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The Multiple Roles of Tyrosinase-Related Protein-2/L-Dopachrome Tautomerase in Melanoma: Biomarker, Therapeutic Target, and Molecular Driver in Tumor Progression

Adina L. Milac and Gabriela Negroiu

Additional information is available at the end of the chapter

Abstract

Cutaneous malignant melanoma (CMM), which is ranked as the 8th most common cancers in the US, makes 4–7% of skin cancers but it causes approximately 80% of skin cancer deaths. CMM is characterized by insidious and fast progression, heterogenic evolution, and significant resistance to numerous therapeutic strategies. CMM is the result of the uncontrolled proliferation of melanocytes, the cells which reside in the basal layer of the epidermis. The most efficient therapy is the surgical removal if the lesion is in an early stage. For metastatic melanomas, there are different strategies, extremely rarely leading to total cure. Tyrosinase-related protein-2 (TRP2) or L-Dopachrome tautomerase (L-DCT) is a member of Tyrosinase-related protein family known for many years for its enzymatic activity in the distal steps of melanogenesis. The modern DCT image is focusing more on processes and mechanisms related to cell development and response to environmental and therapeutic stressors in normal and transformed cell phenotypes. This chapter provides an extended, updated biological status of TRP2/L-DCT encompassing the structural and functional particularities within melanoma molecularity, in the attempt to get new insights into the complex mechanisms of this neoplasm and raise the interest for DCT unexplored yet potential in melanoma diagnosis/prognosis and therapy.

Keywords: tyrosinase-related protein-2, L-Dopachrome tautomerase, melanoma biomarker, structural molecular model, melanoma therapy, melanoma progression, caveolin-1, melanoma signaling pathways

1. Introduction

Cutaneous malignant melanoma (CMM) is a neoplasm generated through the malignant transformation of epidermal melanocytes, the cells which normally reside in the basal layer of the epidermis and produce the skin pigment melanin (**Figure 1A–C**). Noncutaneous melanomas can also develop at other sites populated by melanocytes such as choroidal layer of the eye, respiratory, gastrointestinal, and genitourinary mucosal surfaces, or the meninges. The main incriminating agent for causing CMM remains the UV radiation in interaction with host characteristics (**Figure 1D**). However, CMM may appear in skin areas that are not directly exposed to sun such as palms, soles, or under the nails, which demonstrates a pathogenesis more related to the noncutaneous melanomas. The incidence of CMM has been rising for the last 30 years around the world. Key statistics on CMM released by The American Cancer Society estimate that during 2017, in the US, about 87,110 new melanomas will be diagnosed (about 52,170 in men and 34,940 in women) and about 9730 people are expected to die of melanoma (about 6380 men and 3350 women) [1]. Although CMM makes only 4–7% of skin cancers, this neoplasm causes approximately 80% of skin cancer deaths. CMM is characterized by insidious and fast progression, heterogenic evolution among patients, and significant resistance to diverse therapeutic strategies. CMM is thought to develop in a stepwise manner being initiated with a benign nevus containing cell populations with intense proliferative capacities. Some of these lesions overcome the senescence-inducing signals, exhibit dysplasia (dysplastic nevus), and can progress further toward the malignant stages. The radial growth phase (RGP) is limited to epidermis and has a low invasive potential. In a more advanced stage, the melanoma cells migrate vertically up into epidermis and down into papillary dermis entering a new stage, the vertical growth phase (VGP). In metastatic stage, the tumor cells invade through blood or lymph vessels the distal organs (liver, brain, and lung) where they proliferate, eventually, causing death (**Figure 1D**). The activity of tumor cells is modulated by the complex and dynamic tumor microenvironment that can be extremely heterogenous among tumors of different patients. The multistep process of CMM progression is defined by a plethora of molecular events that are continuously explored, revised, and updated [2, 3].

The only cure for melanoma is the surgical removal of early-stage tumors. For metastatic patients having the median overall survival less than a year, there are different strategies, including combined chemo-/radio- and vaccine therapies, extremely rarely leading to total cure and whose success depends very much on the staging accuracy. Major improvements in the metastatic treatment have been achieved due to advances in understanding the molecularity of this neoplasm. The modern alternative for melanoma evaluation and management is the analysis based on key genes or biomarker(s), pathways, diagnostic technologies, and potentially relevant therapeutics. These tend to replace current limited histological and microscopical evaluation introducing concepts such as "molecular melanoma subtypes" [4], "melanoma disease model (MDM)" [5], or "molecular diagnostic of melanoma" [6], aiming to bring together clinicians, researchers, and pharma for more efficient diagnostic, prognostic, and therapeutic strategies [7, 8]. Tyrosinase-related protein-2 (TRP2, TYRP2) or L-Dopachrome tautomerase (L-DCT) is a member of tyrosinase-related protein (TRP) family known for many years only for its enzymatic activity in the distal steps of melanogenesis. Studies emerging from different
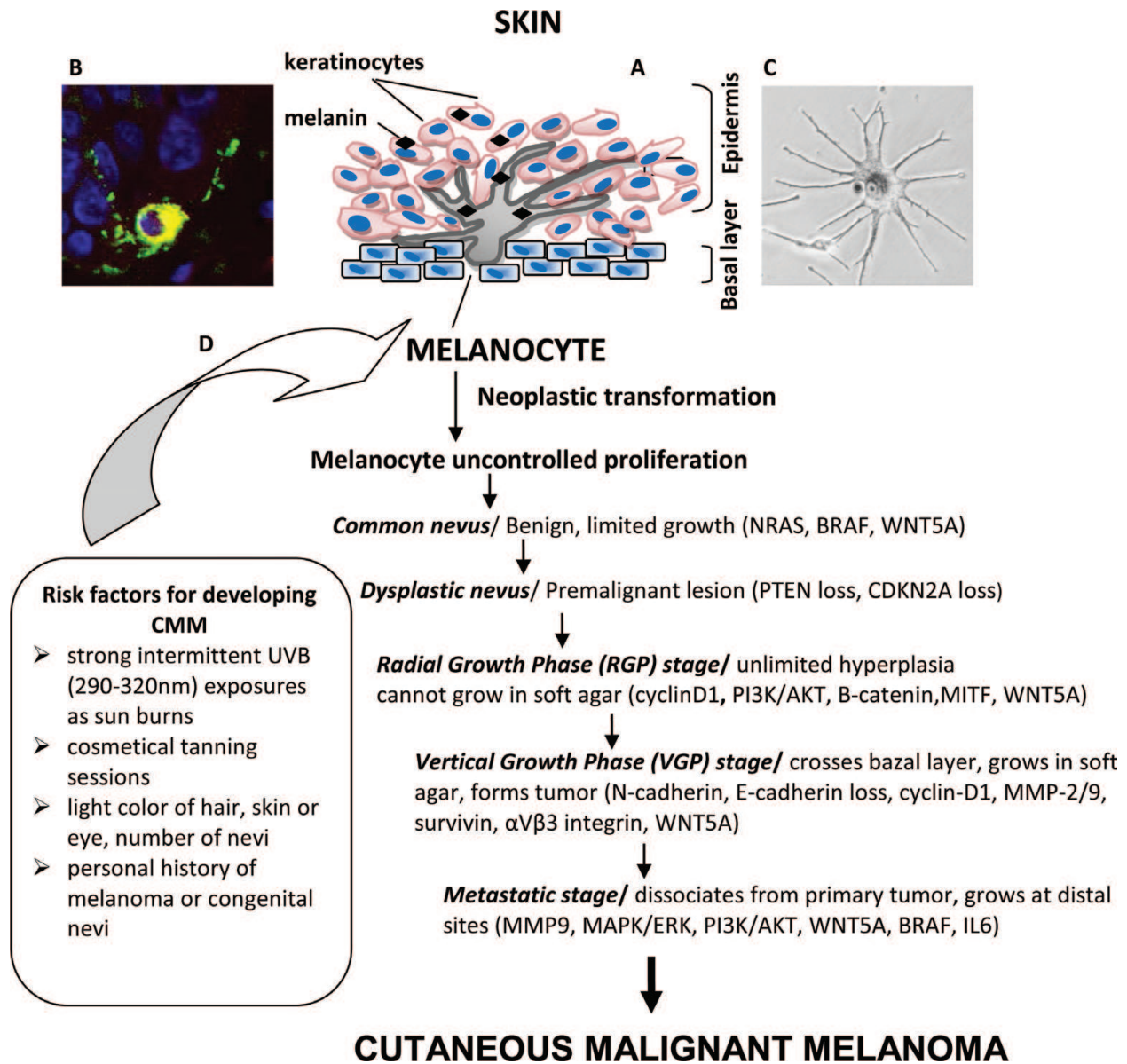
Figure 1. Cutaneous malignant melanoma. (A) Schematic representation of epidermal melanocytes with melanosomes (black dots) exported to the surrounding keratinocytes. One melanocyte and 30–40 keratinocytes form the "epidermal melanin unit". (B) The image of a human melanocyte obtained by confocal fluorescence microscopy of a human skin specimen immunostained for TYR and DCT. The common TYR-DCT staining is in the perinuclear region, whereas TYR staining is visible in dendritic tips too. (C) The image of a human epidermal melanocyte (HEM) in culture, obtained by bright field microscopy. (D) The risk factors for developing cutaneous malignant melanoma and the steps of neoplastic transformation and malignant progression of epidermal melanocytes culminating with the metastatic stage. Several molecular markers and processes emblematic for each tumor stage are indicated.

groups identified TRP2/L-DCT in relation to processes distinct from melanin synthesis (cell protection from environmental and therapeutic stress), melanoma diagnostic (potential biomarker), and therapy (immunotherapeutic target). TRP2/L-DCT is also expressed in precursors of peripheral nervous system associated with developmental processes and in glioma, a brain cancer similar to melanoma in terms of aggressiveness and therapeutic resistance and more recently, unexpectedly, in nonmelanocytic or nonneuronal cellular phenotypes.

This chapter aims to provide an updated status of TRP2/L-DCT in order to demonstrate its multiple implications in melanoma molecularity and therapeutic potential as well as to open up new perspectives for a better understanding of other molecular processes and pathologies. For simplicity, we will further refer to TRP2/L-DCT as DCT.

2. Dopachrome tautomerase: a distinct member of tyrosinase-related protein family

2.1. Structural determinants of DCT

TRPs are type I transmembrane N-glycoproteins. Their polypeptides share significant amino-acid sequence homology and similar patterns of polypeptide chain organization, an amino-terminal signal sequence (residues 1–23 in human DCT) followed by a lumenal domain (aa 24–439), a transmembrane (TM) hydrophobic region (aa 473–493) that inserts the protein into subcellular membranous structures and a carboxi-terminal cytoplasmic (CYT) tail (aa 494–519) interacting with the elements of the sorting and traffic machinery. The lumenal domain encompasses the enzymatic active site shaped by two highly conserved metal-binding regions (MeB1 and MeB2) molded at the core of a four-helical bundle. Interspersed with these two metal-binding regions are two Cys-rich regions (Cys1 and Cys2). Cys1 precedes MeB1 and contains 10 Cys residues conserved only in the human TRPs, and Cys2 located between MeB1 and MeB2 contains six Cys residues of which five are conserved in the human TRPs. Unfortunately, none of the human TRPs have been crystallized, but models of human tyrosinase have previously been developed [9]. Using a similar protocol and based on the high degree of sequence homology among TRPs (about 60% on the entire sequence and 66% in the lumenal domain only), we built a structural model for the lumenal domain of human DCT using as templates the available X-ray structures of tyrosinase proteins from *Bacillus megaterium* [10] (PDB code 3NM8, 3NPY; 2Å resolution) and from *Streptomyces castaneoglobisporus* [11] (PDB code 3AX0; 1.4Å resolution). Alignment between human TRPs and templates sequences (**Figure 2**) was initially generated using CLUSTALW and MULTALIN and further refined by incorporating information on secondary structure elements identified by consensus prediction by several methods, in the case of DCT, and by DSSP assignment in the case of templates.

Despite this high degree of sequence homology between DCT and other human TRPs, distinctive DCT features regarding overall hydrophobicity and charge profiles, active site stereo-chemistry and composition, N-glycosylation, or phosphorylation patterns generate significant differences in protein function, interaction partners, and sorting/trafficking pathways.

