by centrifugation and the DNA pellet was washed with 500μl 70% ethanol. After washing, the pellet was air-dried and the DNA re-dissolved in 30μl TE-buffer (see table 2.15) at room temperature.

Table 2.14: STET-buffer

25ml 1M Tris/HCl pH = 8, final conc. 50mM
50ml 0.5M EDTA pH = 8, final conc. 50mM
40g sucrose
25ml TritonX (added after autoclaving)
dH_2O to a total of 475ml

Table 2.15: TE-buffer (pH = 7.4)

100mM Tris-Cl pH = 7.4
10mM EDTA pH = 8

The plasmids isolated from the picked colonies were checked with a BamHI digest or EcoRI, XhoI double digest in fast digest reaction buffer (Thermo Fisher Scientific), looking for expected fragments after separation of the plasmid digest in an agarose gel electrophoresis. Colonies containing the retroviral expressing plasmid with either wildtype or mutant BRAF open reading frames in the right orientation were chosen for production of BRAF expressing retroviruses.

For obtaining concentrated and purified plasmid working stocks, 100μl of overnight culture were used to generate midipreps using the Promega PureYield plasmid midiprep system. The correct sequence of the BRAF inserts in the retroviral plasmids was verified by DNA sequencing analysis, sending premixed samples containing the appropriate sequencing primers to a commercial service (Eurofins). Additionally, the generated retroviral expression plasmids were transiently transfected into HEK293 cells and the BRAF protein level analysed by western blot analysis.

2.9 Transient transfection of plasmid DNA

One-2x 10^5 cells were seeded into 6-well plates and transfected with Lipofectamin2000 reagent (Thermo Fisher Scientific). First, 250μl serum-free medium were mixed with 10μl Lipofectamin2000 and incubated for 5min. Meanwhile 250μl serum-free medium were mixed with 2.5μg of plasmid DNA. The two prepared components were mixed, then the mix was incubated for 20min at room temperature and added to the cells. After incubation of the cells for 5h in the transfection medium, the cells were washed with PBS and the medium changed to normal culture medium. For transfection in 12-wells the reagents were downscaled, using half the amount of the serum-free medium and the Lipofectamin2000 reagent. After 24h, 48h, 72h and 96h, transfected cells were washed with PBS and scraped either into Trizol reagent for RNA isolation or into LBII lysis buffer (see table 2.16) for protein isolation. For analysis of the transfection efficiency, cells were also transfected with the same plasmid backbone containing either no transgene (empty vector) or encoding EGFP.

2.10 Retroviral transduction of human cancer cell lines

For generation of retroviruses, the retrovirus expression plasmids pQCXIP/BRAFwt and pQCXIP/BRAFV600E were transfected into HEK293 cells. First, 250μl of Opti-Mem medium were mixed with 10μl Lipofectamin2000 and incubated for 5min. Meanwhile 250μl of Opti-Mem medium were mixed with 5μg BRAF expressing pQCXIP plasmid, 3.75μg of plasmid containing a pgag-pol-gpt sequence and 1.25μg of plasmid harbouring a pVSV-G sequence, both constructs essential for generation of pseudotyped retroviruses. Both components were pooled, the transfection mixture incubated for 20min and added to the cells. After 5h of incubation, the transfection medium was changed to normal culture medium. After 3 days the cell supernatants containing the retrovirus were filtered through a 0.22μM cellulose acetate syringe filter and stored at $-20°$C. MEWO melanoma

cells and Caco2 colon cancer cells were incubated with 1ml of retrovirus supernatants in the presence of 0.8μg/ml polybrene overnight. The cells were incubated with either BRAF wildtype or mutant BRAF expressing retroviruses in a 6-well plate. For transfection control the cells were also incubated with retroviruses expressing a fluorescent protein (either EGFP or mCherry) or pQCXIP empty retroviruses without a transgene. Transfected MEWO cells were selected with 0.8μg/ml puromycin and Caco2 cells with up to 10μg/ml puromycin diluted in normal culture medium.

2.11 Protein isolation

Five-10x 10^5 cells were seeded into 6-well plates and incubated for 24h-96h, depending on confluence and experimental background. The attached cells were placed on ice and washed with cold PBS. The cells were scraped in 30-100μl LBII lysis buffer (see table 2.16) and transferred into a centrifugation tube. HEK293 cells were carefully washed at room temperature to avoid detachment of the cells into the PBS. After sonication for 5min in an ultrasonic bath, the cell lysate was centrifuged for 5min at $4°$C with 12200g and the supernatant was transferred to a fresh tube and stored at $-20°$C. Protein concentration was measured with BioRad Protein Assay Dye reagent in a u-shaped 96-well plate. One μl of protein sample was diluted in a volume of 10μl dH_2O. Additionally a standard curve of 0-0.4μg/μl BSA (bovine serum albumin) standards in a total volume of 10μl was generated. The protein assay reagent was diluted 1:5 with dH_2O. Each of the prepared protein samples and BSA protein standards were mixed with 200μl of the diluted reagent in the 96-well plate. The absorbance shift of the Coomassie Brilliant Blue G-250 dye upon protein binding was measured at 595nm.

Table 2.16: Protein lysis buffer II

Stock solution	Final concentration	10ml
100mM EGTA	1mM EGTA	100μl
5M NaCl	150mM NaCl	300μl
50mM Na_3VO_4	1mM Na_3VO_4	200μl
100% TritonX	1% TritonX	100μl
100mM NaF	10mM NaF	1ml
1M Tris pH=8	50mM Tris pH=8	500μl
dH_2O		7.8ml

2.12 SDS-PAGE

For separating the isolated proteins according to their molecular weight (kDa, kilodalton), a polyacrylamide gels were prepared for gel electrophoresis. The polyacrylamide gels were composed of a 10% separating gel and a 5% stacking gel (see table 2.17). The gel was placed into an electrophoresis tank filled with SDS running buffer (see table 2.18) before loading of the protein samples. Thirty μg of isolated protein were diluted in a total amount of 20μl with LBII lysis buffer, mixed with 5μl 5x reducing Laemmli buffer (see table 2.19,

7% β-mercaptoethanol) and denatured at 100°C for 5min. After denaturation, the protein samples and additionally a PageRuler Plus pre-stained protein ladder, 10 to 250 kDa, (Thermo Fisher Scientific) were loaded onto the polyacrylamide gel in the SDS running buffer-filled electrophoresis tank. The gel was run for 15min at 60V then for 2h at 120V in the SDS running buffer.

Table 2.17: Gel composition for protein SDS gel electrophoresis

5% Stacking gel (2 gels)	**10% Separating gel (2 gels)**
3.6ml dH_2O	5.88ml dH_2O
0.625ml Tris 0.5M (pH = 6.8)	3.75ml Tris 1.6M (pH = 8.8)
0.1ml 10% SDS	0.3ml 10% SDS
0.625ml 30% acrylamide/Bis; 29:1(Bio-Rad)	5.025ml 30% acrylamide/Bis; 29:1(Bio-Rad)
50μl APES	37.5μl APES
75μl TEMED	14μl TEMED

Table 2.18: SDS running buffer

25mM Tris base
192mM glycine
0.1% SDS

Table 2.19: 5x reducing Laemmli buffer

300mM Tris.Cl pH = 6.8
60% glycerol
10% SDS
0.025% bromophenol blue
7% β-mercaptoethanol

2.13 Western blot

The proteins size-separated in the polyacrylamide gel were electro-blotted onto a methanol-activated PVDF membrane in transfer buffer (see table 2.20) at 4°C for 1.5-2h at 300mA or overnight at 18V. For a check of the blotted protein bands on the membrane, the proteins on the PVDF membrane were stained for 10-15min with Ponceau- solution (0.1% Ponceau, 5% acetic acid).

Table 2.20: Transfer buffer

13.39g/l glycine
3.03g/l Tris base
50ml/l methanol

The membranes were de-stained and blocked in 5% non-fat dried milk in TBST (TBS (table 2.21) with 0.1% Tween20) and afterwards incubated overnight at 4°C in the primary antibody diluted in blocking solution (see table 2.22). The membranes were washed 3x 10min with TBST and after washing, the membranes were incubated 1-2h in HRP-coupled

secondary antibodies diluted 1:10000 in 5% non-fat dried milk in TBST. After washing 3x 10min with TBST and 1x 10min with TBS, the membranes were incubated with ECL chemo luminescence reagent (Bio Rad Clarity Western ECL Substrate or GE Healthcare Amersham ECL Select) for 1min. The antibody binding was visualized on x-ray films, detecting the chemo luminescence of the ECL, induced by the bound antibodies. Exposure times varied between one second and 60min depending on signal strength.

Table 2.21: TBS – Tris-buffered saline

8g/l NaCl
0.2g/l KCl
3g/l Tris base
HCl to adjust pH to 7.4

Table 2.22: Primary antibodies used for western blot detection

Antibody	Dilution and Diluent	Target size	Assay ID
Rabbit Total BRAF	1:1000 in 3% BSA in TBST	92kDa	Cell Signaling Technology
Mouse Mutant BRAF	1:1000 in 3% BSA in TBST	92kDa	Spring Bioscience
Rabbit anti-Erk 1/2	1:1000 in 3% BSA in TBST	42/44kDa	Cell Signaling Technology
Rabbit anti-pErk 1/2	1:1000 in 3% BSA in TBST	42/44kDa	Cell Signaling Technology
Mouse anti-β-Actin	1:2000 in 3% BSA in TBST	42kDa	Sigma-Aldrich

2.14 Detection of senescence-associated-β-galactosidase (SA-β-gal)

In order to investigate an induction of cell senescence upon overexpression of transduced mutant BRAF in MEWO cells, MEWO cells were again infected with mutant BRAF expressing retroviruses. First, 3x 10^5 cells were seeded into a 6-well plate and incubated for 24h. After 24h, MEWO cells were infected overnight with 1ml culture medium containing 0.8μg/ml polybrene and mutant BRAF expressing retrovirus or empty retrovirus without a transgene. After retroviral infection, the cells were maintained in normal culture medium for 5 days. In parallel, senescence was induced in A375 cells with 5μM vemurafenib diluted in normal growth medium. The A375 cells were treated for 5 days and used for senescence control. For detection of senescence-associated-β-galactosidase (SA-β-gal) in senescent cells, a KAA002 Cellular Senescence Assay kit (Merck Millipore) was used. According to the kit datasheet and manual, the cells were fixed for 15min at room temperature with the fixing solution provided with the kit. The fixed cells were stained in 2ml 1x SA-β-gal detection solution for 12h at 37°C in an incubator without adding additional CO_2. After staining, the cells were washed twice with PBS and stored in 70% glycerol at 4°C.

3 Results

3.1 Regulation of PMCA4b transcript expression by vemurafenib and valproic acid

Recent analysis indicates an increase of the PMCA4b protein level upon inhibition of mutant BRAF in malignant melanoma cells (Hegedus et al, manuscript in preparation). Increase of PMCA4b by vemurafenib also results in an enhanced intracellular calcium clearance and an induced inhibition of migration. Inhibition of migration was also observed when PMCA4b was ectopically overexpressed in melanoma cells. Since increased PMCA4b protein levels upon inhibition of mutant BRAF could result from transcriptional or post-transcriptional regulation, quantitative real time PCR analysis was used to analyze the PMCA4b transcript expression level in melanoma cell lines upon treatment with vemurefenib.

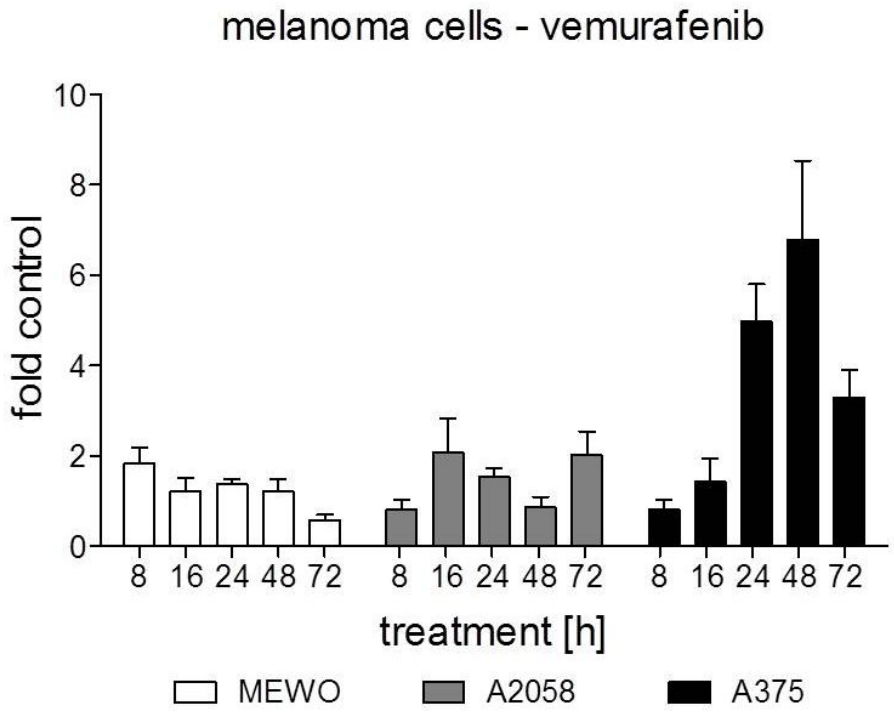

Figure 3.1: Quantitative real time PCR of PMCA4b mRNA in MEWO, A2058 and A375 melanoma cells treated with 0.5μM vemurafenib. Expression change after 8-72h of treatment compared to the respective untreated controls.