Although the two metal-binding regions in the lumenal domain represent a highly conserved feature of TRP family, DCT has a unique preference for zinc instead of copper, as is in the case of TYR. Purified DCT contains two Zn atoms per protein molecule as measured by atomic absorption spectroscopy and Zn^{2+} chelation experiments. Zn^{2+} is the crucial element that accounts for the tautomerization of L-Dopachrome tautomerase [12]. The enzyme DCT reconstituted with
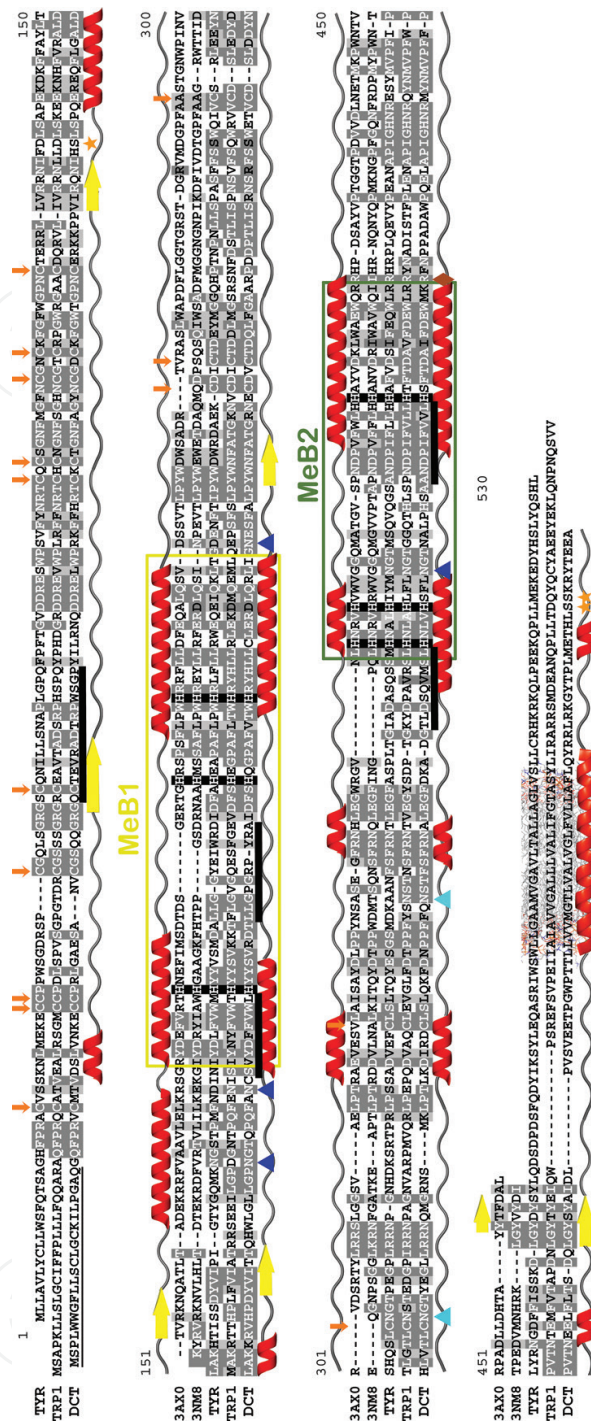
Figure 2. Sequence alignment of human TRPs (TYR, TRP1, and DCT) with the X-ray templates used for modeling DCT (PDB codes 3AX0 and 3NM8). Identical/similar residues between DCT and other sequences are highlighted dark/light gray, metal binding His residues are highlighted black. Assigned/predicted secondary structure elements for templates/DCT are shown above and below the alignment. Membrane pictogram indicates location of (predicted) transmembrane region in all proteins. The rectangles indicate the two Metal-binding regions (MeB1, MeB2). Symbols indicate various functionally relevant residues: stars = phosphorylated residues; diamond = methylated R409 residue; dark triangles = putative N-glycosylation sites, light triangles = experimentally confirmed occupied sites in DCT; arrows = Cys residues. Signal sequence in DCT is thin underlined. The DCT-derived peptides 60–74 [18], 180–188 [19], 197–206 [20], 360–368 [21], 387–395 [22] recognized by CD4+ or CD8+ T-lymphocytes are indicated by thick lines.

Cu^{2+}, which is the cofactor for TYR, or with Fe^{2+}, is inactive, whereas with Co^{2+} is partially active. Unlike the native DCT, which shows a very strict specificity for L-Dopachrome and for which neither dopaminochrome nor D-Dopachrome are suitable substrates, the reconstituted enzyme is stereospecific as well but is also able to rearrange D-Dopachrome into DHI [13]. At this point, it is important to specify that there is also a D-Dopachrome tautomerase (D-DCT, or D-DT) which is decarboxylating D-Dopachrome to DHI. There is no structural or functional relation between L-DCT and D-DT, which is a circulating cytokine, member of macrophage migration inhibitory factor (MIF) protein superfamily with an overlapping functional spectrum with MIF. Within lumenal domain of human DCT, there are 16 cysteine (Cys) residues, clustered into three regions, the first two located N-terminal to MeA and the third between MeA and MeB. In addition to these clustered Cys residues, single Cys residues may be found in the C-terminus cytoplasmic tails of TYR and TRP1 but not of DCT, which indicates a TYR-TRP1 interaction via intermolecular disulfides without DCT participation [14]. This finding is in agreement with our experimental data, showing that DCT does not share common subcellular structures with TYR or TRP1 (see Section 2.3.1.2) and does not support the early theory that all TRPs are possibly interconnected via intermolecular disulfides. Despite the fact that the number of N-glycosylation sites is almost the same in human TYR (seven sites) and DCT (six sites) and they are all located in the lumenal domain, glycosylation pattern is significantly different between TYR and DCT. In the case of human TYR, occupancy of six of the seven sites was demonstrated by site-directed mutagenesis [9], while in the case of DCT, only two sites (N300 and N342) have been experimentally confirmed to be occupied [15] by MALDI/TOF of a truncated version of protein expressed in insect cells. Both N-glycosylated sites in DCT are located in close vicinity (on opposite sides) of the metal containing active site, possibly influencing ligand access within, but only N300 is conserved in all human TRPs while equivalent of N342 is found only in TRP1 not in TYR. The first two N-sites of TYR, which are required for TYR entry in the CNX cycle [16] are not present in DCT, which further supports the idea that TYR and DCT take different intracellular processing pathways. Indeed, our experimental data confirmed that folding pathways, which in all TRPs are dependent on the step of N-glycan processing, are differently regulated within the same cell phenotype and have further distinct impact on their trafficking and stability (see Section 2.3.1.2). Additional unique characteristics of DCT post-translational modifications refer to the methylated residues. A recent large-scale mass spectrometry analysis of arginine-methylated peptides in human T cells [17] demonstrated methylation of R409 in DCT (indicated by a diamond in the alignment in **Figure 2**), located at the end of the second metal-binding region. Structurally, this positively charged residue is positioned in the luminal domain and oriented toward the melanosomal membrane (**Figure 3**), thus likely to interact with the negatively charged head groups of membrane phospholipids. Addition of a methyl group to R409 would shield the positive charge and decrease probability of luminal domain interacting with membrane. Surprisingly, although this residue is conserved in all human TRPs, the same study could not identify similar modification of corresponding residues in the other members of the family. This post-translational modification of DCT could have an impact on interactions between DCT and sorting/traffic machinery and subsequently on DCT intracellular routes. The same study [17] demonstrates that changes in arginine methylation stoichiometry during cellular stimulation in a subset of proteins are critical to T cell differentiation. DCT is a tumor antigen, and several peptides
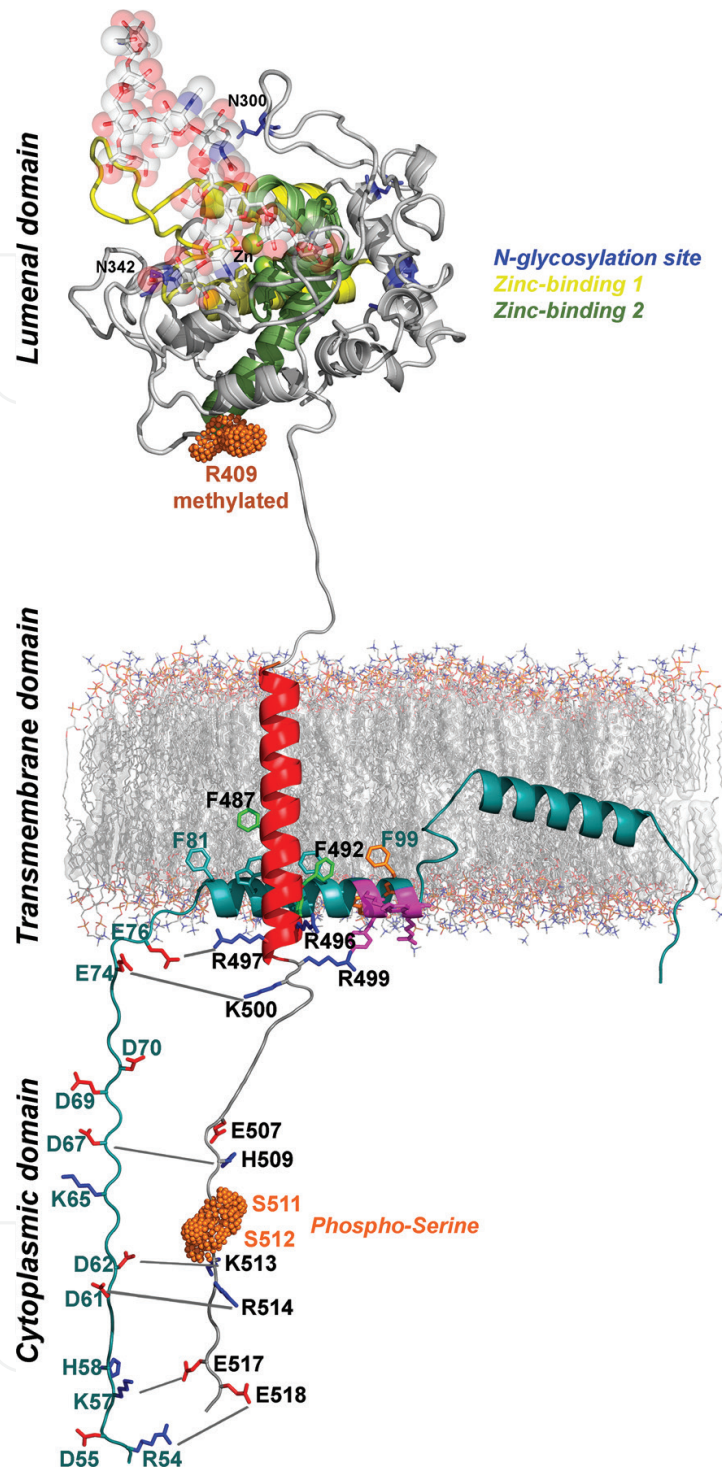
Figure 3. Structural model of DCT protein (cartoon representation) interacting with membrane bilayer and caveolin-1 (Cav1). In the lumenal domain (above membrane) the helical segments indicate the two metal-binding regions, containing two Zn^{2+} ions (shown as opaque spheres). Putative N-glycosylation sites are depicted using thick sticks. Representative structural models of N-glycans (shown as transparent spheres) are attached to glycosylation sites experimentally shown to be occupied (N300 and N342). Methylated R409 (within lumenal domain) and phosphorylated S511, S512 (within cytosolic membrane) are shown as dotted spheres. Within DCT transmembrane region, aromatic residues F487, F492 (thick sticks) and Y495 (behind helical structure) form the Cav1-binding motif. Charged residues in the cytosolic regions of DCT and Cav1 are labeled and shown as sticks, and putative salt bridges are depicted by thin gray lines connecting oppositely charged residues.

derived from it were identified as targets of CD4+ or CD8+ T-lymphocytes, and their position within DCT sequence is presented in **Figure 2** [18–22]. Whether DCT-methylated peptides could be a part of the peptide-methylated pool involved in triggering T-cell differentiation in melanoma would represent a subject worthwhile to be further investigated. Other distinctive features of DCT TM domain are the presence of cholesterol (CRAC) and caveolin-binding motifs, which supports the idea of an interaction with these membrane components. Our detailed computational analysis using various sequence bioinformatics, structural modeling, and molecular simulation approaches allowed us to generate the first complete structural model of DCT in interaction with caveolin-1. This model revealed DCT-specific structural determinants involved in interaction with membranes having specific compositions and possibly regulating its enzymatic activity and intracellular trafficking, as well as its participation in complex processes as signaling pathways [23] (**Figure 3**). The overall model advocates for an interaction between Cav1 and DCT mediated by two distinct regions, one within the membrane (hydrophobicity-driven interaction) and the second cytosolic (electrostatics-driven interaction). The CYT DCT domain is predicted to adopt an extended, possibly disordered conformation and has a net positive charge (7 basic and 3 acidic residues out of 26) whose distribution is complementary to that of Cav1 cytosolic region carrying a negative formal charge, which strongly supports the electrostatic interaction between these regions, facilitated by salt bridges (**Figure 3**, thin lines). Interestingly, the DCT charge distribution in the CYT domain may be modified by the phosphorylation state of two adjacent serine residues (S511, S512 pointed by stars in **Figure 2** and indicated by dotted van der Waals spheres in **Figure 3**) whose phosphorylation was experimentally confirmed by mass spectrometry [24]. We can speculate that phosphorylation of these unique sites may represent a control mechanism for modulating DCT interaction with Cav1 or with other molecules involved in trafficking/sorting/signaling pathways. However, the presence of these interactors would need to be confirmed by additional experimental approaches.