Real time PCR data confirmed a noticeable increase of PMCA4b expression in at least one of the two mutant BRAF expressing cell lines upon treatment with vemurafenib. A375 cells showed a 3 – 6-fold increase of PMCA4b expression during 72h of treatment, while A2058 cells showed only modest PMCA4b expression changes (see figure 3.1).

Next, the HDAC inhibitor valproic acid was used to investigate a potential role of HDACs in the regulation of PMCA4b expression in melanoma cells. Melanoma cells were treated with valproic acid alone and in combination with vemurafenib to analyze an epigenetic regulation of PMCA4b transcript expression and possible synergistic effects of vemurafenib in combination with valproic acid. The HDAC inhibitor valproic acid is supposed to inhibit classI HDACs and to induce histone hyperacetylation in human cancer cells.

MEWO cells (expressing wildtype BRAF) revealed an early 3 – 5-fold increase of PMCA4b in response to treatment with valproic acid and in response to the combination of valproic acid with vemurafenib. In A375 mutant BRAF expressing cells, valproic acid treatment induced a noticeable increase of PMCA4b expression after 48h of treatment. Combination treatments showed an enhanced PMCA4b expression in both mutant BRAF expressing cell lines. A375 and A2058 cells both showed a 3 – 7-fold increase of PMCA4b expression. In the A2058 cell line, the PMCA4b expression increase, observed with the combination, derives from a synergistic effect of vemurafenib and valproic acid, since both compounds alone have only minor effects on the PMCA4b transcript levels. In the A375 cells the enhanced PMCA4b expression is mainly induced by vemurafenib, since the combination treatment mimics the expression change of A375 cells treated with vemurafenib alone. (see figure 3.2)

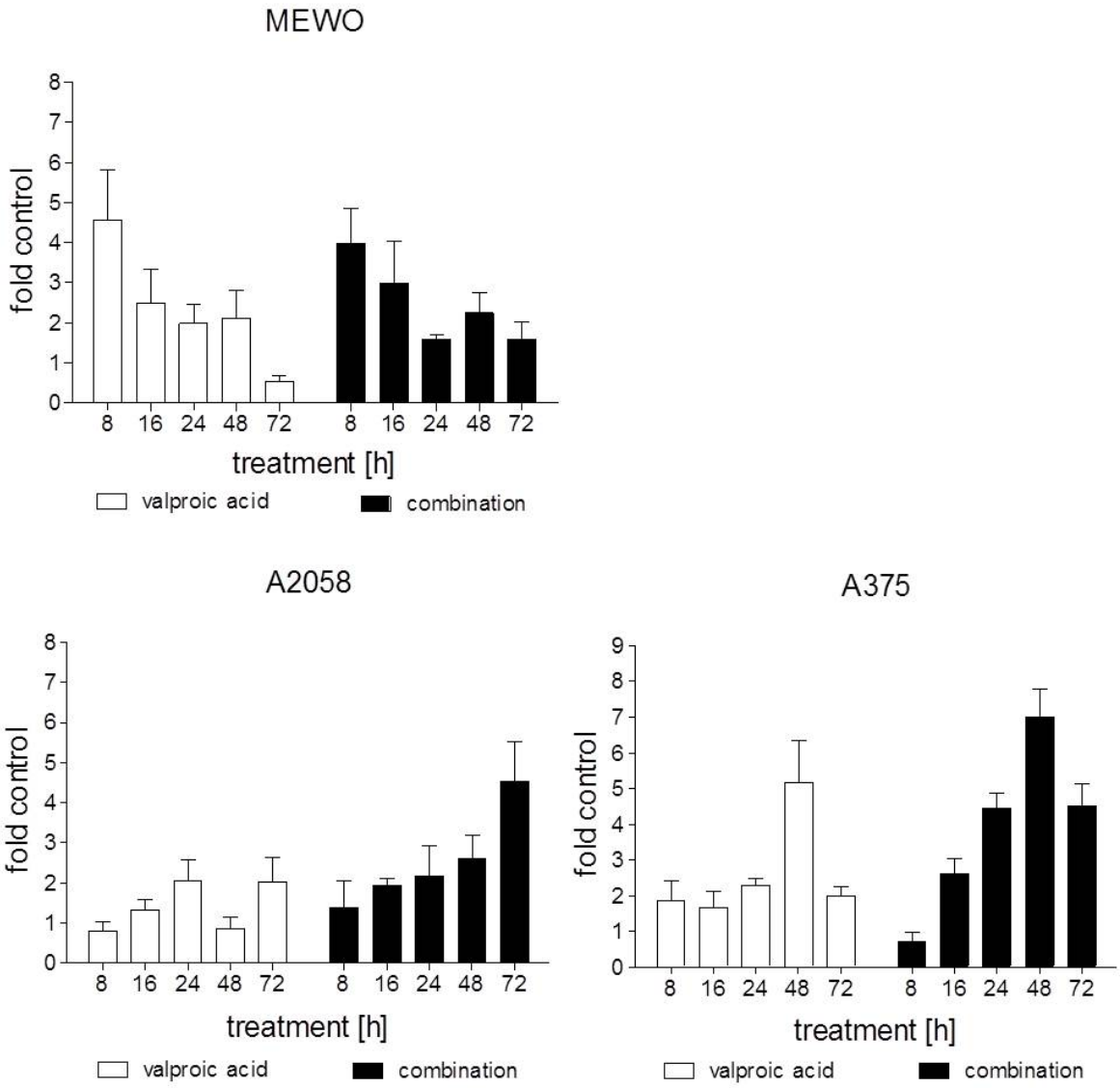

Figure 3.2: Quantitative real time PCR of PMCA4b mRNA in MEWO, A2058 and A375 melanoma cells treated with 2mM valproic acid alone and in combination with 0.5μM vemurafenib. Expression change after 8-72h of treatment compared to the respective untreated controls.

3.2 Regulation of transcript expression of other calcium channels by vemurafenib and valproic acid

As shown above, a noticeable increase of PMCA4b transcription upon treatment with vemurafenib or valproic acid was observed in melanoma cells. Since regulation of intracellular calcium homeostasis involves a complex network of several calcium channels, the expression regulation of other calcium channels was also investigated in vemurafenib and valproic acid treated melanoma cells. Vemurafenib treatment in general showed mostly

unchanged expression levels of calcium channels, except for an upregulation of TRPM1 in mutant BRAF expressing A2058 cells. While TRPM1 expression is low in the untreated A2058 cells, treatment with vemurafenib restored TRPM1 expression in mutant BRAF expressing A2058 cells (see figure 3.3). TRPM1 was undetectable in A375 cells with or without treatment.

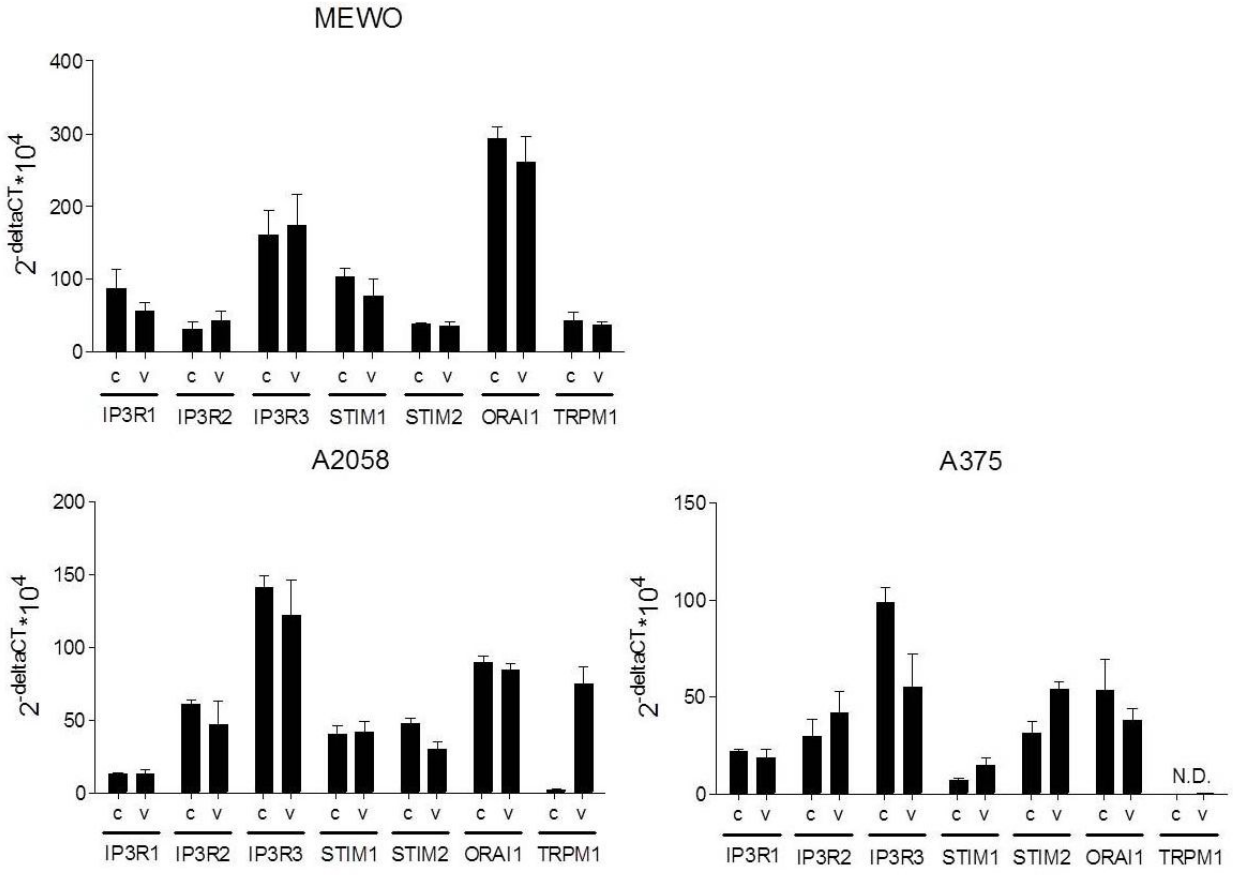

Figure 3.3: Quantitative real time PCR of calcium channels in MEWO, A2058 and A375 melanoma cells treated with 0.5μM vemurafenib (v) and in untreated controls (c). Inositol triphosphate receptor proteins 1-5 (IP3R), stromal interaction molecule protein 1 and 2 (STIM), calcium release-activated calcium channel protein 1 (ORAI1) and transient receptor potential channels (melastatin) 1 (TRPM1) were amplified with respective primers (see table 2.8). Expression levels were determined after 48h of treatment. In A375 cells TRPM1 was not detectable (N.D.).

3.2.1 Regulation of the intracellular IP3R calcium channels

IP3R intracellular calcium channels are activated by binding of InsP3 downstream of G-Protein coupled receptors or receptor tyrosine kinases. Active IP3R channels release intracellular-stored calcium from the ER into the cytosol and increase the cytoplasmic calcium concentration (Hogan et al. 2003; Foskett 2007). Since vemurafenib treatment indicated an unchanged expression of IP3R 1, 2 and 3 in melanoma cells, in addition,

the epigenetic regulation of the IP3R expression was analyzed upon treatment with valproic acid alone and in combination with vemurafenib. Inhibition of classI HDAC proteins revealed a noticeable expression increase of IP3R channels in mutant BRAF expressing melanoma cells (see figure 3.4). Valproic acid and combination treatment with vemurafenib increased IP3R1 expression in A375 cells and enhanced all three IP3R variants in A2058 cells. In A375 cells, IP3R2 expression was only enhanced by combination of vemurafenib and valproic acid. Except for expression of IP3R2 channels in A375 cells, the expression increase of IP3R channels in mutant BRAF expressing melanoma cells is mainly driven by the effect of valproic acid.

3.2.2 Regulation of STIM1 and STIM2, activators of transmembrane CRAC calcium channels

IP3R channels in the membrane of the ER mediate the release of intracellularly stored calcium from the ER into the cytoplasm (Soboloff et al. 2012). Depletion of ER-stored calcium by IP3R channels activates calcium sensitive STIM proteins in the ER membrane. Active STIM proteins aggregate with the pore-forming ORAI1 protein in the plasma membrane and induce SOCE through CRAC channels (Soboloff et al. 2006). Since an expression increase of IP3R channels is induced by valproic acid treatment in mutant BRAF expressing melanoma cells, an increase of STIM and ORAI1 expression by valproic acid was investigated in melanoma cells. Treatment with valproic acid and combination treatment with vemurafenib showed a noticeable increase of STIM1 and STIM2 expression levels in mutant BRAF expressing melanoma cells (see figure 3.4).

While A2058 cells revealed a modest increase of STIM expression, enhanced expression levels of STIM1 and STIM2 were observed in A375 cells after treatment with valproic acid and with combination treatment. Increase of STIM expression mainly seemed to derive from the effect of valproic acid. In parallel with a STIM expression increase, valproic acid also enhanced the ORAI1 expression in at least one of the two investigated mutant BRAF expressing melanoma cell lines (see figure 3.4).

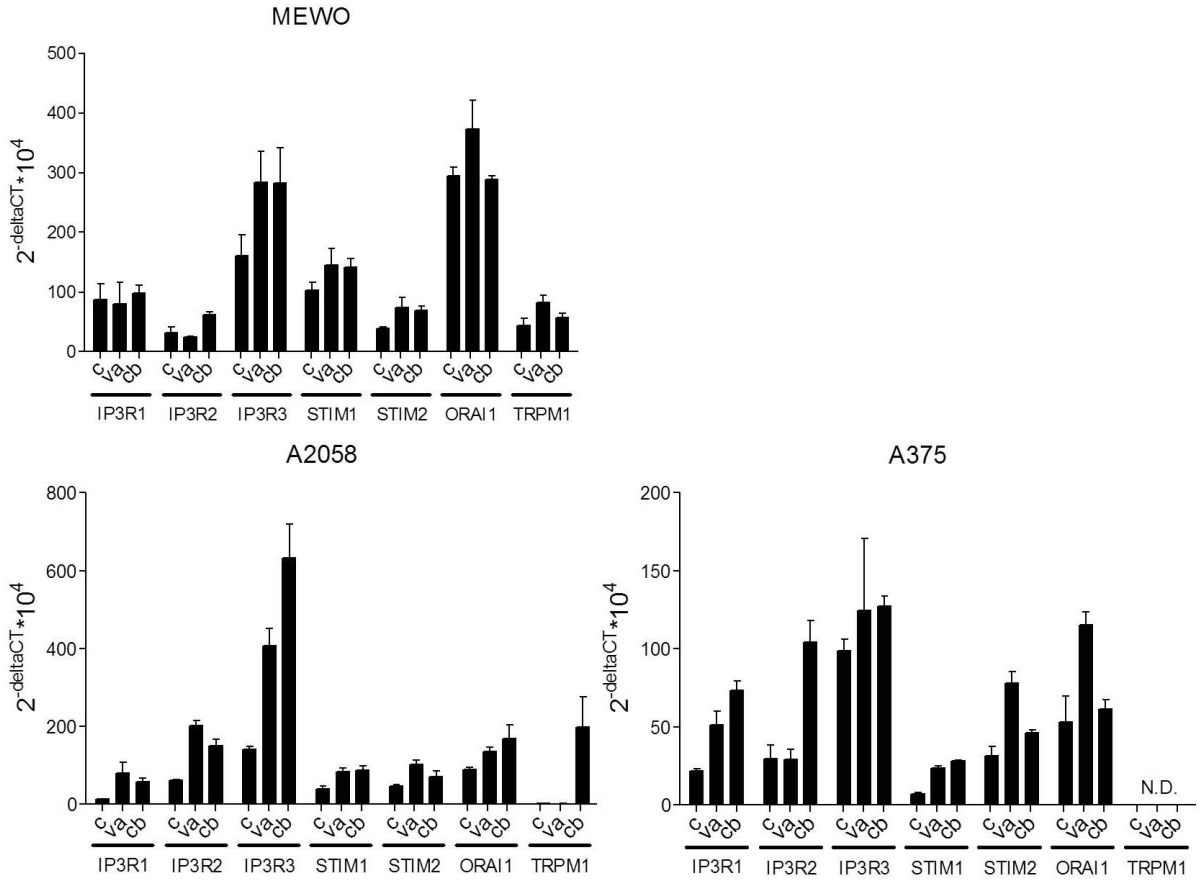

Figure 3.4: Quantitative real time PCR of calcium channels in MEWO, A2058 and A375 melanoma cells treated with 2mM valproic acid alone (va) and in combination with 0.5μM vemurafenib (cb) and in untreated controls (c). Inositol triphosphate receptor proteins 1-5 (IP3R), stromal interaction molecule protein 1 and 2 (STIM), calcium release-activated calcium channel protein 1 (ORAI1) and transient receptor potential channels (melastatin) 1 (TRPM1) were amplified with respective primers (see table 2.8). Expression levels after 48h treatment are shown. In A375 cells, TRPM1 was not detectable (N.D.).

3.3 Regulation of transcript expression of NFAT, MITF and of the AP1 complex elements c-fos, c-jun and Fra1

Increase of PMCA4b transcript expression upon inhibition of mutated BRAF indicates a suppression of PMCA4b expression by the constitutively activated MAPK pathway in melanoma cells. For a closer look into the regulatory mechanism of PMCA4b expression downstream of the MAPK pathway in malignant melanoma cells, expression of the transcription factors NFAT1-4 and the AP1 regulatory complex were analyzed upon treatment with vemurafenib and valproic acid.

NFAT2 has been discovered to stimulate PMCA transcription in osteoclasts. Furthermore PMCA4b proteins are able to interact with the NFAT activator protein calcineurin and

inhibit activation of the NFAT signaling pathway (Kim et al. 2012). Since NFAT activation depends on increased calcium concentrations in the cytoplasm and is linked to PMCA4b activity, NFAT expression was monitored after treatment with vemurafenib and valproic acid. All three melanoma cell lines indicated low levels of NFAT1, 2, 3 expression and high levels of NFAT4. The vemurafenib treated melanoma cells showed unchanged NFAT transcript expression levels (see figure 3.5). Both mutant BRAF expressing melanoma cell lines revealed a modest increase of NFAT4 expression upon treatment with valproic acid or combination with vemurafenib (see figure 3.7).

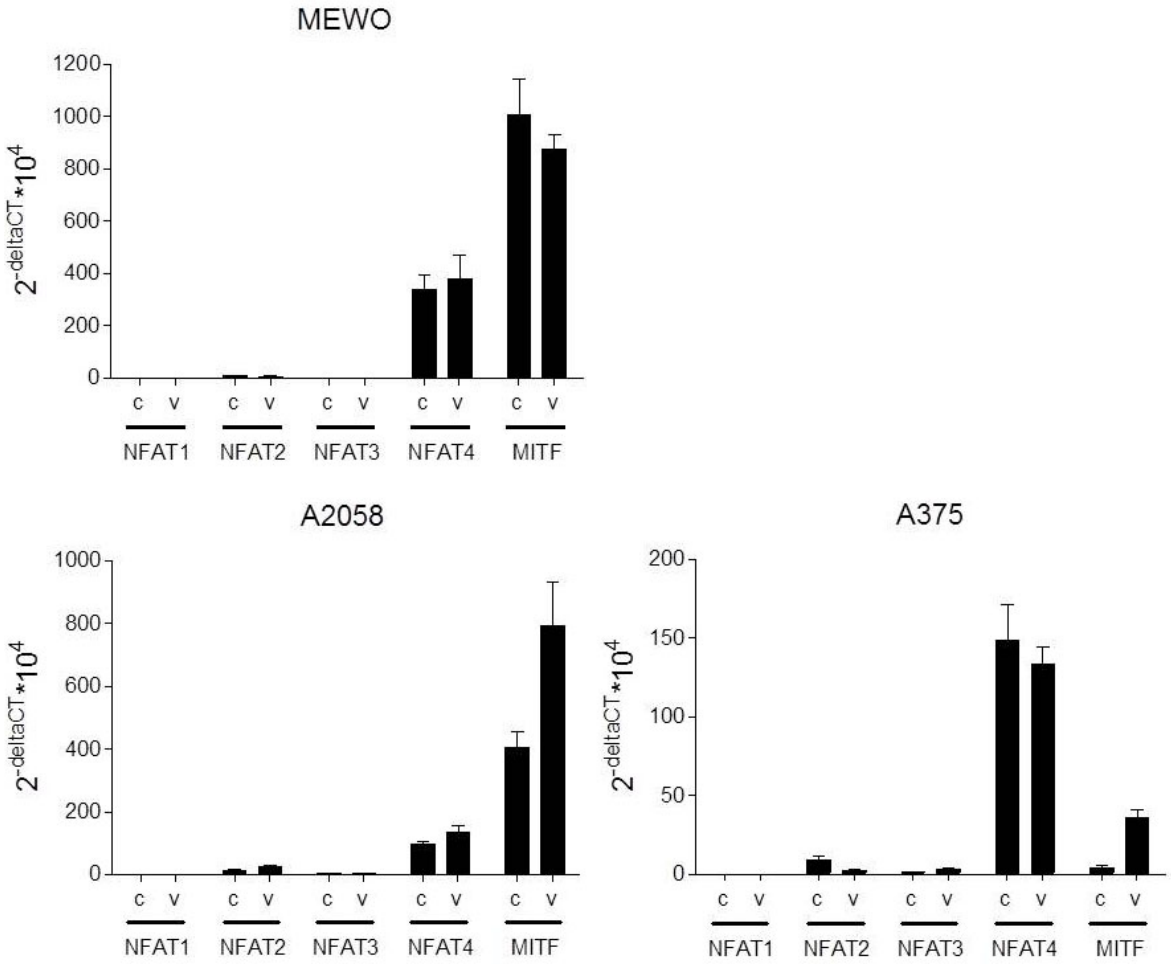

Figure 3.5: Quantitative real time PCR of transcription factors NFAT1-4 and MITF in MEWO, A2058 and A375 melanoma cells upon treatment with 0.5μM vemurafenib (v) and in untreated controls (c). Expression levels after 48h of treatment are shown.

NFAT transcription factors form cooperative complexes with AP1 transcription regulators (Hogan et al. 2003). Since AP1 complexes, composed of c-fos, c-jun and Fra1 dimers, are regulated downstream of the MAPK pathway, expression changes of these AP1 fac-

tors were analyzed upon inhibition of mutant BRAF. Quantitative real time PCR analysis indicated low expression levels of c-jun and c-fos in MEWO and A375 melanoma cells. Fra1 transcript expression showed a strong regulation in both cell lines by vemurafenib treatment. In MEWO wildtype BRAF expressing cells Fra1 revealed a 10-fold expression increase upon treatment with vemurafenib. In contrast to the MEWO cells, the A375 mutant BRAF expressing cells showed a 9-fold downregulation of Fra1 transcript expression upon treatment with vemurafenib (see figure 3.6).

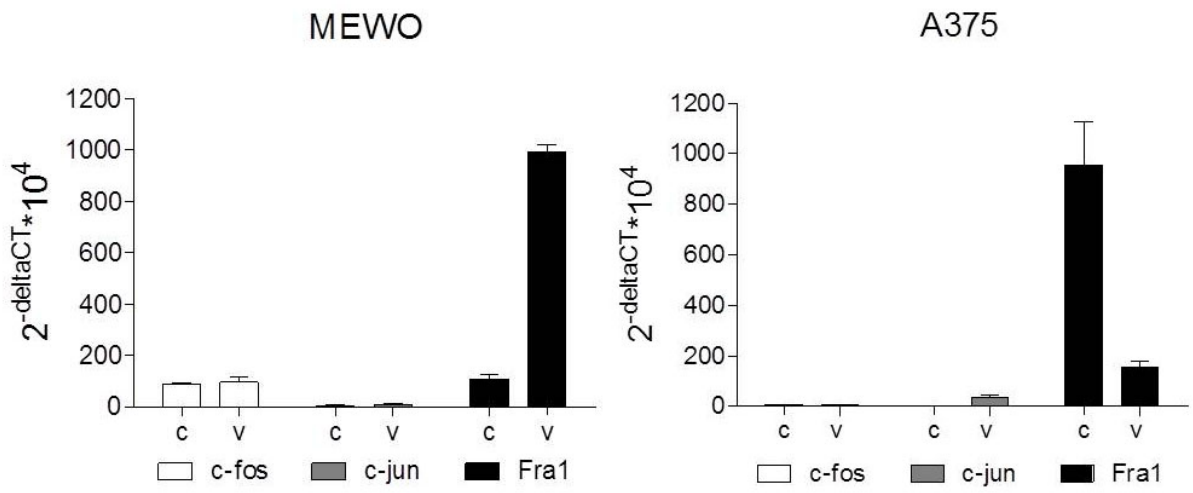

Figure 3.6: Quantitative real time PCR of c-fos, c-jun and Fra1, elements of the AP1 complex, in MEWO and A375 melanoma cells treated with 0.5μM vemurafenib or in untreated controls (c). Expression levels after 48h of treatment are shown.