To understand more deeply the specific behavior of TRPs in interaction with cholesterol-rich membranes, we performed molecular dynamics simulations (60 ns) of TYR and DCT TM segments embedded in 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) lipid bilayers in the presence and absence of cholesterol. The 3D structures of TM domains were modeled ab initio as α-helices whose length was based on sequence hydrophobicity and helix propensity profiles which indicated that TYR TM is slightly longer (~4 residues, one helical turn) than DCT TM. Although the two TM domains had identical initial positions and orientations in the membrane bilayer, and the overall helical structure is maintained throughout the entire 60 ns simulations, the TYR TM adopted a more tilted inclination (measured by the angle between α-helix central axis and axis normal to bilayer plane) compared to DCT (upper panels in **Figure 4A**). The magnitude of this tilting effect is likely correlated with the length of the hydrophobic helix segment that needs to fit within the membrane thickness; therefore, the orientation of shorter DCT helix is closer to normal axis while TYR is more tilted (see plot in **Figure 4B**). As expected, tilting is less pronounced in cholesterol-containing membrane due to its increased thickness (lower panels in **Figure 4A**). Surprisingly, cholesterol affects helix translation within membrane in a different manner: while in the cholesterol-free membrane both proteins experience similar levels of helix translation, in cholesterol-containing membrane, DCT translation
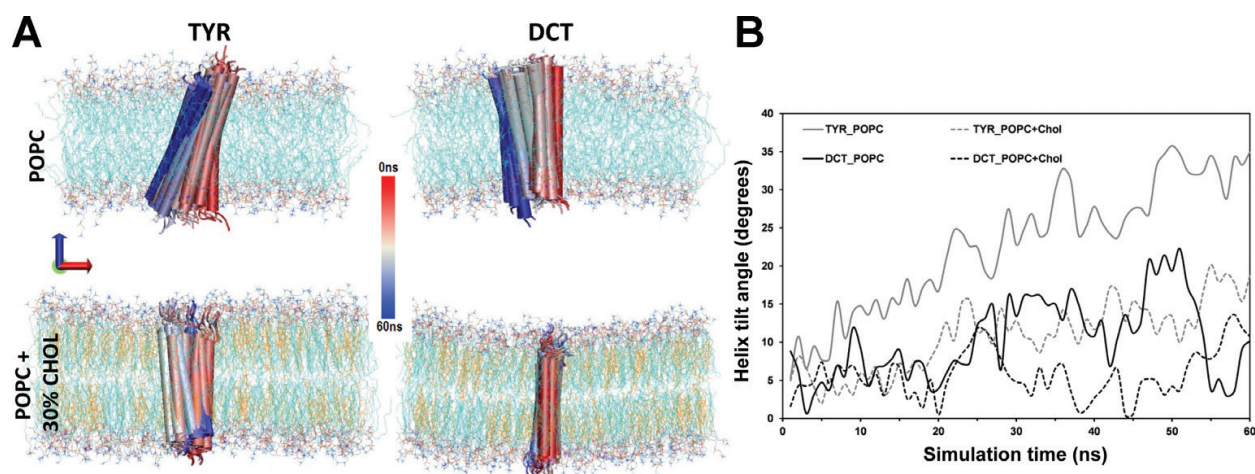
Figure 4. Molecular dynamics simulations (60ns) of transmembrane segments of human TYR/DCT embedded in pure POPC bilayer compared to cholesterol-containing membranes. (A) Structural representation of the transmembrane helix (shown as cylinder) every 2ns in the membrane bilayer; and (B) plot of the helix tilt angle variation during simulation, indicating higher tilt for TYR compared to DCT.

is highly restricted while TYR translation is only slightly affected, suggesting that cholesterol interacts more tightly with DCT, possibly due to the presence of CRAC signature. This would explain the preferential DCT sorting into CRD domains and distinct trafficking along the secretory pathway (see Section 2.3.1.2). This study, presented here for the first time, is one of the few simulation studies on the importance of cholesterol for TM type I protein stability and trafficking. The DCT structural determinants account for its distinct intracellular processing and biological functions.

2.2. DCT cellular expression

DCT is expressed preponderantly in melanocytes, which originate from neural crest cells (NCC) and migrate during embryonic development to different regions (**Figure 5**). There are also melanocytes in retinal pigmented epithelium (RPE) that originate from the forebrain neuroepithelium and in which DCT expression has also been confirmed [25]. DCT is detected in melanoblast, the progenitor of melanocyte, at embryonic day (E) E9.5, in a SOX10-melanoblast/glial bipotent progenitor, together with microphthalmia-associated transcription factor (MITF) and KIT, whereas TYR or TRP1 are expressed later in the development [26]. In hair follicle, DCT expression has been associated with a pool of melanocytes having stem cell traits of self-renewal and multipotency within the lower permanent proliferation portion of this tisssue [27]. In the precursors of peripheral nervous system which derive also from NCC, the spatial and temporal profiles of DCT expression correlate with neurogenesis during embryonic development and enhance the proliferation of cortical neural progenitor cells and neuroblast migration [28]. A unique cell population called melanocyte-like cells, found within murine and human hearts, that is distributed to the pulmonary veins, atria, and atrioventricular canal, also expresses DCT but has transcriptional profiles distinct from dermal melanocytes. The presence of these DCT-positive cells has been connected with the clinical syndrome of atrial ectopy initiating atrial fibrillation, autonomic dysregulation, and oxidative
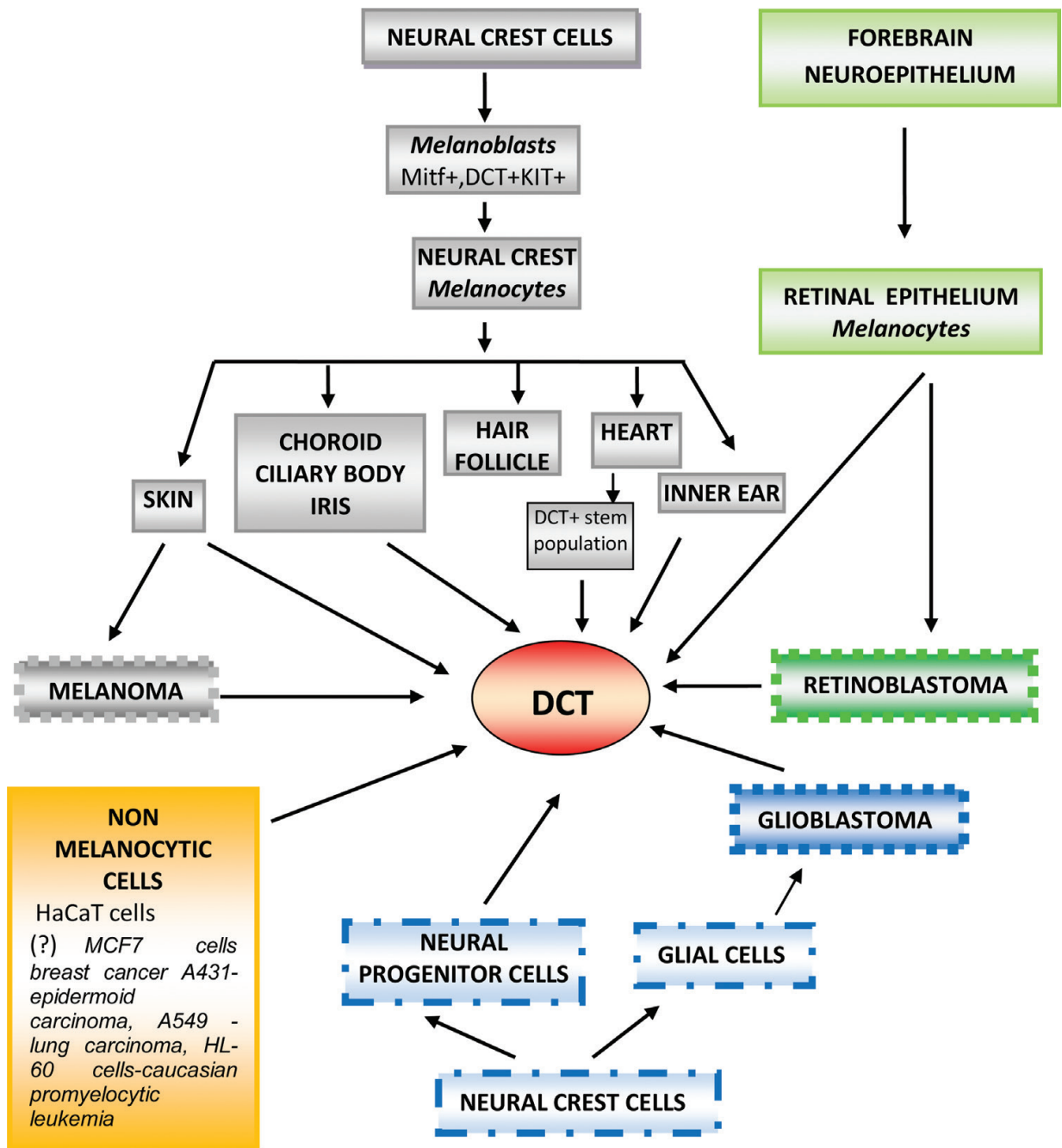
Figure 5. DCT cellular and tissular expression. DCT is primarily expressed by melanocytic (continous line) and neuronal (interrupted line) cells and by their malignant counterparts (dotted line). DCT possible expression in cells of nonmelanocytic origin is also indicated.

stress. It seems that DCT-cardiac melanocytes are involved in maintaining the normal balance of oxidative species in the myocardium [29]. The DCT expression is also retained in the malignat counterparts derived from melanocytes and neuronal cells as melanoma retinoblastoma [30], glioma [31], and glioblastoma [32]. Moreover, the neoplastic cells express different DCT transcripts and in higher amounts compared with the normal cells. For example, in patients with glioma, the DCT mRNA transcripts are in excces of 100,000-fold over that in healthy brain [33]. In amelanotic melanoma cells, in which TYR and TRP1 are downregulated

or enzymatically inactive, DCT is well expressed [34] and during melanoma malignant progression, DCT expression, unlike TYR or TRP1, remains constant [35]. A recent study presents that DCT is endogenously expressed in HaCaT cells (basal keratinocytes) [36] which has an electrophoretic pattern comparable with DCT in RPE lysate, but distinct from the 68/80 kDa DCT doublet expressed by melanoma cell lines [37, 38]. A significant number of commercially available anti-DCT antibodies include in their technical data sheets, as positive controls for endogenous DCT, cell lysates, or histopathological specimens from cell lines or neoplasms in which DCT is not expected to be expressed such as A431-epidermoid carcinoma (Sigma-Atlas); NBT-II-Nara bladder tumor cells, WEHI-231 B cell line, CTLL-2-cytotoxic lymphocyte (Santa Cruz), human liver cancer tissue lysate, K562 (leukemia) lysate, K-562-chronic myelogenic leukemia, A549-lung carcinoma, HeLa-cervical cancer (Abcam); MCF7 cells-breast cancer, HL-60 cells-caucasian promyelocytic leukemia (Proteintech Group); human cervical cancer tissue (OriGene). Most of them show in WB analysis bands of approximately 50 kDa or/and 30 kDa. Two hypotheses can explain these data: (1) the 50/30 kDa bands are not DCT but possibly contaminants detected due to antibodies cross-reactivity. This would be very unlikely because these antibodies have been raised against different DCT sequences, by different technologies, in different laboratories. However, as many of these antibodies do not show data on these cells having "DCT gene" downregulated or amplified (with specific primers for DCT mRNA), their specificity is still questionable and may induce false-positive results with severe consequences especially in clinic; (2) the 50/30 kDa in nonmelanocytic/-neuronal cells or tissues are indeed derived from DCT (possibly isoforms or degradation products). DCT is expressed in neural crest progenitors that generate multiple cell lineages during development. The demonstrated DCT involvement in anti-apoptotic and stress-resistance pathways (Section 2.4) would qualify it for activated expression in cellular niches of different normal or transformed phenotypes where it would be requested to sustain specific processes. For example, osteopontin, primarily expressed in bone cells (osteoblasts) has become a well-known marker for various neoplasms, including melanoma, where its expression is associated with tumor progression [39]. HaCaT is an immortalized keratinocyte cell line with a high capacity to differentiate and proliferate in which endogenous DCT has detoxification biological activities similar to those already described in melanocytic lineage [36]. These new data consolidate the theory that DCT expression may encompass, indeed, multiple cell phenotypes where it accomplishes, very likely, functions related to cell protection. How is DCT expression activated and modulated in nonmelanocytic/-neuronal cells are questions whose clarification require additional studies. Morevoer, the DCT expression in nonmelanocytic lineages would raise the question whether DCT can still be considered a specific biomarker for the diagnosis of melanocytic lesions.

2.3. Regulation of DCT expression and intracellular processing in melanoma

2.3.1. Intracellular regulation

2.3.1.1. Transcriptional level

The human DCT gene (h-DCT) has 55-kb and was mapped to the chromosomal region 13q31-q32 with a coding region of eight exons all encompassing the open reading frame of the protein [40].

The h-DCT is controlled by the two separate regulatory regions: the 32-bp element and the proximal region [41]. The 32-bp element is a composite enhancer having potential binding sites for transcription factors that contain a basic helix-loop-helix structure (including Microphthalmia-associated transcription factor—MITF), a high-mobility-group (HMG) domain (the TCF/LEF-1 or SOX family), or an Ets domain [42]. MITF is a master regulator of pigmentary system [43], and there is a selective requirement for MITF-M isoform for melanocyte development. The promoter region of MITF-M contains CREB, SOX10, PAX3, and LEF-1 binding sites. The presence within DCT promotor of the 32-bp element containing a CAATTG motif do not produce significant transactivation by MITF, as in case of the other TRPs, suggesting that the mechanism for melanocyte-specific transcription of the DCT gene is different from that of the other TRPs [44]. In addition to MITF, DCT is regulated by SOX10, which is a high-mobility-group transcription factor that plays a critical role in many processes in neural crest cells, including multipotency, proliferation, apoptosis, survival, and commitment to defined neural crest-derived lineages. SOX10 transiently regulates DCT expression during early melanocyte development, independently of MITF function [45] and synergistically with MITF that enhances SOX10-dependent activation of the DCT promoter [46]. Another member of the SOX family, SOX5, inhibits the SOX10-stimulated activity of the DCT promoter in melanocytes [47]. A synergistic transactivation of DCT gene promotor results also from cooperation between TLEF-1 and MITF or between TLEF-1 and TFE3, a MITF-related protein [48]. The TCF/LEF-1 family regulates target gene transcription in response to Wnt signals. The transcriptional regulation of DCT involves also PAX3, a member of a highly conserved family of transcription factors essential to the development of many tissue types throughout embryogenesis and vital to the maintenance of several stem cell niches. Unlike MITF which is an activator of DCT expression, PAX3 inhibits both DCT expression and the ability of MITF to bind to the DCT promoter. PAX3 forms a repressor complex with LEF1 and GRG4 on the DCT enhancer sequence and actively blocks MITF binding. In the presence of beta-catenin, LEF1 forms a complex with MITF and beta catenin and displaces PAX3 from DCT enhancer [49]. Oppositely, SOX10 does not cooperate with PAX3 to activate DCT in combination with PAX3 [50]. OTX2 is a transcription factor that regulates the specific expression of DCT gene in REP. OTX2 binds to the DCT gene promoter *in vivo*, whereas repression of endogenous OTX2 expression results in the decrease of DCT protein content [25]. Our most recent data introduces Cav1 as the newest regulator of the DCT [23] (detailed in Section 5). Several DCT isoforms resulted from translation of introns of DCT are reported. One sequence contains exons 1–4 with retention of intron 2 and part of intron 4 (DCT/TRP-2-INT2) [51], another is from the same sequence except for an extended 3'-untranslated region originating by alternative polyadenylation (Tyrosinase-protein-2 long tail), and the third isoform results from the 3'-untranslated region containing the alternatively spliced last DCT exon (Tyrosinase-protein-2-8b) [52]. Importantly, unlike the fully spliced DCT mRNA expressed in normal skin melanocytes, retina, and melanomas, the DCT/TRP-2-INT2 mRNA is detected only in melanomas, whereas the Tyrosinase-protein-2 long tail and Tyrosinase-protein-2-8b mRNAs are expressed in both melanocytes and melanomas. The DCT/TRP-2-INTL isoform is recognized by a CTL clone and has potent therapeutic value due to its specific and elevated expression in melanoma. Another sequence that containes two novel exons alternatively spliced from the sixth intron between exons 6 and 7 of TRP-2/DCT generates a novel TRP/DCT-2-6b mRNA. The TRP2/DCT-6b isoform is also recognized by a TIL clone and may play a role in tumor regression [53].

2.3.1.2. Post-translational level

TRPs follow the general secretory pathway: TRP-polypeptide synthesis and folding in endo-plasmic reticulum (ER), the N-glycan maturation along the Golgi complex and transport to the steady-state destination, the melanosomes, the site of melanin synthesis and storage. In parallel with our early research on TRP1 intracellular processing in murine melanoma cells [54], studies of other groups were presenting a specific drug-and UV-resistance mediated by TRP2/DCT in melanoma [55–57]. In this context, we considered that deciphering the intra-cellular processing pathways of DCT would bring fundamental knowledge and possible exploitable information into melanoma development and therapy. The immunofluorescence microscopy images and ultracentrifugation data reveal a unique pattern of DCT subcellular distribution. Unexpectedly, DCT is detected in high amounts in a perinuclear position, co-localizing with the TGN marker, syntaxin 6, and in substructures at plasma membrane (PM), showing weak overlapping with late melanosome markers TRP1 and Rab27a. The matura-tion kinetics and traffic along the secretory pathway show that ER DCT 68 kDa precursor containing high-mannose N-glycans moves along the Golgi where it acquires complex struc-tures, gradually turning into the DCT 80 kDa mature protein, within approximately 3 h [37] compared to 45 min in which TRP1 becomes a fully glycosylated 75 kDa protein [54]. Similar to TYR and TRP1, DCT interacts with the ER lectin chaperone calnexin that assists normal polypeptide folding of all TRPs [37]. In N-glycoproteins, the glycan procesing in ER interferes with polypeptide folding. The step of N-glycan trimming by glucosidase I and II results in the formation of a monoglucosylated precursor that interacts with the ER lectin chaperones, cal-nexin, or calreticulin, which assist the polypeptide folding. The inhibition of glucosidase I and II with N-butyldeoxynojirimycin (NBDNJ) perturbs N-glycosylation, resulting in a trigluco-sylated precursor unable to interact with calnexin. In NBDNJ-treated cells, TRP1 folds in the absence of interaction with calnexin, being rescued by another ER chaperone BiP, leaves ER, and moves along Golgi [54], whereas in the same cells, TRP2/DCT conformation is severely altered, and the misfolded protein is targeted to proteasomal degradation [37]. A more recent study reports that the treatment of Melan-a cells, with the chemical compound, A3B5, results also in proteasomal degradation of DCT but not of TYR [58]. Whether DCT from A3B5-treated cells is targeted to proteasome from the ER, via the well-known retrotranslocation pathway or from a post-ER compartment remains to be further investigated. In any case, this is an addi-tional proof that, indeed, DCT fate in melanoma is distinctly regulated from the other TRPs. Additional information about the DCT biosynthetic pathway came from our investigations of the two human amelanotic melanoma cell lines, MelJuSo (MJS) and SKMel28 (SK28) [23]. In SK28, as in other amelanotic cell lines, pH homeostasis is altered, and TYR is retained in the secretory pathway and prematurely, proteasomally degraded [59]. Importantly, in both MJS and SK28, amelanotic cell phenotypes DCT appears at steady state as a mix of the fully processed protein and the partially glycosylated precursor. This pattern indicates that a sig-nificant DCT amount is able to overcome the pH-induced blockade being sorted from the early steps of its biosynthetic pathway in a different cargo than TYR. Our experimental data demonstrate that DCT maturation between ER and Golgi is interrupted or pertured in the presence of nystatin [23] or monensin [60], two pharmacological agents that disrupt CRDs or insert in Golgi CRDs, respectively. A significant amount of DCT is detected by co-localization

and co-immunoprecipitation experiments in complexes with Cav1, an abundant component of CRDs. The association of DCT with Cav1 and cholesterol is supported by our structural analysis (detailed in Section 2.1). Cav1 downregulation has a profound regulatory impact on DCT and subsequently on its entire biosynthetic pathway [23] (detailed in Section 5.3). Our theory is that a significant fraction of DCT is sorted in the early secretory pathway, possibly from ER, in CRDs with Cav1, in a cargo without TYR and trafficked on a route less sensitive to amelanotic acidic pH. Our data is supporting the concept of the selective ER exit sites and ER-Golgi transport [61] and that production of specific lipids might have a regulatory role in cargo recruitment and export from ER [62]. Another cellular parameter regulating DCT processing, between ER and Golgi is the intravesicular pH. The treatment of B16F1 pigmented melanoma cells with bafilomycin (Baf), a specific inhibitor of v-ATPases and pH corrector, slightly increases the amount of DCT mature complex protein [60]. This demonstrates that pH of the secretory pathway is altered in pigmented phenotypes as well, but to a less extent than in amelanotic cells and that only a DCT fraction is trafficked on a route sensitive to pH alterations too. We also found that DCT maturation between ER and Golgi is interrupted by microtubule depolymerization agent nocodazole (NCZ) when DCT is prevented to reach medial Golgi and remains in the form of the 68 kDa precursor [unpublished data]. Post-Golgi, the membrane composition and the interaction of the sorting and trafic machinery with the CYT tail of TRPs decide their destination [63, 64]. The di-Leu motif (QPLLMD) present in both cytoplasmic tails of TYR and TRP-1 and specifically requested for the interaction with the AP-3/AP-1 sorting elements in post-Golgi compartments is absent from DCT CYT domain which has Tyr-like motif (YRRL). The detection of DCT in TGN area and at PM in both murine and human melanoma cell lines with two distinct antibodies and the low amounts in mature melanosomes [23, 37] support the theory that post-Golgi DCT is trafficked on a distinct route than TYR or TRP-1, possibly being recycled from PM via a recycling endosomal (RE) compartment. Interestingly, in GL261 mouse glioma cell line DCT is also detected at PM, which may indicate a post-Golgi common route for DCT in different tumor cells [65]. We discovered an unexpected effect of the lysosomotropic agent chloroquine (CQ) on DCT stability, from both murine and human cell lines. CQ, a well-known pharmacologic agent that accumulates within acidic compartments, usually recommended as inhibitor of lysosomal enzymatic machinery [66] was expected to block DCT constitutive degradation. Instead, we found that DCT amount synthetized within 30 min (pulse), after 3 h (chase), in the presence of added CQ is diverted to a premature degradation pathway, whereas TRP1 stability is not affected in the same cell line. This is not an artifact, given that DCT degradation can be prevented in CQ-treated cells if Baf is present in the system. It is worth mentioning that DCT degradation is significantly decreased if CQ is added at 6 h chase, when probably DCT is in a more protected compartment. The effects of CQ in living systems are pleiotropic, and many of its action mechanisms or targets are still unknown. CQ interferes with the trafficking [67] and recycling processes from PM [68] or with the fusion vesicular processes, by enhancing the rate of the phagolysosomal fusion [69]. Our theory about CQ impact on DCT fate is that in our experimental conditions (mild CQ concentration, 50 μM and short time period treatment of 2 h), CQ potentiates the fusion between a DCT-positive post-Golgi endosomal compartment with a still proteolytically active one, most likely the lysosomes. It will be also interesting to identify which other proteins share the DCT fate in CQ- treated melanoma cells or if the effect of CQ is similar in other cells phenotypes expressing endogenous DCT.