The MITF transcription factor is a key regulator of specific gene expression in melanogenesis and has a critical role in development of melanoma. In mutant BRAF expressing melanoma cells, MITF activity is suppressed by the MAPK pathway. Hyper-activated ERK kinases phosphorylate MITF proteins and target MITF proteins for proteasomal-degradation in melanoma cells with oncogenic BRAF. Degradation and decrease of MITF levels support proliferation and survival in melanoma with mutant BRAF (Wellbrock et al. 2008). Since MITF mediates melanogenesis and is suppressed by pERK downstream of mutant BRAF, inhibition of the constitutively activated MAPK pathway suggests an increase of MITF in mutant BRAF expressing melanoma cells. Treatment with vemurafenib and valproic acid induced a noticeable increase of MITF expression in A375 cells (see figure 3.5 and 3.7). In A0258 cells the MITF expression was increased by treatment with vemurafenib alone and in combination with valproic acid. The combination of vemurafenib and valproic acid indicated a synergistic effect on MITF expression in A2058 cells (see figure 3.7).

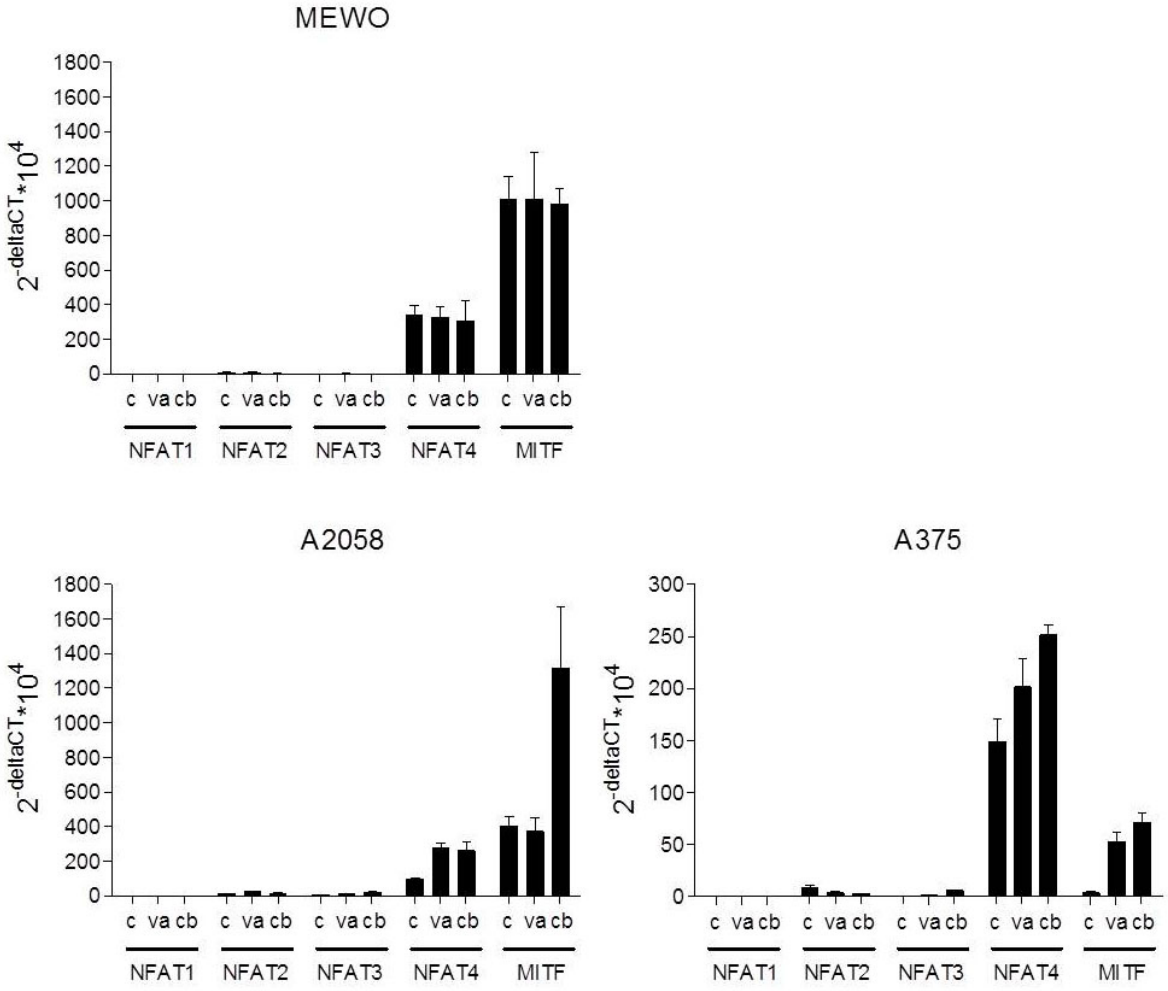

Figure 3.7: Quantitative real time PCR of transcription factors NFAT1-4 and MITF in MEWO, A2058 and A375 melanoma cells upon treatment with 2mM valproic acid (va) alone or in combination with 0.5μM vemurafenib (cb) and in untreated controls (c). Expression levels after 48h of treatment are shown.

3.4 Regulation of calcium channels and transcription factors by PMCA4b overexpression

To directly study the impact of elevated PMCA4b in BRAF mutant melanoma cells, A375 cells, stably overexpressing a functional GFP-PMCA4b chimeric protein, were generated. The GFP-PMCA4b overexpressing A375 cells were established with a sleeping beauty transposon system (Hegedus et al, manuscript in preparation). Two clones of PMCA4b overexpressing A375 cells (A375-GFP-PMCA4b-I, A375-GFP-PMCA4b-II) and one control clone expressing only GFP (A375-GFP) were used to investigate the consequences of forced overexpression of PMCA4b in melanoma cells. In order to analyze the regulation of cal-

cium channels IP3R, STIM1-2, ORAI1 and TRPM1 and transcription factors NFAT and MITF upon PMCA4b overexpression, expression levels were investigated in PMCA4b overexpressing A375 melanoma cells. Similar to mutant BRAF inhibition, PMCA4b overexpression resulted in an expression increase of IP3R channels and STIM proteins in A375 melanoma cells (see figure 3.8). In contrast to the A375 cells treated with vemurafenib, overexpression of PMCA4b in A375 revealed an unchanged expression of MITF transcription factor and an expression increase of NFAT4.

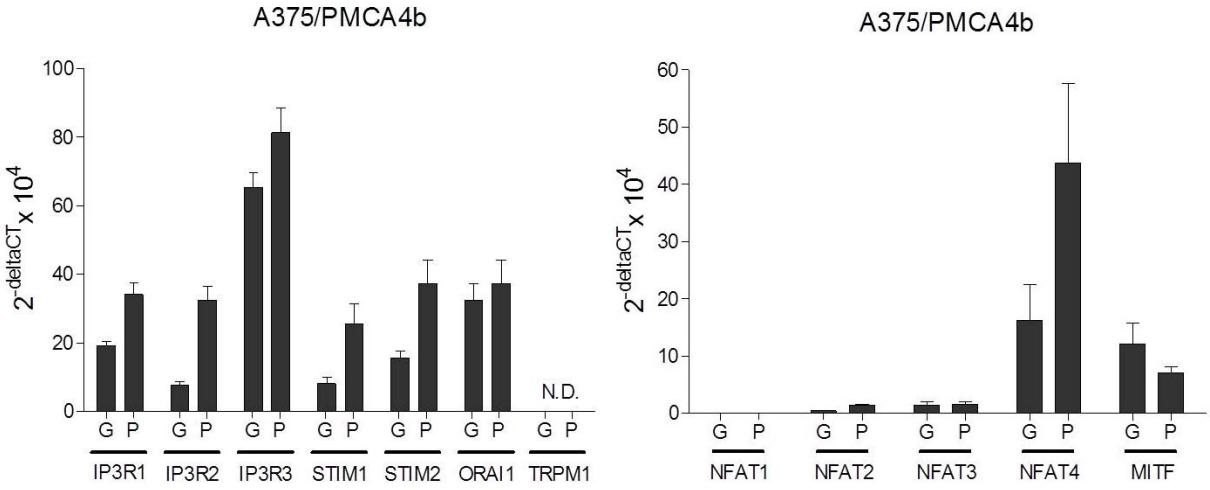

Figure 3.8: Quantitative real time PCR of inostitol triphosphate receptor 1-5 (IP3R) channels, stromal interaction molecule 1 and 2 (STIM), transient receptor potential channels 1 (TRPM1), calcium release-activated calcium channel protein 1 (ORAI1), nuclear factors of activated T-cells 1-4 (NFAT) and microphthalmia-associated transcription factor (MITF) in PMCA4b overexpressing (P) and in GFP overexpressing control (G) A375 melanoma cells. TRMP1 was not detectable (N.D.).

3.5 Forced overexpression of wiltype and mutant BRAF

Since inhibition of oncogenic BRAF kinases and histone deacetylases in mutant BRAF expressing cells resulted in an increase of PMCA4b expression, mutant BRAF was hypothesized to have a repressive effect on PMCA4b expression. For analysis of PMCA4b expression in BRAF overexpressing human cancer cells, the open reading frames of BRAF variants were cloned into retroviral vectors and used for transduction of cancer cells with wildtype or mutant BRAF.

3.5.1 Construction of BRAF expressing retroviruses

For generation of BRAF expressing retroviruses, the open reading frame of either wildtype or mutant BRAF was cloned into the retroviral expression plasmid pQCXIP. The correct orientation and sequence were confirmed by DNA sequencing analysis, using specific BRAF

primers (Eurofins) (see figure 3.9). The mutant BRAF variant in the retroviral expressing plasmid backbone showed the expected substitution of a glutamic acid for a valine at the residue 600 and did not show reading frame shifts or additional mutations.

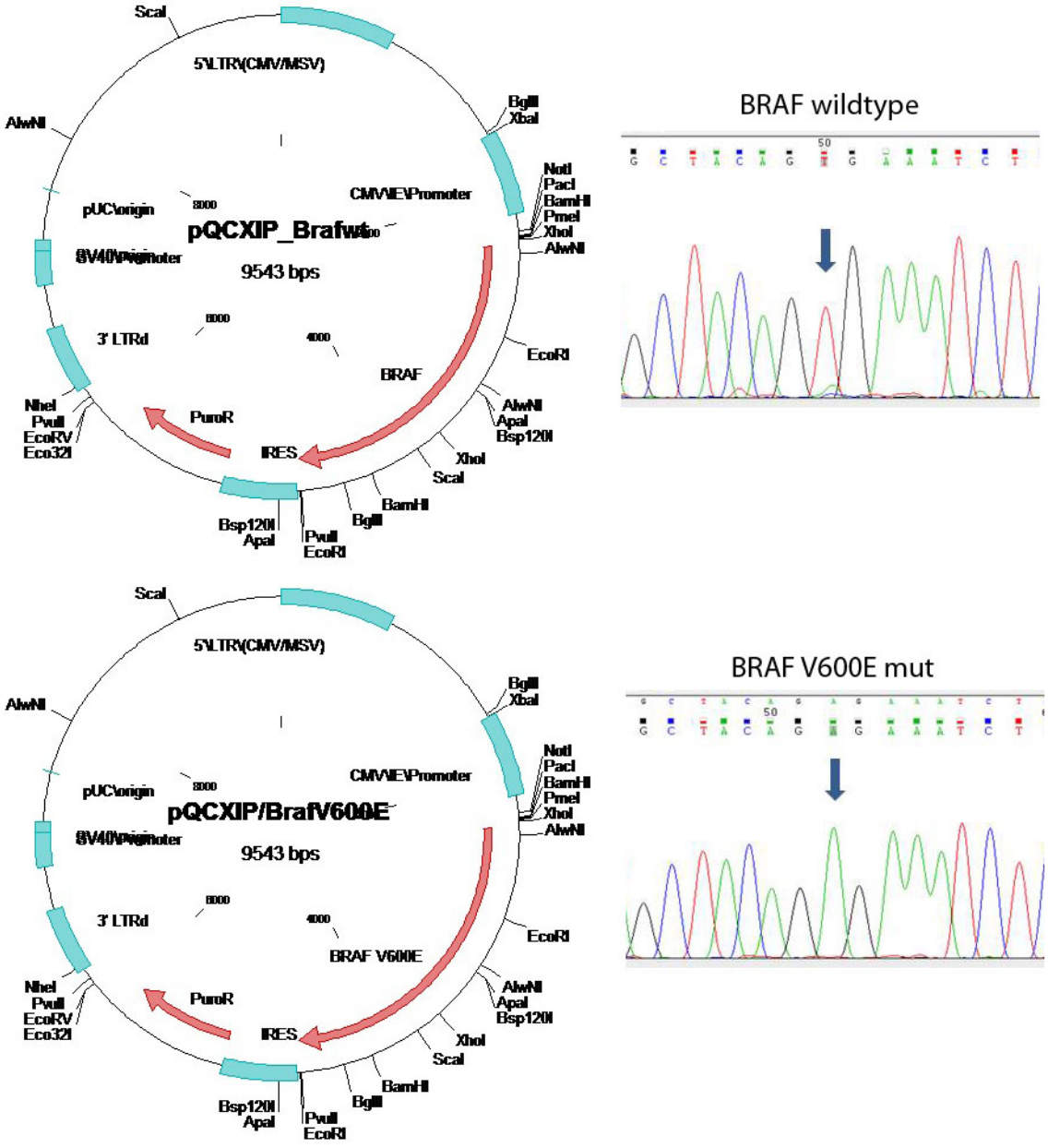

Figure 3.9: Retroviral expression vector pQCXIP with inserted wildtype (pQCXIP/BRAFwt) or mutant BRAF with a V600E mutation (pQCXIP/BRAFV600E) (left). Sequencing analysis of inserted wildtype and mutant BRAF open reading frames (right). The chromatogram shows the expected nucleotide substitution in the mutant BRAFV600E insert (arrow) leading to a replacement of valine with glutamic acid at the residue 600 in the activation domain.