2.3.2. Extracellular regulation

One of the early events in neoplastic transformation of melanocytes is the uncontrolled proliferation. During this step, tumor cells secrete numerous cytokines and growth factors, which can regulate back the tumor cells activities, by binding to self-receptors (autocrine stimulation) or receptors of neighboring cells (paracrine stimulation) and self-sustaining tumor growth signals. In addition, the nutrient deprivation and numerous homotypic cell-cell contacts, established as a result of the alterations that occurred in cell adhesion molecule repertoire, result in activation of multiple signaling cascades. A similar situation to autocrine/paracrine stimulation is simulated in an *in vitro* experimental approach when we cultured three different melanoma cell lines, MNT-1 (pigmented, metastatic stage), MJS (amelanotic, VGP stage), and SK28 (amelanotic, metastatic stage), for various time periods representing subconfluent (48 h), semi-confluent (72 h), and confluent (96 h) stages and when the dramatic increase of DCT mRNA and protein are registered [23] (see also Section 5). Surprisingly, in MNT-1 cells that express all TRPs, only DCT is increasing, whereas neither TYR nor TRP-1 expressions are changed. The process was further dissected in MJS phenotype when subconfluent cells grown in exhausted medium resulted from a confluent culture as well as in nutrient deprivation conditions (2% instead of 10% fetal calf serum) showed also the DCT overexpression [23]. All these data demonstrate that only DCT is the target of autocrine/paracrine stimulation. The DCT increase is more abrupt in MJS than in MNT-1 or SK28 and may be a process which is distinctly controlled in VGP stage phenotypes. The VGP cells express a variety of growth factors for autocrine and paracrine stimulation that enable them with survival and proliferation capacities in growth-factor free medium and with increased invasiveness potential through basement membranes [70]. It appears that DCT remains under the control of extracellular factors even in advanced stages of tumor progression as its increasing expression still persists in the two metastatic cell lines. The identification of the cytokines and growth factors, secreted during intense proliferative step, to which DCT overexpression is activated would be a further important step in elucidating how the expression of this antigen is modulated. Altogether these data demonstrate that DCT fate in melanoma is controlled by multiple and specific factors that do not act in the biosynthetic pathways of the other TRPs. There are several checkpoints in DCT life-cycle: (1) in ER, the DCT stability, controlled by early step of N-glycan processing and polypetide folding assisted by calnexin; (2) between ER and Golgi, the DCT precursor trafficking and maturation, controlled by Cav1 expression, integrity of CRDs and microtubules and pH of the secretory pathway; (3) beyond Golgi, the DCT stability, controlled by CQ; and (4) at transcriptional level, the DCT mRNA and protein, controlled by Cav1 expression, nutrient deprivation and secreted growth factors and cytokines during proliferation step (**Figure 6**).

2.4. DCT: a regulator of melanogenesis, cellular detoxification, and stress-resistance pathways

Melanins represent a group of polymers produced by both normal and transformed melanocytes. The skin melanins are synthetized and deposited within melanocyte-specialized cellular organelles called melanosomes that are finally transferred into epidermal keratinocytes ensuring not only skin pigmentation but also UV light absorption and scattering, free radical
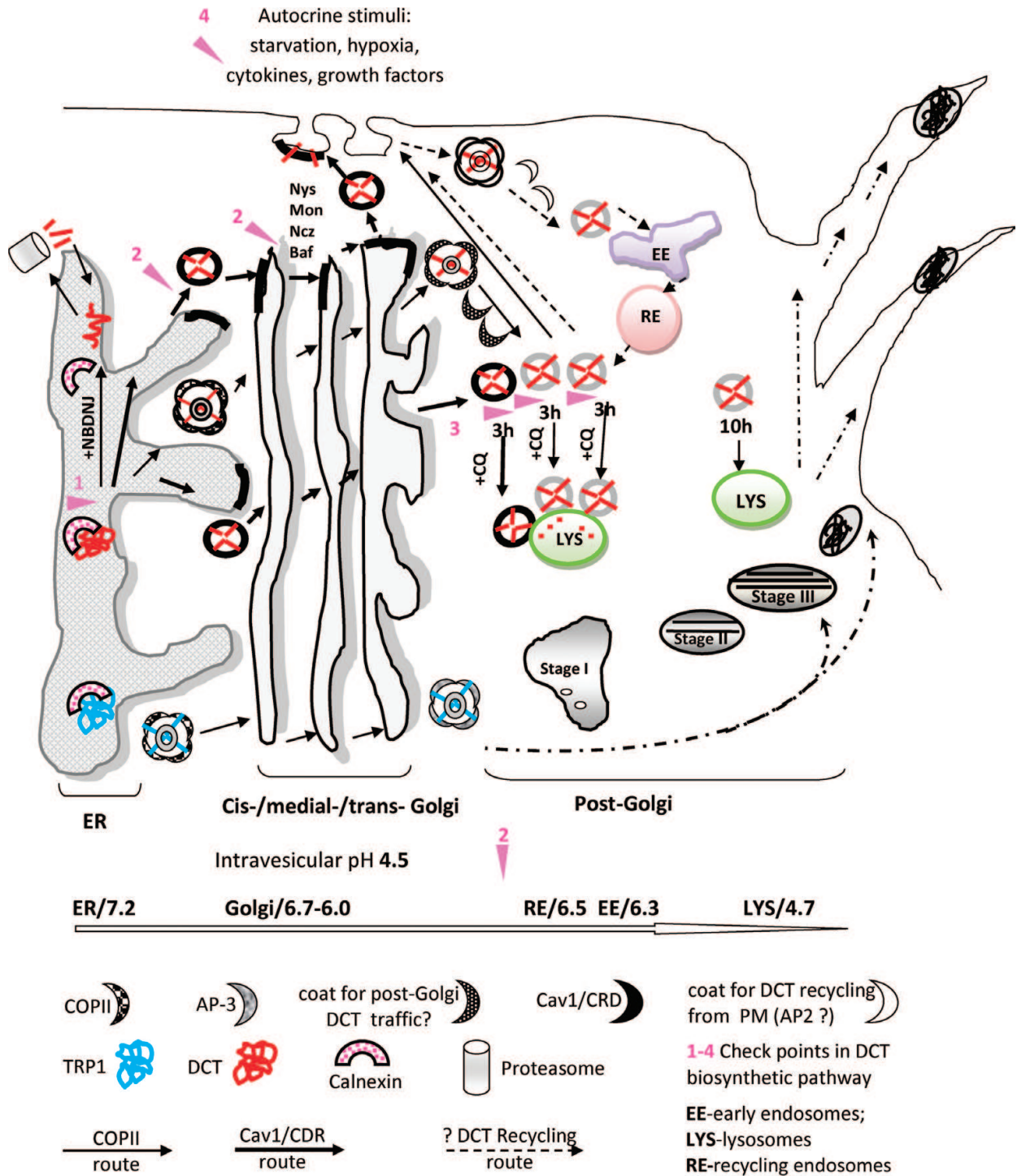
Figure 6. The intracellular journey of DCT in melanoma cells. The DCT biosynthetic pathway within a melanoma cell is schematically presented. All checkpoints along this route are indicated by triangle symbol. The DCT polypeptide is synthetized and folded in ER assisted by lectin chaperone calnexin (Clx). The interruption of N-glycan processing in ER with NBDNJ prevents interaction with Clx. TRP1 is further processed beyond the ER, whereas DCT is targeted to proteasomal degradation (1st checkpoint). Between ER and Golgi, DCT maturation is blocked by disrupting agents of cholesterol-rich domains (CRD) (nystatin-Nys, monensin-Mon) and microtubules (nocodazole-NCZ), intravesicular pH (bafilomycin—Baf) and caveolin-1 (Cav1) downregulation (2nd checkpoint). Post-Golgi, DCT, unlike TRP1, is diverted into a premature degradation pathway induced by CQ treatment (3rd checkpoint). Nutrient deprivation, secreted factors during proliferation and Cav1 gene down regulation are activators of DCT, not of TYR or TRP1, expression (4th checkpoint). Possible DCT recycling route from PM is presented as segmented line. TRP1 post-Golgi route to melanosomes stage III/IV is shown as intrerupted line.

scavenging, coupled oxidation-reduction reactions, and ion storage [71]. TRPs are the main regulators of principal steps of melanin polymer formation (**Figure 7**). TYR is the key-enzyme of melanogenesis that catalyzes the hydroxylation of L-Tyrosine to L-3,4-dihydroxyphenyl alanine (L-DOPA). L-DOPA is rapidly oxidized to DOPAquinone that spontaneously undergoes cyclization to Dopachrome. In the absence of any enzymatic activity, Dopachrome loses carboxylic acid generating 5,6-dihydroxyindole (DHI). TRP2 or L-Dopachrome tautomerase (DCT) acts downstream of TYR by rearranging Dopachrome into DHI-2-carboxylic acid (DHICA) that is further oxidized to the corresponding quinone by the activity of TRP1 in mouse or by TYR in humans. In 1992, Jackson and colab reported the cloning and sequencing of mouse cDNA corresponding to the region of the mice coat color mutation slaty. The gene product was named tyrosinase-related protein-2 (TRP-2) due to its high degree of amino acid identity with the other TRPs [72] or Dopachrome tautomerase (DCT) due to enzymatic activity on Dopachrome [73]. DCT is now well acknowledged as the modulator of melanin qualities. L-Dopachrome is the second branch point which under the unique L-DCT action is transformed into DHICA (**Figure 7**). Melanin derived from oxidation and polymerization of DHI, formed in the absence of DCT are black and insoluble, whereas the DHICA-enriched melanins that contain a higher proportion of carboxylated versus noncarboxylated indolic monomers are brown and more soluble [74]. Despite of numerous mutations identified in other melanosomal proteins, with consequences on pigmentation, no mutations have been described in human DCT, suggesting this is a conserved protein. However, in mouse, mutant alleles of DCT are associated with pigment dilution, producing the slaty (R194Q substitution in the MeA binding domain) and slaty light (G486R substitution in the TM domain) phenotypes. DCT mutations increase pheomelanin and reduce eumelanin produced by melanocytes in culture showing that the enzymatic activity of DCT play a role in determining whether pheo-or eu-melanin pathway is preferred [75]. The intermediates generated during melanogenesis have genotoxic [71] and immunosuppressive properties [76]. DHI is a cytotoxic melanin precursor [77], whereas DHICA is an antioxidant molecule [78], a diffusible chemical messenger [79], and DHICA unlike DHI melanins exhibit potent hydroxyl radical-scavenging activity (**Figure 7**). Moreover, eumelanins bind calcium with an affinity similar to calmodulin and thus interfere with the intracellular calcium regulation [80]. DCT, as a specific limiting factor of DHI concentration and DHICA-eumelanins formation becomes thus a modulator of different processes in melanocyte in which DHICA and DHICA-melanins are involved. To establish the general impact of DCT on a living organism, the DCT gene was targeted during mouse embryonic development [81]. The DCT-KO mice are viable, have a diluted coat color phenotype, due to reduced melanin content in hair but do not show any decrease in melanocyte numbers. However, under chronic UVA-induced oxidative stress in skin of DCT-KO mice compared with wild-type, the level of reactive oxygen species (ROS) and the numbers of apoptotic cells are increased, whereas the amount of eumelanin is decreased [82]. This demonstrates that, in melanocytes, DCT is involved in regulating a protective pathway in response to environmental stressful conditions. The DCT protective effect seems not to be exerted only via its enzymatic activity. The extremely low growth rate for the DCT-slaty and DCT-slatylight melanocytes could not be abrogated in the presence of catalase, added to culture medium to overcome effects of H_2O_2 resulted from DHI excess due to inactivity of mutated DCT [83]. In transformed melanocytes, DCT is a tumor protector as well. In pigmented melanoma, as in melanocytes, DCT generates DHICA and further
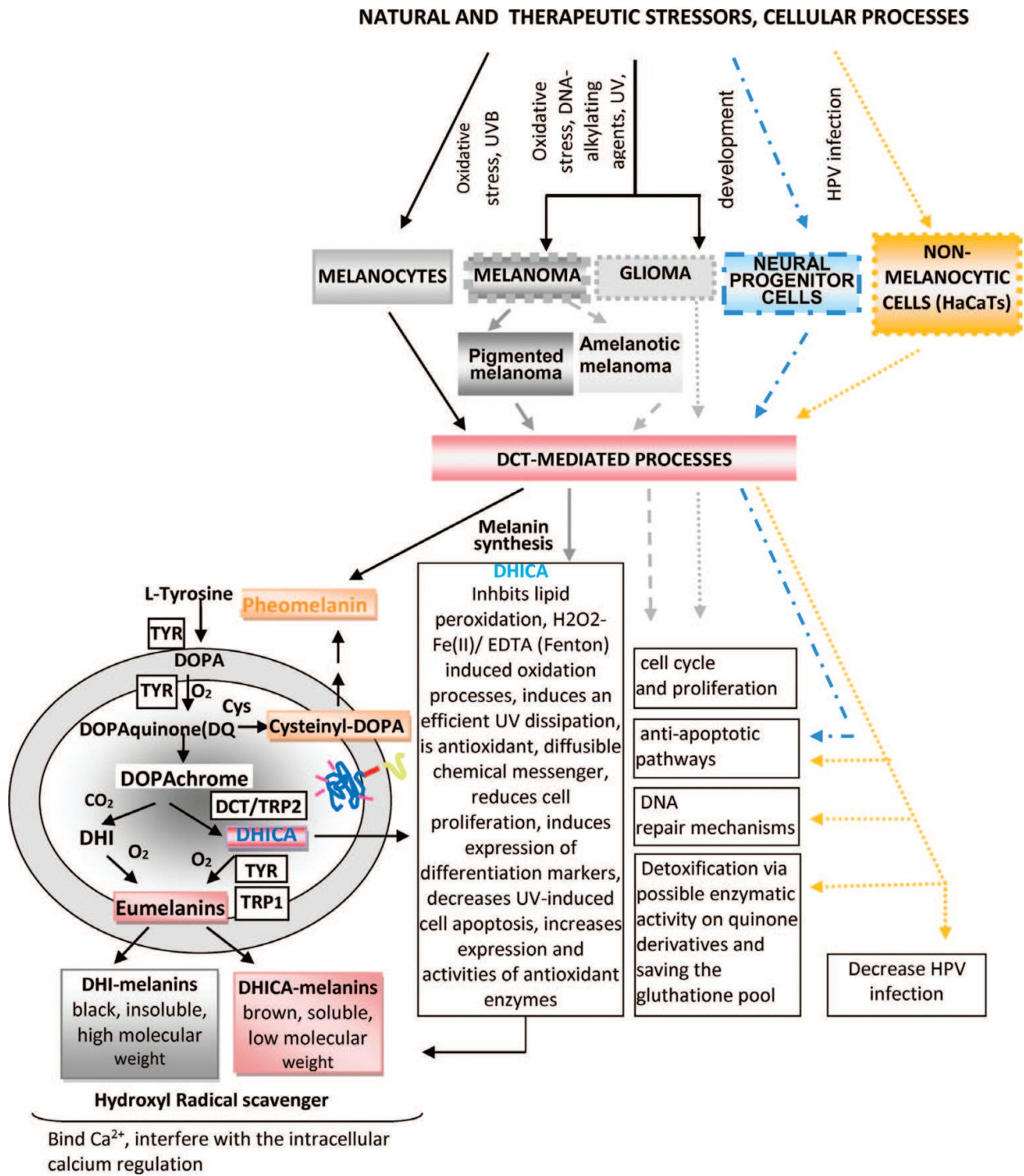
NATURAL AND THERAPEUTIC STRESSORS, CELLULAR PROCESSES

Figure 7. The processes mediated by DCT in different normal and malignant cell phenotypes.

DHICA-eumelanins, both exerting the antioxidant properties (**Figure 7**). However, DCT protective activity is independent of melanin pathway, and this is in good-agreement with finding that DCT is well-expressed in amelanotic cell lines and tumors [34, 35]. In a process of identification of genes associated with cis-diamminedichloroplatinum (II)(CDDP)-and X-ray resistance in the amelanotic melanoma cell line WM35, Bed-David's group found that DCT expression was upregulated in both CDDP- and X-ray resistant mutants compared with the

parental line [84]. On the other hand, DCT ectopic overexpression in melanoma cells abrogates UVB-induced apoptosis [57]. DCT-drug resistance-mediated pathway is related to anti-tumorals that interferes with DNA replication as CDDP, carboplatin, or methotrexate and is not effective to the ones acting on microtubule formation as paclitaxel. In correlation with our data about DCT intracellular processing, we can speculate that DCT-mediated tumor resistance to the microtubule depolymerizing agents, unlike the one to DNA-alkylating agents, requires mature DCT and not DCT precursor which is the only DCT glycoform in cells treated with microtubule depolymerization agents (Section 2.3.1.2). DCT-radiation resistance is addressed to both X- and UVB-radiation that act on DNA by creating DNA strands and causes the formation of pyrimidine dimers, respectively, and are independent of TYR or TRP1 expression or melanin content [85]. DCT protective effect may be explained by either interference with DNA repair mechanisms or the regulation of anti-apoptotic pathways. DCT anti-apoptotic activity has also been reported in AJS sensory neurons in *C. elegans* [86]. A possible DCT mechanism suggested by the authors would be the activation of the ERK/MAPK stress pathway in response to high DHICA content produced as result of DCT overexpression and enzymatic activity after radiation [84]. However, this will not explain the DCT protective effect in WM35 amelanotic cell line used in these experiments or in other amelanotic phenotypes where melanogenic pathway is interrupted and Dopachrome, DCT natural substrate, is not produced due to TYR inactivity. Specific melanoma protective DCT-mediated effects, independently to melanogenesis, have been demonstrated also in amelanotic melanoma cell line WM35, expressing inducible DCT and subjected to oxidative stress conditions [87]. DCT endogenous expression increases cell viability and intracellular glutathione (GSH)—a key factor of ROS detoxification, whereas ectopic DCT expression decreases nonmelanocytic cell sensitivity to quinone compounds [88]. The DCT detoxification action is dependent of the tautomerase enzymatic activity, is lineage-specific, and is in conjunction with specific metabolites that naturally appear in melanocytes, neural/glial cells, and in melanoma and glioma. A possible mechanism by which DCT prevents quinone toxicity could be explained by the shared homology between quinones derivatives with DCT natural substrate, L-Dopachrome, and to the fact that DCT may have a possible oxidoreductase activity. This, however, is not supported by the presence of Zn^{2+} in DCT catalytic site, as Zn^{2+} has no redox properties, and is unable to catalyze oxidative reactions [12] but possible if the presence of the ferrous-iron would be accepted in the DCT catalytic site [89]. Such a DCT protective mechanism could function in glioma, in which, after targeting DCT by immunotherapy, the tumor cells become more sensitive to chemotherapy [90].

Although the object of this chapter is DCT in melanoma, we consider that it is of importance to discuss the role of DCT in other cell lineages. We have argued about DCT expression in HaCaT cells (basal keratinocytes) [36] (Section 2.2). The effects of DCT downregulation in HaCaTs are similar to the ones reported so far in melanocytic cells, namely increased ROS levels, DNA damage, and altered cell cycle, which furthermore compromise the infection of these cells with HPV. There are several common processes, mainly related to cell protection, with which DCT interferes, regardless the cell phenotypes in which it is expressed. However, these processes are involved in cell-specific responses to different aggressors (e.g., therapeutic stressors in melanoma and viral infection in basal keratinocytes).