The western blot analysis further confirmed the correct expression of the generated

BRAF expressing retroviral plasmids upon transient transfection in HEK293 cells. High levels of wildtype and mutant BRAF protein were detected in HEK293 cells upon transient transfection of the respective plasmid constructs (see figure 3.10). Since the transfection of BRAF variants in HEK293 cells showed high efficiency, HEK293 cells were used for generation of BRAF expressing retroviruses.

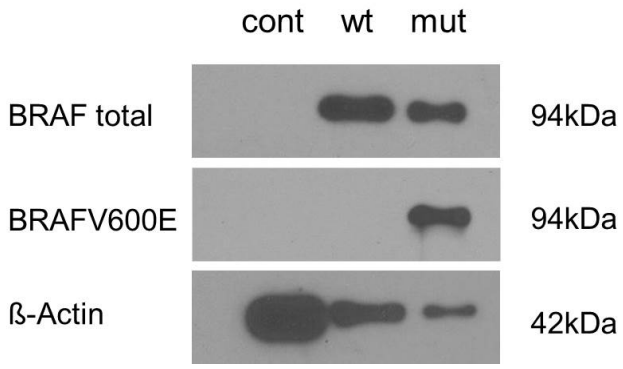

Figure 3.10: Western blot analysis of HEK 293 cells, transiently transfected with wildtype (pQCXIP/BRAFwt) or mutant BRAF (pQCXIP/BRAFV600E) expression vectors. Total protein was harvested and loaded on the polyacrylamide gel. Protein levels of total BRAF (BRAF total), mutant BRAF (BRAF V600E) and β-actin reference protein were detected with respective primary antibodies (see table 2.22) in HEK293 cells transfected with wildtype BRAF (wt) or mutant BRAF (mut) and in untransfected control cells (cont).

3.5.2 Transient overexpression of wildtype and mutant BRAF in melanoma cells

First, the BRAF expressing retroviral vectors were used to transiently transfect wildtype and mutant BRAF into MEWO cells. Twenty four- 48h after transient transfection, the GFP control cells already showed clear fluorescence and confirmed successful transfection in 40% of the cells (see figure 3.11). Overexpression of wildtype and mutant BRAF could be verified on mRNA level and protein level 24h-92h post transfection. The transfected MEWO cells showed an 800-fold and 200-fold expression increase of BRAF (see figure 3.11). Control cells showed only a modest transcript expression of BRAF.

Western blot analysis confirmed high protein levels of transfected wildtype and mutant BRAF. BRAF protein was not detectable in the EGFP expressing control cells and the control cells transfected with empty retroviral expression plasmids. High protein levels of ERK and pERK could be seen in the wildtype or mutant BRAF transfected samples and in the EGFP and empty vector control. The levels of ERK and pERK protein were not altered upon forced short-term overexpression of wildtype and mutant BRAF in MEWO cells (see figure 3.11).

During 92h post-transfection the mutant BRAF overexpression more rapidly decreased to a basal level in comparison to the wildtype BRAF overexpression in the MEWO cells. DNA sequencing analysis of the purified real time PCR products confirmed a V600E point mutation in the kinase domain of the transfected mutant BRAF.

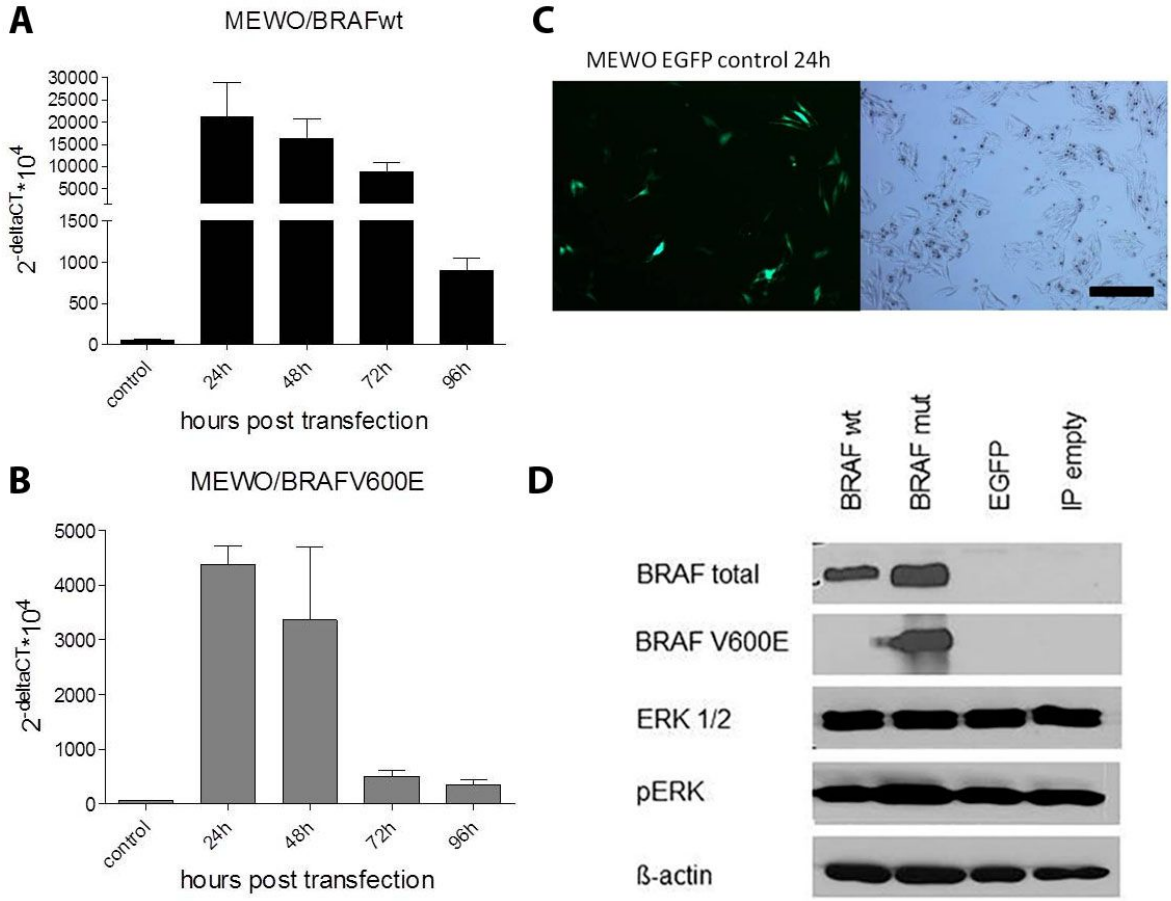

Figure 3.11: Transient overexpression of wiltype and mutant BRAF upon transient transfection with wildtype (BRAFwt) or mutant (BRAFV600E) BRAF expressing plasmids. A,B. Expression levels of wildtype and mutant BRAF 24-96h post transfection. C. Image of EGFP positive MEWO cells 24h post transfection. Scalebar 200μm D. Western blot analysis 24h post transfection with wildtype BRAF (BRAFwt), mutant BRAF (BRAFmut), EGFP expressing plasmids and empty vectors without a transgene (IP empty). Protein levels of total BRAF (BRAF total), mutant BRAF (BRAFV600E), ERK (ERK1/2), phosphorylated ERK (pERK) and β-actin reference protein were detected with respective antibodies (see table 2.22).

Since distinct overexpression of wildtype and mutant BRAF in the transfected MEWO cells could be confirmed, the regulation of PMCA4b expression was analysed upon BRAF overexpression. Quantitative real time PCR data showed no expression change of PMCA4b in wildtype and mutant BRAF overexpressing MEWO cells. PMCA4b expression levels were not affected by BRAF overexpression, but increased with high confluence of MEWO cells 72h and 96h post transfection (see figure 3.12).

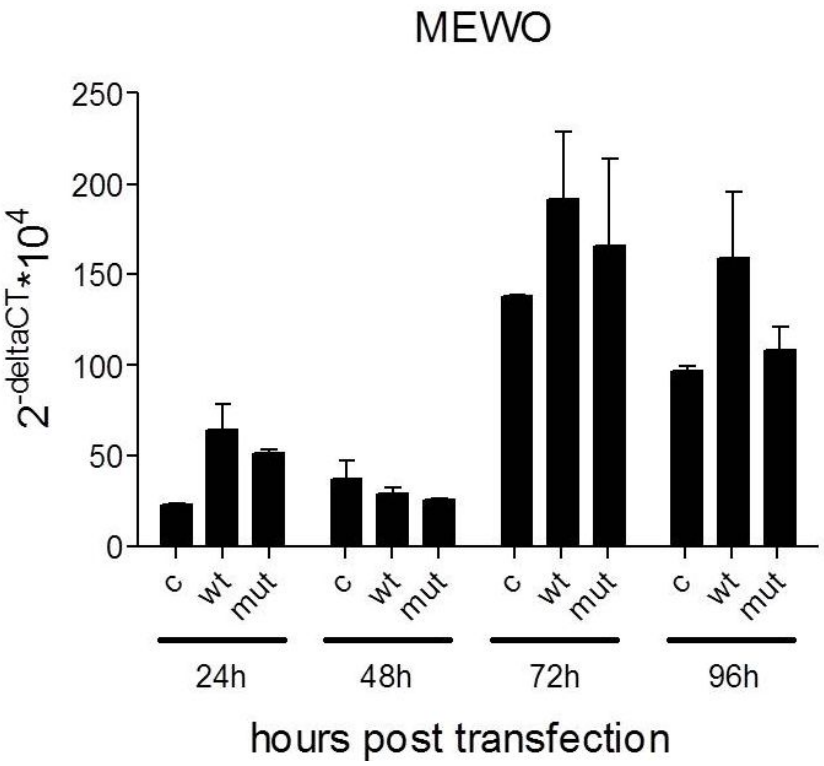

Figure 3.12: Quantitative real time PCR analysis of PMCA4b expression levels in MEWO melanoma cells. Expression levels 24-96h post transfection with wildtype (wt) or mutant (mut) BRAF expressing plasmids and in untransfected control cells (c).

3.5.3 Stable overexpression of wildtype and mutant BRAF in melanoma cells

Since PMCA4b expression was unaffected by short-term overexpression of wildtype and mutant BRAF, retroviruses were generated to establish stable wildtype and mutant BRAF overexpression in MEWO melanoma cells and Caco2 colon cancer cells. The stably transduced MEWO cells with respective retroviruses showed well defined puromycin-resistant cell colonies, while the MEWO cells of the negative control without retroviral infection died after adding 0.8μg/ml puromycin selection. The transduction controls, infected with either GFP or mCherry expressing retroviruses already showed fluorescent cells 24h post transduction. After completion of selection, the quantitative real time PCR data clearly showed a 60-fold expression increase of wildtype BRAF in stably transduced MEWO cells upon infection with respective retroviruses (see figure 3.13).

The western blot analysis revealed high level of wildtype BRAF protein upon transduction with respective retroviruses and confirmed stable overexpression in MEWO cells. The MEWO cells transduced with mutant BRAF expressing retrovirus, however, showed no overexpression of mutant BRAF (see figure 3.13). Mutant BRAF protein was only detected in A375 control cells. ERK and pERK antibodies indicated high protein levels in each samples. The ERK levels of the transduced cells did not differ from the protein level of the untransduced control cells. MEWO cells with stable wildtype BRAF overexpression

showed a slightly higher pERK level. Quantitative real time PCR of the mutant BRAF transduced cells showed a modest increase of BRAF expression upon stable transduction (see figure 3.13).