3. DCT value in the assessment of melanocytic lesions

The diagnostic and prognostic of CMM is in general evaluated histopathologicaly. In particular cases, when it is difficult to discriminate between melanocytic lesions and other resembling tumors as sarcomas, lymphomas, or neuroendocrine tumors, the expression of melanocytic biomarkers is requested, and they are commonly assessed by immunohistochemistry. For patients with unambiguous tumor histologic features, the CMM prognostication relied on Breslow's index, the level of invasion in skin layers (Clark's level), growth pattern (nodular, superficial spreading, etc.), dimensions, and presence/absence of ulceration information proves to be statistically significant in very large clinical cohorts [91]. The panel of melanoma markers is continuously revised and improved in accordance with the new discoveries related to the molecular mechanisms and pathways in melanoma progression [92]. One of the most challenging is the thin melanoma subset, defined by Breslow depth, 1.0 mm representing patients with early-stage disease. Despite that most are thought to have an excellent clinical outcome (85% survival during a 10-year period) and can be treated effectively, 15% of melanoma deaths result from metastases of thin lesions. Furthermore, the clinical outcome of patients with melanoma of intermediate thickness (2.0–4.0 mm in Breslow depth) is less predictable. Clearly, identifying a high-risk population with thin melanomas remains a challenge, and new markers to assist this patient population are expected in order to establish more accurate risk groups with subsequent more aggressive therapeutic approach and tighter follow-up [93]. Our group assessed for the first time, the expression of DCT comparatively with the one of TYR in a panel of formalin-fixed, paraffin wax-embedded benign and malignant melanocytic lesions. The DCT and TYR proteins were analyzed by immunohistofluorescence microscopy in human specimens by simultaneous triple staining, with anti-DCT/-TYR antibodies, followed by secondary antibodies AlexaFluor-labelled and with DAPI for nuclei [38]. This technique allows to follow DCT and TYR expressions in identical cells within different tumor components. In tumor progression, the expressions of melanoma antigens are often lowered [94], and their immunodetection in histological specimens may be enhanced using antibody populations that recognize more than one epitope. In this study, the DCT expression was assessed with a novel anti-DCT antibody raised in our laboratory against the luminal domain of human DCT and in which the bioinformatic analysis identified multiple potential antigenic sites [38]. There is a heterogeneity in the expressions of the two antigens in benign tumors represented by junctional (JNs), compound (CNs), or dysplastic nevi (DNs) and malignant melanomas represented by superficial spreading (SSMs), nodular (NMs), achromic (ACMs), acralentiginous (ALMs) melanomas. Specimens expressing both antigens, only one and negative for both, were present in different numbers in each melanoma subgroup that was analyzed. The melanocyte neoplastic transformation and malignant progression is well correlated with the dissociation of DCT and TYR expression in distinct cell populations. In **Figure 8A** is presented an example of DCT and TYR dissociated expression in distinct tumor cells in a specimen representing a nodular melanoma. Within the double-positive category, we have identified in some specimens a subtype named by us "DCT-phenotype" in which DCT and TYR expressions specifically distributed within cell populations of tumor components create a tumor-specific architecture, with cells Tyr+/DCT- in the subepidermal layer, whereas DCT+/Tyr- cells segregate into deep dermis. The DCT-phenotype was found
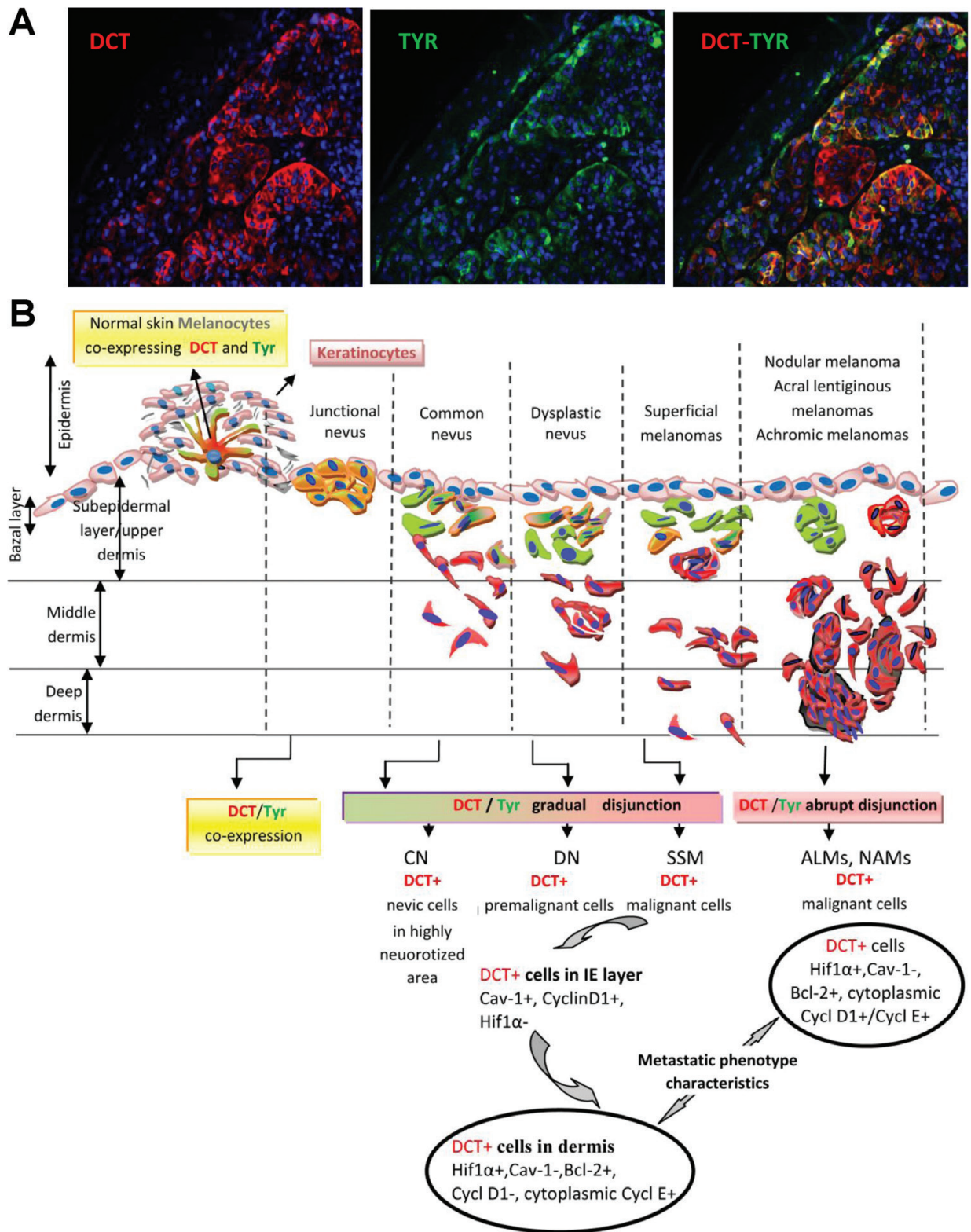
Figure 8. DCT in melanocytic lesions. (A) A nodular melanoma specimen immunostained for DCT and TYR expressions and analyzed by fluorescence microscopy. Tumor cells co-expressing both markers and cells DCT+/TYR- or DCT-Tyr+ can be observed. Unlike TYR, DCT is well expressed in numerous cells. (B) Schematic representation of DCT and TYR dissociation in melanocyte transformation and melanoma progression and molecular anatomy of DCT-phenotype. The switch in molecular repertoire of markers of tumor progression and bad prognosis in DCT+ cells in intraepidermal (IE) layer is indicated. DCT+ cells in deep dermis acquire molecular parameters of metastatic phenotypes [38].

in benign specimens with high neurotization and also in some early malignant ones having low Breslow/Clark indexes but with ulceration. Our theory is that DCT-phenotype is emblematic for a long-lasting, "die-hard" phenotype. The DCT-intense expression is observed in large areas of compound neurotized nevi contributing probably to the well-acknowledged enhanced stability and low proliferation rate of these nevus cells [95] and may not represent a life-threatening problem in benign tumors. However, the superficial malignant melanomas, with low indexes Clark or Breslow but having DCT-phenotype could be a warning signal for considering those specimens as ones of high risk with a possible unfavorable prognostic. The DCT-clones selected in inner dermis of early malignant lesions acquire the expression and subcellular distribution of molecular markers reported to be associated with different types of neoplasms, including melanoma, with extended migratory capacities (caveolin-1-), survival in stressful conditions (cytoplasmic Hif-1α+), activated anti-apoptotic mechanisms (cytoplasmic cyclin D+ and Bcl-1+), angiogenic, and metastatic potential (cytoplasmic cyclin E+) (**Figure 8B**). Several ALMs or ACMs advanced melanomas diagnosed by anatomopathological analysis with bad prognostic detected DCT as the unique melanosomal antigen. The ALMs distinguish themselves from other melanoma types in terms of a worse prognosis, enhanced aggressiveness, and by a more advanced stage at diagnosis [96], whereas some ACMs are characterized by a peculiar and aggressive evolution [97]. It is very possible that DCT expression in ALMs and ACMs mediates tumor stress resistance pathways and contributes to the malignant characteristics of these melanoma categories. DCT could be an useful adjunct marker increasing sensitivity of tumor cell detection in specimens having downregulated other melanoma antigens, and the DCT-phenotype could represent a parameter associated with high-risk for bad disease outcome.
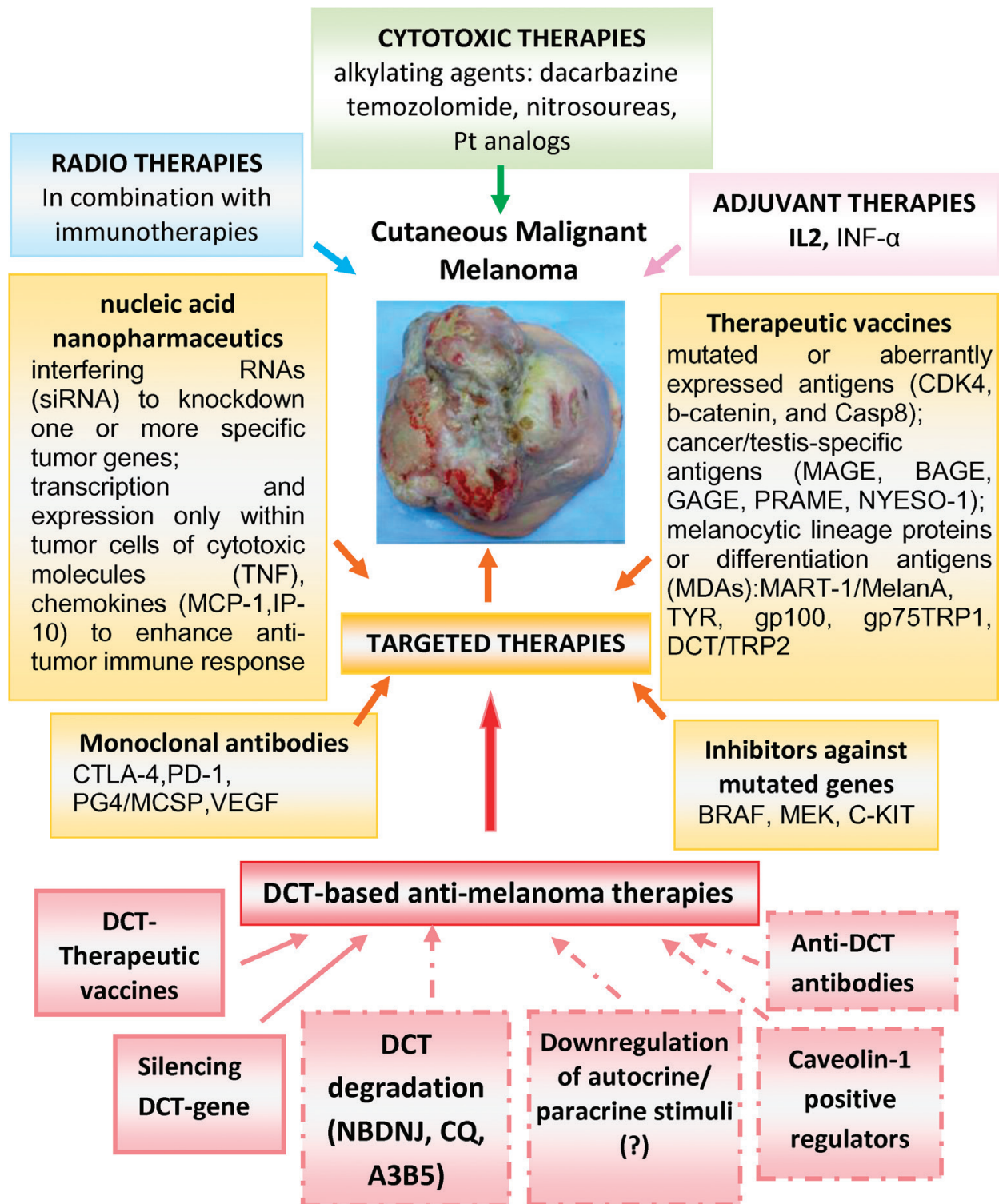
4. DCT as target in melanoma therapy

4.1. Anti-melanoma therapies

The surgical removal is the only cure for melanoma with the condition that the excised lesion be in an early stage. However, the micrometastases cannot be addressed exclusively by the surgery and therefore, combinatorial therapeutical strategies are applied in the attempt to extend survival rates. The treatment options in melanoma are continuously revised, and there are several excellent reviews about this topic [98–100]. The schematic representation of the treatment of metastatic melanoma including different approaches is shown in **Figure 9**.

4.2. Anti-melanoma immunotherapeutic strategies involving DCT

The identification of different T-cell clones in melanoma patients recognizing peptides derived from DCT (**Figure 2**) raised the interest for this antigen in the development of anti-melanoma immunotherapeutical strategies. The cellular vaccine engineered to co-express a DCT epitope, with IFN-γ in the same gene by replacing the IFN-γ signal peptide with a DCT epitope-expressing signal peptide, resulted in decreased B16 tumorigenicity and enhanced immunogenicity after gene transfer. More importantly, irradiated transiently, TRP-2 epitope-expressing, IFN-c

Figure 9. The therapies in melanoma. The different anti-melanoma treatment strategies are presented. DCT-based therapies are integrated part of the targeted therapies. The solid lines indicate the already existing therapies, whereas the dotted lines are proposed as possible adjuvant therapies based on the molecular studies about DCT intracellular processing and stability in melanoma cells. The melanoma specimen is an ulcerated nodular melanoma of a 26-year-old man, from lumbar region (by courtesy of Dr. S. Zurac, Department of Pathology, Colentina University Hospital, Bucharest, Romania).

gene-modified B16 cells worked efficiently as a cellular vaccine to protect animals from parental wild-type tumor challenge [101]. The VacciMax® (VM), a liposome-based antigen delivery platform, has been used to deliver DCT 181–188 in combination with p53-derived peptides. A single administration of VM was capable of inducing an effective CTL response to multiple tumor-associated antigens. The responses generated were able to reject 6-day old B16-F10 tumors [102]. Another plasmide liposome DNA vaccine targeting the DCT in combination with chemokine CCL2 as an adjuvant used xenogeneic (human) DCT in a mouse model and resulted in induction of strong anti-DCT cell-mediated immunity after two vaccinations [103]. A novel vaccine system designed from a long TRP2/DCT peptide with a CD8 epitope (TRP2/DCT 180-88) and a CD4 epitope (TRP2/DCT 88-102) together with α-galactosyl ceramide, a lipid antigen representing a new class of promising vaccine adjuvants into cationic liposomes tested on mice tumors resulted in the enhanced production of IFN-ϒ and increased cytotoxic T-cell responses [104]. Importantly, the antitumor immune activity involving MDAs as immunotherapeutic targets may have as side effects the damage (depigmentation) of the normal tissues that also express the MDAs [105]. However, in a patient receiving infusion with TIL586 (recognizing the DCT 109–205 peptide), tumor regression was observed, but not depigmentation [20], which demonstrates that immunotherapy directed against some DCT epitopes is specific and does not affect normal tissues. In another study, the inoculation of plasmid DNA encoding murine DCT elicited antigen-specific CTLs that recognized the B16 mouse melanoma and protected the mice from challenge with tumor cells. Moreover, mice that rejected the tumor did not develop generalized vitiligo, indicating that autoimmunity is not automatically triggered by administrating therapeutic MDA-based vaccines [106]. The vaccination with bone marrow-derived dendritic cells loaded with DCT peptide resulted in activation of high avidity CTLs mediating protective antitumor immunity *in vivo* without the development of adverse autoimmunity [107]. In a murine therapeutical model, four of seven mice with pre-established tumor remained tumor-free for 80 days after therapeutic vaccination with mouse DCT gene-modified dendritic cells, using a HIV-1-based lentiviral vector demonstrating again that DCT gene transfer to dendritic cells is a potent therapeutic strategy in melanoma [108]. A very important aspect is DCT immune-based therapy in glioma. DCT is expressed in glioma cells naturally, and DCT-specific CTLs have been detected in patients' peripheral blood mononuclear cells [109]. On the other hand, DCT overexpression is associated with tumor cell resistance to chemo- or radio-therapeutic treatments. The theory that DCT is a key player in the synergy between chemotherapy and immunotherapy was demonstrated in a clinical study in which tumor cells escaped from vaccination against DCT were more sensitive to chemotherapy with DNA-damaging drugs.