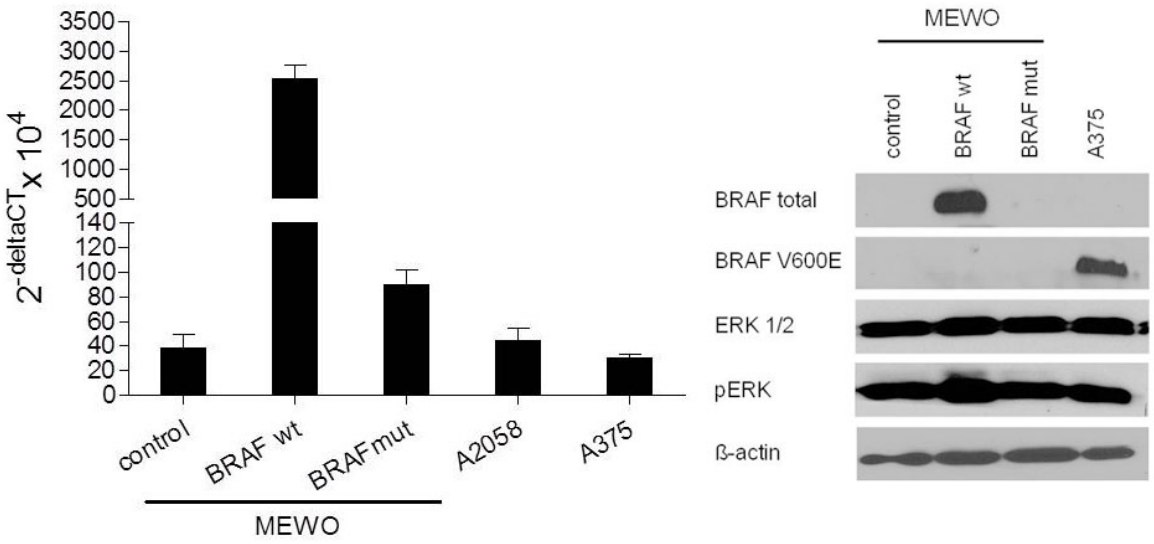

Figure 3.13: Quantitative real time PCR analysis of BRAF expression levels (left) and western blot analysis of BRAF protein levels (right) in MEWO melanoma cells stably transduced with wildtype (BRAFwt) or mutant (BRAFmut) BRAF expressing retroviruses, untransduced cells (control) and in mutant BRAF expressing A2058 and A375 cells. Protein levels of total BRAF (BRAF total), mutant BRAF (BRAFV600E), ERK (ERK1/2), phosphorylated ERK (pERK) and β-actin reference protein were detected with respective antibodies (see table 2.22).

MEWO cells transduced with mutant BRAF retrovirus continued to grow in the selection medium and revealed resistance to puromycin. MEWO cells apparently did not tolerate overexpression of mutant BRAF, but kept the puromycin resistance. To test whether the reason for this observation might be that overexpression of mutant BRAF leads to senescence, a staining for senescent cells was performed in MEWO cells following transduction with mutant BRAF. The senescence control showed clear blue staining of SA-β-gal in 10% of vemurafenib treated A375 melanoma cells (see figure 3.14). Mutant BRAF transduction revealed only five SA-β-gal positive MEWO cells per well, thus indicating that senescence is not a major cause for loss of mutant BRAF expression in MEWO melanoma cells transduced with the mutant BRAF retrovirus.

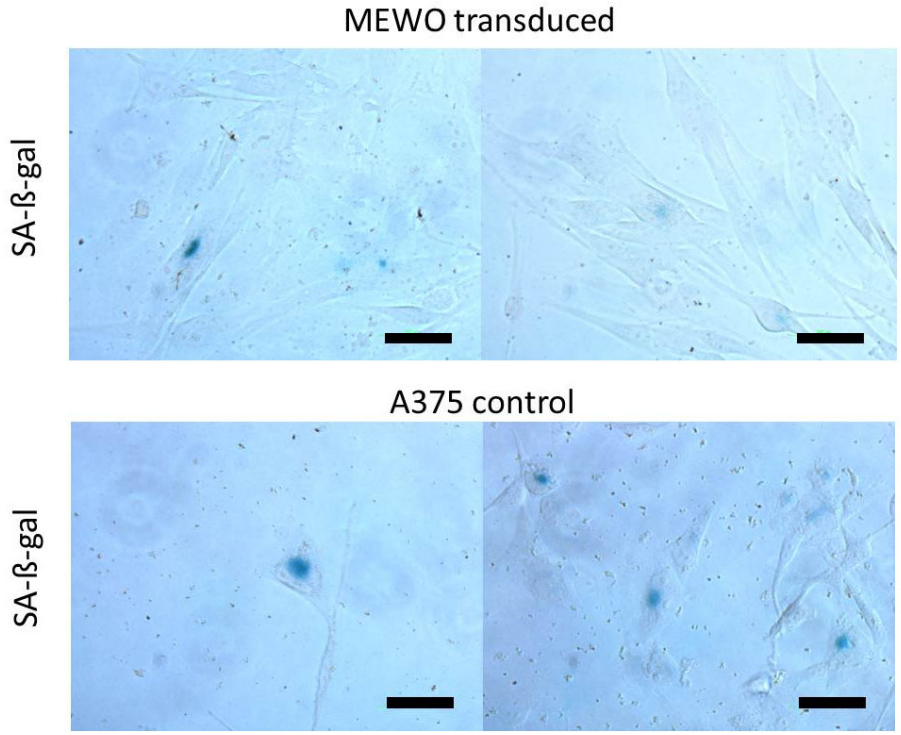

Figure 3.14: Senescence-associated-β-galactosidase (SA-β-gal) staining of the MEWO cells 5 days post transduction with mutant BRAF expressing retrovirus and of A375 cells treated for 5 days with 5μM vemurafenib. Scalebar 500μm.

3.5.4 Transient and stable overexpression of wildtype and mutant BRAF in colon cancer cells

Oncogenic BRAF is one of the most frequent gene mutations in melanoma and occurs in several other malignant cancer types such as colorectal cancer (Hertzman Johansson & Egyhazi Brage 2014). Transient transfection of Caco2 colon cancer cells with wildtype and mutant BRAF expressing plasmids was performed to induce overexpression of wildtype and mutant BRAF in colon cancer cells. Overexpression of transient wildtype and mutant BRAF in Caco2 colon cancer cells was confirmed by real time PCR analysis 24h post transfection with BRAF expressing retroviral plasmids. Transfected Caco2 cells indicated a 300-fold expression increase of wildtype and mutant BRAF 24h post transfection (see figure 3.15).

In order to establish stable overexpression of wildtype and mutant BRAF in Caco2 cells, Caco2 cells were infected with the respective retroviruses. The cells revealed puromycin resistant cell colonies upon transduction with the respective retroviruses, however, the untransduced control cells also showed resistant cell colonies even after increasing the pyromycin concentrations to 10μg/ml. The transduction control with mCherry expressing retroviruses nevertheless resulted in a clear fluorescence in the Caco2 cells 24-48h post transfection and also after completion of selection. Western blot analysis, however, showed

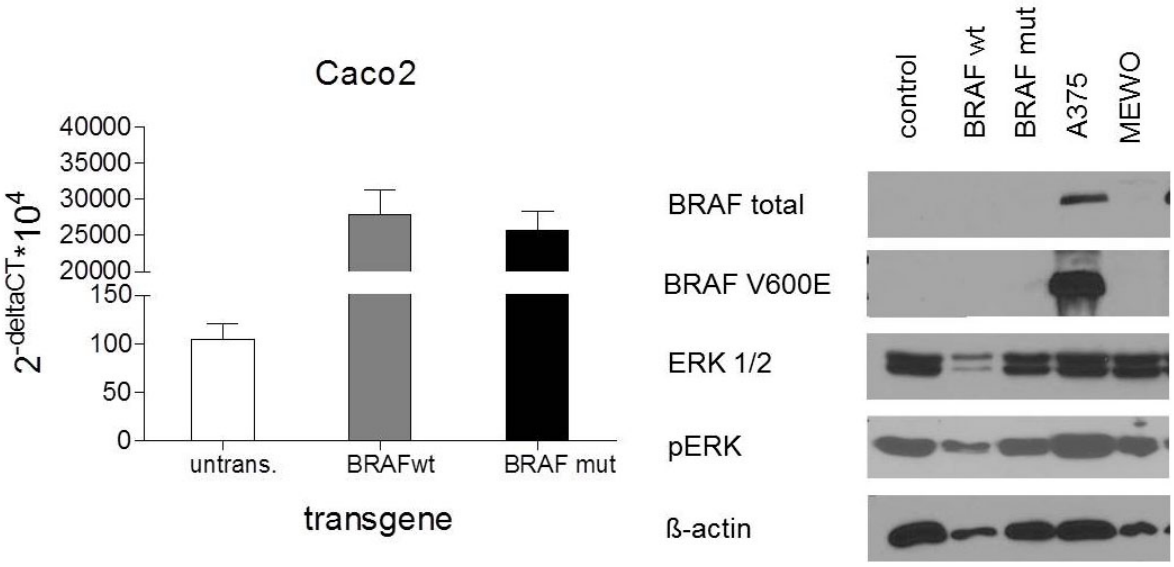

Figure 3.15: Quantitative real time PCR analysis of BRAF expression levels in Caco2 colon cancer cells 24h after transient transfection with wildtype (BRAFwt) or mutant (BRAFmut) BRAF expressing plasmid (left). Western blot analysis of Caco2 colon cancer cells stably transduced with wildtype or mutant BRAF expressing retroviruses (right). Wildtype BRAF expressing cells (MEWO) and mutant BRAF expressing cells (A375) were used as BRAF control. Protein levels of total BRAF (BRAF total), mutant BRAF (BRAFV600E), ERK (ERK1/2), phosphorylated ERK (pERK) and β-actin reference protein were detected with respective antibodies (see table 2.22).

no stable overexpression of wildtype or mutant BRAF in the Caco2 cells (see figure 3.15). In the MEWO control cells, the total BRAF antibody was not sensitive enough to detect endogenous BRAF protein, but the A375 control cells indicate a high level of mutant BRAF protein. The ERK level of the Caco2, MEWO and A375 cells was roughly equal. In comparison to Caco2 and MEWO cells the pERK protein level was slightly increased in the A375 mutant BRAF expressing cells (see figure 3.15).

4 Discussion

4.1 Regulation of calcium channels by wildtype and mutant BRAF

Melanoma is still one of the most deadly cancers worldwide with very poor prognosis once the tumor has metastasized. In approximatly 66% of melanoma patients, mutations can be found in the BRAF oncogene (Houben et al. 2004; Hertzman Johansson & Egyhazi Brage 2014). Treatment of metastatic melanoma with the mutant BRAF specific inhibitor vemurafenib results in therapeutic benefit. Nevertheless development of drug resistances and limited response to therapies with BRAF inhibitors require discovery of additional molecular targets in malignant and metastatic melanoma (Yadav et al. 2012).

Treatment with vemurafenib inhibits ATP-binding of the mutant BRAF kinase and prevents phosphorylation and activation of the MAPK kinase MEK and consequently of ERK. Inhibition of mutant BRAF activity and decrease of the constitutively activated MAPK pathway in melanoma cells induce remodeling of ERK-regulated target gene expression. Aberrant PMCA4b expression and dysregulation of intracellular calcium homeostasis were discovered in mutant BRAF expressing human cancer cells (Ribiczey et al 2007, Varga et al 2014). Recent analysis indicates an increased protein level of PMCA4b upon inhibition of mutated BRAF in malignant melanoma cells. Both, upregulation of PMCA4b upon mutant BRAF inhibition and an induced overexpression of PMCA4b result in enhanced intracellular calcium clearance and reduced cell migration (Hegedus et al, manuscript in preparation). The results by Hegdus et al indicate that PMCA4b probably acts as an unrecognized metastatic suppressor downstream of oncogenic BRAF.

In this thesis vemurafenib was used to inhibit mutated BRAF in malignant melanoma cells. Expression regulation of PMCA4b calcium pumps and other intracellular calcium channels downstream of the inhibited MAPK cascade was investigated. The transcript expression analysis showed that upon treatment with vemurafenib PMCA4b transcript expression was increased only in one of the two mutant BRAF expressing melanoma cell lines. Vemurafenib treatment of A2058 mutant BRAF expressing melanoma cells revealed only a modest change of PMCA4b expression. A2058 cells harbour an additional mutation in the tumor suppressor gene PTEN. Beside NRAS and BRAF mutations, in about 30-40% of melanoma cells a mutation in the tumor suppressor PTEN is found (Wu et al 2003).

The phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumor suppressor gene that encodes for a lipid and protein phosphatase (Carracedo & Pandolfi 2008; Georgescu 2010). The main function of PTEN is the buffering of the PI3K-Akt

pathway, which is a frequently activated survival pathway in cancer. The primary function of the phosphatidylinositol-3-kinase (PI3K) is the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) by phosphorylating PIP2. PTEN is an antagonist of PI3K and hydrolyzes PIP3 to PIP2 (Georgescu 2010). Thereby PTEN acts as a tumour suppressor and negatively regulates the PI3K-Akt pathway. Upon activation by the second messenger PIP3, Akt regulates several downstream factors that are involved in the control of cell survival, growth, proliferation and other cellular processes (Altomare & Testa 2005). The loss and mutation of PTEN in various cancers leads to hyperactivation of the PI3K-Akt signaling (Carracedo & Pandolfi 2008).

PTEN regulates cell cycle control proteins such as cyclin proteins and cyclin-dependent kinases (CDKs) and MAPK in the growth signal-induced MAPK signaling pathway. In cancer cells, ectopic expression and activity of PTEN inhibit phosphorylation levels of MEK and ERK in the MAPK cascade in absence of extracellular stimuli (Wu et al 2003). Inhibition of MEK and ERK kinases by PTEN indicates that the phosphatase PTEN intersects with constitutively active MAPK signaling. It is possible that the mutated PTEN plays an additionally unrecognized role in regulation of PMCA4b expression and acts as an antagonist of oncogenic BRAF in melanoma. The expression of the other investigated calcium channels was not affected upon vemurafenib treatment, except an induced expression increase of melanocyte-specific TRP channel variant TRPM1 in A2058 mutant BRAF expressing melanoma cells. TRPM1 expression is steadily lost in progression of cutaneous melanoma (Miller et al. 2004). A2058 cells showed only a modest increase of PMCA4b transcript, but demonstrated an induced re-activation of lost TRPM1 expression upon vemurafenib treatment.

An increase of PMCA4b transcript levels in vemurafenib treated melanoma cells suggests that the constitutively active MAPK pathway suppresses PMCA4b expression in mutant BRAF expressing melanoma cells. Furthermore, in melanoma cells with an additional mutation in the tumor suppressor PTEN, lost expression of TRPM1 could be restored upon inhibition of mutant BRAF. Regulation of PMCA4b and TRPM1 by PTEN in BRAF mutant melanoma is a hypothesis that still has to be substantiated. To investigate the role of wildtype or mutant PTEN in regulation of calcium channels in melanoma, additional cell models will be required to investigate expression of calcium channels in mutant PTEN expressing melanoma cells and upon forced PTEN overexpression.

Cancer development and progression are likely to require both, an accumulation of cancer promoting somatic mutations and an epigenetically induced repression of tumor suppressors and activation of oncogenes in certain signaling pathways and gene regulatory networks. HDAC proteins are co-repressors of gene transcription and promote a heterochromatic DNA packaging (Wang et al. 2010; de Ruijter et al. 2003). Extensive research in cancer epigenetics indicates a gene regulatory role of HDAC proteins during development and progression of cancer (Kim & Bae 2011; Andreoli et al. 2013). Nuclear classI HDAC proteins, HDAC1 and HDAC2, are highly involved in transcription regulation by inducing chromatin remodeling and by interaction with transcription factors. Specific inhibition of classI HDACs leads to induction of hyperacetylation and reactivation of

gene expression upon chromatin relaxation and regulation of transcription factor activity (Kraemer et al. 2003). Inhibition of HDAC proteins in human cancer cells induced cell differentiation and was shown to result in upregulation of PMCA4b calcium pumps in colon and breast cancer cells (Ribiczey et al. 2007, Varga et al. 2014). In order to investigate the role of HDAC proteins in the regulation of the observed calcium channels, melanoma cells were treated with the HDAC inhibitor valproic acid alone and in combination with vemurafenib. Valproic acid treatment increased the PMCA4b transcript expression in wildtype BRAF and in mutant BRAF expressing melanoma cells and confirmed an epigenetic regulation of PMCA4b expression by HDAC proteins. Vemurafenib alone showed only a modest increase of PMCA4b transcript expression in A2058 cells. In combination treatment with valproic acid, vemurafenib revealed a synergistic effect, which resulted in an enhanced PMCA4b expression in A2058 cells. While treatment with vemurafenib only modestly affected PMCA4b expression in A2058 cells with an additional PTEN mutation, PMCA4b transcript expression could be noticeably increased by combination treatment with valproic acid.

Regulation of intracellular calcium homeostasis requires not only PMCA calcium pumps, but involves intracellular IP3R channels in the Endoplasmic Reticulum, store-operated CRAC channels and TRP ion channels. Altered expression and activity of calcium channels stimulate dysregulation of intracellular calcium signaling and promotes tumor progression (Chen et al. 2013). In addition to PMCA4b increase, treatment with valproic acid and combination with vemurafenib induced an expression increase of IP3R channels, CRAC activator proteins STIM1 and STIM2 in mutant BRAF expressing melanoma cells. Like an increase of PMCA4b by vemurafenib treatment, PMCA4b overexpression induced an expression increase of IP3R and STIM in A375 cells with mutated BRAF.

The observed upregulation of PMCA4b calcium pumps in combination with an increase of IP3R channels and STIM proteins in the ER-membrane suggests that valproic acid alone and combined with vemurafenib stimulates an increase of ER-stored calcium release accompanied by an enhanced calcium clearance through an enriched abundance of PMCA4b calcium pumps.

4.2 MAPK pathway-dependent regulation of Fra1 and MITF transcription factors in melanoma cells

In order to investigate transcription factors that are associated with regulation of PMCA4b expression and calcium signaling, expression of the transcription factors NFAT1-4 was analysed upon treatment with vemurafenib and valproic acid. In osteoclasts NFAT2 proteins stimulate PMCA expression (Kim et al. 2012). NFAT2 and 4 are expressed in metastatic melanoma and activated by oncogenic BRAF (Flockhart et al. 2009). Since NFAT proteins are regulated by oncogenic BRAF in melanoma and a NFAT2 binding site was described in the promoter region of the PMCA gene, an expression regulation of PMCA4b calcium pumps by increased NFAT levels was suggested in melanoma cells.

Furthermore, NFAT proteins interact with AP1 protein dimers, forming cooperative transcription regulator complexes (Hogan et al. 2003). AP1 complex formation is regulated downstream of the MAPK pathway upon activation of the MAPK proteins ERK and JNK (Karin 1995). Treatment with vemurafenib and valproic acid did not induce a noticeable expression change of NFAT proteins, but vemurafenib induced an expression change of the AP1 element Fra1 in melanoma cells, dependent on the BRAF mutation status. While vemurafenib treatment increased the Fra1 expression in MEWO BRAF wildtype cells, in mutant BRAF expressing A375 cells the Fra1 expression was downregulated upon inhibition of the oncogenic BRAF in line with a previous study (Michaloglou et al. 2005).

Inhibition of the constitutively active MAPK pathway in mutant BRAF expressing A375 cells decreased the expression level of Fra1 and suggests that AP1 complexes with incorporated Fra1 proteins possibly suppress PMCA4b expression in melanoma with mutant BRAF.

MITF transcription factors regulate melanogenesis and play a critical role in development of melanoma. In mutant BRAF expressing melanoma cells MITF activity is suppressed by the MAPK pathway. Upon phosphorylation of MITF by ERK, phosphorylated MITF proteins get degraded in melanoma cells with oncogenic BRAF. Decreased MITF protein levels stimulate uncontrolled proliferation and survival of melanoma cells (Wellbrock et al. 2008). Treatment with vemurafenib, valproic acid and a combination of both drugs enhanced the MITF expression in mutant BRAF expressing melanoma cell. Reduced activation of ERK upon inhibition of BRAF in melanoma cells suggests that increased production of MITF transcription factors stimulates expression of tryrosinases and tyrosinase-related protein1 and induces cell differentiation of melanoma cells. Furthermore in melanocytes and melanoma cells MITF was found to target and increase expression of TRPM1 ion channels (Miller et al. 2004). In line with this observed crosstalk, in A2058 melanoma cells an increase of TRPM1 expression is linked to transcriptional regulation by an enhanced level of MITF transcription factors.

In A375 PMCA4b overexpressing cells MITF expression was not affected, but NFAT4 showed a modest increase. This result suggests that MITF expression is regulated by mutant BRAF in melanoma cells independent of its effect on PMCA4b. In contrast to vemurafenib and valproic acid treatment, PMCA4b overexpression induced a slight upregulation of NFAT4. Since NFAT4 transcription is not affected by vemurafenib and valproic acid treatment in melanoma cells, it appears that NFAT4 plays no major role in regulation of PMCA4b expression.

4.3 Mutant BRAF overexpression in melanoma cells

Inhibition of mutant BRAF resulted in an increase of PMCA4b calcium pumps in melanoma cells. In order to investigate regulation of PMCA4b transcript expression upon overexpression of wildtype and mutant BRAF in human cancer cells, respective retroviruses were generated for stable transduction. Open reading frames of wildtype and mutant BRAF were successfully cloned into retroviral expression plasmids. The generated BRAF

expressing retroviral plasmid vectors were first used to transiently transfect the MEWO BRAF wildtype cell line with wildtype and mutant BRAF. Transient overexpression of wildtype and mutant BRAF did not change the PMCA4b expression level in melanoma cells.

Since short-term overexpression of the BRAF variants did not result in the expected decrease of PMCA4b expression in melanoma cells, respective retroviruses were used to establish stable overexpression of wildtype and mutant BRAF in melanoma cells. Stably transduced MEWO cells showed an overexpression of wildtype BRAF, but did not overexpress mutant BRAF. Expression levels of transiently transfected wildtype and mutant BRAF in MEWO cells already confirmed that within 96h post-transduction, forced expression of mutant BRAF declines more rapidly back to the basal level than forced wildtype BRAF expression. In line with these results, stable expression of mutant BRAF in MEWO may also decreases early after retroviral infection and may be switched off by the cells until completion of selection. Previous research demonstrated that transduction and sustaining of mutant BRAF expression in melanoma cells reduced proliferation and induced cell cycle arrest and senescence (Michaloglou et al. 2005). Induction of senescence by transduced mutant BRAF overexpression, however, was not observed in the MEWO melanoma cells. Staining of SA-β-gal revealed an induced senescence in A375 melanoma cells upon treatment with high concentrations of vemurafenib, but did not indicate a noticeable amount of senescent MEWO melanoma cells upon transduction with mutant BRAF. Although MEWO cells did not show noticeable senescence upon transduction with mutant BRAF, the cells did not seem to tolerate high levels of mutant BRAF over a longer period of time.

4.4 Mutant BRAF overexpression in colon cancer cells

Oncogenic BRAF is also expressed in colon carcinoma, albeit at a lower frequency than in melanoma (Brose Marcia S et al. 2002). For analysis of forced BRAF overexpression in colon cancer, Caco2 cells were transiently transfected and stably transduced with wildtype and mutant BRAF. First, Caco2 colon cancer cells were transfected with either wildtype or mutant BRAF expression plasmids. Similar to MEWO cells, Caco2 cells showed transient overexpression of wildtype and mutant BRAF 24h post transfection. Next, stable transduction of Caco2 cells was attempted with the respective retroviruses. Due to an observed resistance of untransduced Caco2 cells to high levels of pyromycin, wildtype or mutant BRAF transduced cells could not be selected and cultivated in sufficient quantities. Since Caco2 cells showed strong resistance to puromycin, stable transduction needs to be performed with an alternative retroviral vector, which harbours a resistance for neomycin instead of puromycin.

5 Conclusion and outlook

To sum up the data, inhibition of mutant BRAF increased the transcript expression of PMCA4b calcium pumps in mutant BRAF melanoma cells. Treatment with the HDAC inhibitor valproic acid alone and in combination with mutant BRAF inhibition enhanced the PMCA4b expression in wildtype and mutant BRAF melanoma cells. Valproic acid alone and in combination with vemurafenib increased the transcript level of IP3R channels and SOCE regulating proteins STIM1 and 2 in melanoma cells with mutant BRAF. Upregulation of IP3R channel variants and STIM subunits of the CRAC channels upon HDAC inhibition indicates evidence for enhanced release of intracellular ER-stored calcium, followed by an increased calcium clearance through high abundance and activity of PMCA4b calcium pumps. Decreased ERK activation downstream of the oncogenic BRAF in mutant BRAF expressing melanoma cells results in an upregulation of the melanogenesis regulating transcription factor MITF and a reactivation of the MITF-regulated TRPM1 channel expression. Treatment with vemurafenib and valproic acid suggests that upregulation of PMCA4b calcium pumps, IP3R channels and of CRAC channels supports intracellular calcium clearance and suppression of melanoma cell migration. In line with these observations the expression increase of MITF transcription factors upon treatment with vemurafenib and valproic acid is able to restore the expression of TRPM1 calcium channels and may induce cell differentiation programs in malignant melanoma cells.

Within the wide range of different types of cutaneous neoplasia, malignant melanomas have the greatest mortality rates and the highest potential of tumor dissemination (Sandru et al. 2014). The 5-year overall survival of patients with metastatic melanomas is approximately 5-19% depending on the location and number of metastases. Once metastasis spread in the lymph nodes, the 5-year survival rate is approximately 23%, depending on the number of affected lymph nodes (Sandru et al. 2014). With visceral metastasis, the 5-year survival drops to approximately 6-10% and the median survival from time of diagnosis is only 7.5 months (Chudnovsky et al. 2005). The introduction of vemurafenib in the treatment of metastatic melanoma improved the therapeutic option for melanoma patients with a BRAF mutation. Nevertheless, because of tumor resistance to vemurafenib and other BRAF-targeting agents (dabrafenib) and despite the recent advances in immunotherapeutic treatment of melanoma, discovery of additional therapy targets will be essential.

Enhanced calcium clearance by increased PMCA4b calcium pumps and intracellular calcium channel activity in combination with a reactivation of cell differentiation programs by MITF transcription factors could be promising targets for anti-metastasis therapies in malignant melanoma. Within this thesis the HDAC inhibitor valproic acid was used in ad-

dition to vemurafenib to induce histone hyperacetylation and expression increase of epigenetically controlled calcium channels and transcription factors. Usage of HDAC inhibitors could be an additional approach in treatment of melanoma in order to remodel intracellular calcium signaling and thereby induce cell differentiation. Investigation of intracellular calcium clearance and an inhibition of cell migration and invasion upon treatment with valproic acid alone and in combination with vemurafenib could substantiate an effect of HDAC inhibition on the metastatic potential of melanoma cells. Furthermore, in vivo experiments could be performed to confirm metastatic suppression by valproic acid alone or in combination with vemurafenib in melanoma. Since valproic acid is only one candidate within the large panel of small molecule inhibitors of HDACs, treatment of melanoma with other HDAC inhibitors such as SAHA or Trichostatin A should be kept in mind.

6 Appendix

6.1 Abbreviations

AP1 - Activator protein 1

APES - Ammonium persulfate

ATP - Adenosine triphosphate

BSA - Bovine serum albumin

BRN2 - POU domain transcription factor

bZIP - Basic region-leucine zipper

CDK - Cyclin-dependent kinase

CRAC - Store-operated Ca^{2+} -release-activated calcium channels

DAG - Diacylglycerol

DMEM - Dulbeccos Essential Eagle Medium

DNA - deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

EGTA - Ethylene glycol tetraacetic acid

EMT - Epithelial-mesenchymal transition

Erk1/2 - Extracellular signal regulated kinases 1/2

FCS - Fetal calf serum

FDU - FastDigest Unit

FGF - Fibroblast growth factor

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GRB2 - Growth factor receptor bound protein 2

GTP / GDP - Guanoside triphosphate / guanosine diphosphate

HAT - Histone acetylase

HDAC - Histone deacetylase

InsP3 - Inositol-1,4,5-trisphosphate

IP3R - intracellular Inositol triphosphate receptor protein

JNK - c-jun N-terminal kinase

LB - Luria Bertani

LBII - Lysis buer II

MAPK - Mitogen activated protein kinase

MET - Mesenchymal-epithelial transition

MEK - Mitogen activated protein kinase

MEME - Minimum essential eagle medium

MITF - Microphthalmia-associated transcription factor

MyoD - Myogener Faktor 3

NES - Nuclear export signal

NFκB - Nuclear factor kappa-light-chain-enhancer of activated B-cells

NFAT - Nuclear factor of activated T-cells

NuRD - Nucleosome remodeling and deacetylating complex

ORAI - Ca^{2+} -release-activated calcium channel protein

PBS - Phosphate buered saline

PCR - Polymerase chain reaction

PI3K - phosphatidylinositol-3-kinase

PIP2 - Phosphatidylinositol-4,5-biphosphate

PIP3 - Phosphatidylinositol-3,4,5-trisphosphate

PKC - Protein kinase C

PLCγ - Phospholipase C-gamma

PLCβ - Phospholipase C-gamma

PMCA - Plasma membrane Ca^{2+} ATPase

PTEN - Phosphatase and Tensin homolog

qPCR - Quantitative real-time polymerase chain reaction

RGP - Radial growth phase

RNA - Ribonucleic acid

RPMI - Roswell Park Memorial Institute

RTK - Receptor tyrosine kinase

SAHA - Suberoylanilide hydroxamic acid

SDS page - Sodum dodecyl sulfate polyacrylamide gel electrophoresis

SH2 - Src homology 2

Sir - Silent information regulator

SOCE - Store-operated calcium entry

SOS - Son of Sevenless

STAT - Signal transducers and ctivators of transcription

STET - NaCl/Tris/EDTA/TritonX-100

STIM - Stromal interaction molecule

TBE - Tris/Borate/EDTA

TBS - Tris buered saline

TE - Tris-EDTA

TEMED - N, N, N, N-tetramethylenethylenediam

TPA - phorbol 12-O-tetradecanoate-13-acetate

TRP - Transient receptor potential channel

TSA - Trichostatin A

TYRP - Tyrosinase-related protein

VEGF - Vascular endothelial growth factor

VGP - Vertical growth phase

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List of Figures

List of Tables

Zusammenfassung

Eine V600E-Mutation im BRAF-Onkogen tritt in 8% aller Krebserkrankungen und 66% aller Melanomen auf und fördert in Melanomen die Proliferation und das Überleben von Tumorzellen. Der Einsatz von Vemurafenib, einem spezifischen Inhibitor von mutiertem BRAF, zeigt therapeutische Erfolge in der Behandlung des metastasierten Melanoms mit dieser Mutation. Auf Grund von Resistenzentwicklung, sind jedoch zusätzliche Therapieansätze erforderlich. Gestörte Ca^{2+}-Homöostase und die erhöhte Migrationsfähigkeit von Melanomzellen sind unter anderem auf eine Fehlregulierung der Plasmamembran Ca^{2+}-ATPase PMCA4b zurückzuführen. Inhibierung von mutiertem BRAF in Melanomzellen führt zu einem PMCA4b-Anstieg und in der Folge zu einem erhöhten Ca^{2+}-Ausstrom. Weiters zeigte sich, dass sowohl eine Behandlung mit Vemurafenib als auch eine künstliche Überexpression von PMCA4b zu einer deutlichen Verminderung der Migration von Melanomzellen führte. Neben onkogenem BRAF wurde auch eine epigenetische Regulation der PMCA4b-Expression durch Histon-Deacetylasen (HDAC) beschrieben.

Ziel: Ein PMCA4b-Anstieg durch Inhibierung des mutierten BRAF zeigt einen Zusammenhang zwischen onkogenem BRAF und der Ca^{2+}-Homöostase in Melanomen. Diese Masterarbeit untersucht den Einfluss von okogenem BRAF und von HDAC-Inhibierung auf die mRNA-Expression von Ca^{2+}-Kanälen und von Transkriptionsfaktoren, die im Zusammenhang mit Ca^{2+}-Signalwegen in Melanomzellen stehen.

Methoden: Melanomzellen mit unterschiedlichem BRAF-Status wurden mit Vemurafenib und mit dem HDAC-Inhibitor Valproinsäure behandelt. Expressionsänderungen wurden mittels quantitativer PCR (qPCR) gemessen. Zur Untersuchung der Hypothese, dass das Vorhandensein von mutiertem BRAF die PMCA4b-Transkription unterdrückt, wurden BRAF-exprimierende Retroviren hergestellt und Krebszellen mit diesen Retroviren (Wildtype oder BRAFV600E) transduziert. BRAF-Expression wurde mit Western Blot und qPCR überprüft. Durch SA-β-gal-Färbung wurde Seneszenz von Melanomzellen auf Grund einer Überexpression von onkogenem BRAF untersucht.

Ergebnisse: Vemurafenib erhöhte die PMCA4b-Expression in Melanomzellen mit mutiertem BRAF unterschiedlich stark je nach Zelllinie. Valproinsäure alleine oder kombiniert mit Vemurafenib förderte nicht nur eine PMCA4b-Expression sondern auch die Transkription von IP3R und CRAC-Kanälen in Melanomzellen mit onkogenem BRAF. Dies deutet auf einen erhöhten Ca^{2+}-Transport aus dem ER und einen gestärkten Ca^{2+}-Ausstrom aus den Zellen hin. Eine reprimierte Fra1-Expression in Vemurafenib-behandelten Melanomzellen mit mutiertem BRAF könnte auf eine mögliche PMCA4b-Regulierung durch AP1 hinweisen. Weiters förderten Vemurafenib und Valproinsäure die Expression von MITF. Ein Expressionanstieg von MITF, einem zentralen Transkriptionsfaktor in der Melanozyten-Differenzierung, deutet auf eine Reaktivierung von Zell-Differenzierungsprogramen hin.

Fazit: Die Daten dieser Masterarbeit zeigen, dass Vemurafenib, Valproinsäure und eine Kombination aus beiden Inhibitoren die Expression von Ca^{2+} -Kanälen und der Transkriptionsfaktoren Fra1 und MITF beeinflussen. Die Wiederherstellung der Ca^{2+}-Homöostase durch HDAC Inhibitoren und Inhibitoren von onkogenem BRAF könnte zur reduzierten Metastasierung von Melanomen beitragen.

Abstract

Background: The BRAF mutation V600E occurs in about 8% of human tumors and 66% of melanomas and has an oncogenic influence on cell survival and proliferation in melanomas. Treatment with the mutant BRAF-specific inhibitor vemurafenib has therapeutic value in metastatic melanoma with mutant BRAF. Nevertheless, drug resistance and limited therapy response require discovery of additional molecular targets. Decreased Ca^{2+} efflux upon repression of the plasma membrane Ca^{2+} ATPase PMCA4b results in altered Ca^{2+} homeostasis and promotes migratory ability and metastatic activity in melanoma. Inhibition of mutant BRAF with vemurafenib in melanoma cells was recently found to increase protein levels of PMCA4b and enhance intracellular Ca^{2+} clearance. Moreover, vemurafenib-treatment or ectopic expression of PMCA4b in melanoma cells with mutant BRAF decreased their migratory and lung colonizing ability. Besides oncogenic BRAF, epigenetic regulation of PMCA4b expression by histone deacetylases (HDAC) was described.

Aim: Enhanced PMCA4b protein levels upon mutant BRAF inhibition in melanoma cells suggest a link between oncogenic BRAF and Ca^{2+}-homeostasis in melanoma. The aim of this thesis was to investigate in more detail the role of mutant BRAF and of HDAC inhibition in the regulation of intracellular calcium channels and of transcription factors involved in remodeling of intracellular calcium signaling in melanoma.

Methods: Melanoma cell lines with different BRAF status were treated with vemurafenib and in addition with the HDAC inhibitor valproic acid to investigate their impact on calcium channels and melanoma-associated transcription factors. Transcript expression changes were analyzed with quantitative real time PCR (qPCR). To investigate the hypothesis that mutant BRAF overexpression represses PMCA4b, respective retroviruses were generated to establish wildtype and mutant BRAF overexpressing cancer cell lines. BRAF overexpression was confirmed by western blot and qPCR analysis. SA-β-gal-staining was used to investigate senescence in mutant BRAF transduced melanoma cells.

Results: Vemurafenib treatment increased the transcription of PMCA4b in melanoma cells with mutant BRAF in a cell line-dependent fashion. Valproic acid alone and in combination with vemurafenib enhanced PMCA4b expression in wildtype and mutant BRAF melanoma cells and increased intracellular IP3R channels and activator proteins of CRAC channels in melanoma cells with mutant BRAF. The combined effect suggests an enhanced release of ER-stored calcium by IP3R channels accompanied by an increased Ca^{2+} clearance through PMCA4b calcium pumps. Mechanistically, downregulation of the AP1 complex element Fra1 upon vemurafenib treatment indicates a possible repression of PMCA4b expression by AP1 complexes downstream of mutant BRAF. In addition, increase of the melanogenesis-regulating transcription factor MITF by vemurafenib and valproic acid suggests reactivation of cell differentiation programs in melanoma cells.

Conclusion: The results of this thesis show that vemurafenib or valproic acid and combination of both compounds induce expression changes of calcium channels and of the transcription factors Fra1 and MITF. Restored calcium homeostasis by inhibition of oncogenic BRAF and HDAC inhibition could play a role in suppression of melanoma metastasis.