4.3. Anti-melanoma therapies targeting DCT gene or protein: current status and perspectives

Despite the already acknowledged DCT involvement in melanoma drug-resistance, there are no reports so far, to our knowledge, about melanoma therapies targeting directly the DCT gene or protein. There is, however, a patent claiming the treatment of melanoma cells *in vitro* with antisense nucleotides targeting DCT mRNA in conjunction with DNA-alklylating anticancer drugs [110].

Our data about the intracellular processing and the main checkpoints in DCT fate in tumor cells (Section 2.3) indicate that pharmacological agents that impact DCT stability could represent also potential adjuvants in melanoma therapy. For example, NBDNJ or A3B5 produce specific DCT proteasomal degradation possibly sensitizing tumor cells to therapeutic stress and could also generate DCT-peptides suitable for MHCI presentation and immune response. The selective premature DCT degradation induced in melanoma cells following CQ treatment is another possible way to decrease tumor cell resistance to therapies. CQ has been found to strongly potentiate the inhibitory effect of radiation on tumor cell proliferation [111], to be effective in eliminating chemotherapy-resistant cancer cells and to significantly improve the median survival in glioblastomamultiformis patients [112]. Moreover, the DCT detection at PM by us in melanoma cells [23] and by others in glioma cells [65] introduces DCT as a suitable molecule for targeting tumor cells with specific antibodies. If studies will confirm that DCT is internalized from the PM, this will open interesting perspectives of coupling anti-DCT antibodies with nanocarriers loaded with various antitumor agents. And finally by downregulating DCT (by siRNA or CRISPR/Cas9 system), it can be targeted the Cav1 stability and architecture and possibly some Cav1-mediated pathways including ones involved in tumor progression. The DCT-mediated therapeutic strategies are presented as integrated part of anti-melanoma treatments in **Figure 9**.

5. DCT: a novel molecular driver in melanoma progression

Our most recent studies in two distinct amelanotic melanoma cell lines representing different tumor phenotypes, MJS and SK28, demonstrate a molecular crosstalk, between DCT and caveolin-1 (Cav1), with structural and functional implications [23].

5.1. DCT is associated with Cav1 membranes

DCT and Cav1 are present in common structures in cytoplasm or decorating segments of PM (**Figure 10A**). Both Cav1 monomers/oligomers and DCT-precursor/mature forms have the same distribution along a density gradient in an ultracentrifugation experiment. Moreover, Cav1 has been identified in western blot and mass spectrometry analysis of the immuno-precipitates obtained with anti-DCT antibody from MJS cell lysates [23]. These experimental data are strongly supported by the structural analysis of DCT and Cav1 and by DCT-Cav1 structural model presented in Section 2.1.

5.2. DCT regulates Cav1 assembly and stability and possibly Cav1-mediated cellular processes

The transient downregulation of DCT expression (si-DCT) in MJS and SK28 cells increased the amount of Cav1 protein by its redistribution into more stable, insoluble membrane aggregates with altered morphologies [23] (**Figure 10A**). This is the first report about a melanosomal protein that regulates Cav1 assembly. We postulate that DCT may regulate Cav1- and/or lipid raft structures by competing either with different signaling molecules for Cav1

binding or with Cav1 monomers for Cav1 oligomerization domain or for cholesterol binding. Both caveolae and Cav1-scaffolds are associated with lipid rafts, which are membrane domains with a very dynamic structure abundant in cholesterol, sphingolipids recruiting different molecular players of signaling platforms, and controlling numerous and diverse cellular processes [113]. Either directly or indirectly, DCT as a major regulator of Cav1- or cholesterol-membrane architecture is thus expected to impact also different cellular events mediated by Cav1 (**Figure 10C**). For example, the interaction of membrane/lipid rafts, with the cytoskeleton, has impact on trafficking and sorting mechanisms, formation of platforms for cell anchorage to ECM, transduction of signaling cascades across the PM, cell growth and migration, entry of microorganisms (viruses/bacteria), and toxins or nanoparticles [114]. Indeed, we also observed that in MJS cells having downregulated DCT expression, there was an increase in cell volume, a significant redistribution of actin filaments in cell periphery, and a dramatic decrease in cell proliferation by 20 at 48, 60 at 72, and 75% at 96 h coupled with the cell cycle arrest in G1 [unpublished data]. Interestingly, these effects were less prominent in SK28 phenotype that indicates that DCT-mediated processes are tumor phenotype specific. Importantly, our mass spectrometry analysis of immunoprecipitates obtained from MJS cell lysates with anti-DCT antibodies against N- or C-terminus epitopes has identified as potential DCT interactors, regulators of small GTPases (Arf, Rho and Ras) and numerous proteins involved in anti-apoptotic, proliferative, migration, and invasion mechanisms and pathways [unpublished data]. The structural analysis pointed also the possibility that two Ser residues within DCT CYT subdomain to be phosphorylated (Section 2.1). Our theory based on all these data and preliminary information is that DCT, placed in a molecular environment with Cav1, is a key-molecular player acting on one or more signaling pathways involved in tumor cell survival and morphology, either by itself, as a potential target of the phosphorylation cascades, or as modulator of Cav1 or other participants in regulatory processes (**Figure 10C**). The numerous potential interactors present DCT as a possible new molecular scaffold. Further experimental studies are required to validate these interactions and place DCT in the exact pathway(s) where it operates.

5.3. Cav1 controls DCT gene expression, protein processing, and subcellular distribution

The Cav1 downregulation (si-Cav1) has a dramatic impact on DCT in MJS cells. There is a 20-fold increase over 96 h of Cav1 silencing on DCT mRNA level. Accordingly, there is also a protein increase detected by western blot, and the deglycosylation experiments showed that DCT synthetized in si-Cav1 cells is mainly DCT-precursor. The imagistic studies of confocal immunofluorescence microscopy and Tissue FAXS cytometry quantitative analysis revealed a 7-fold increase in a DCT-population with intense cytoplasmic, but no PM, DCT staining, the "DCT-high clones" (**Figure 10B**). This is the first report about a melanosomal protein/melanoma antigen-regulated by Cav1 and a novel target gene for Cav1. Cav1 is a regulator of several genes as CyclinD or folate receptor promoters [115] or for survivin, a member of the Inhibitor Apoptosis Protein-family [116]. In melanoma, Cav1 function is still ambiguous. In some studies, Cav1 is associated with tumorigenicity [117], whereas others present Cav1 as a tumor suppressor by inhibiting Wnt-β-catenin-TCF/LEF [118], Src/FAK [119] pathways, or attenuating tumor cell motility by disrupting glycosphingolipid GD3-mediated malignant signaling [120]. In the context of DCT-mediating pro-survival and resistance pathways and
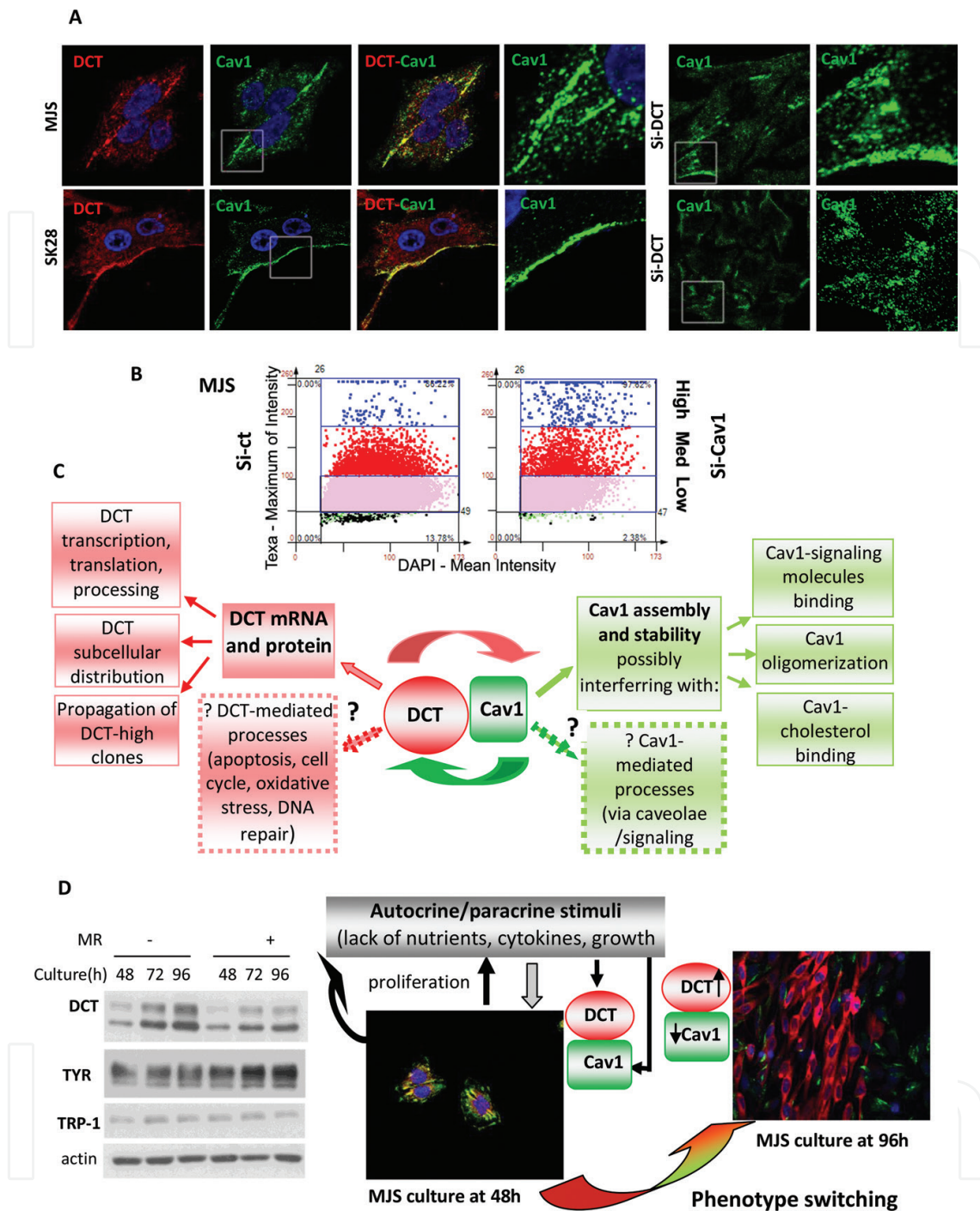
Figure 10. The structural and functional relationship between DCT and Cav-1. (A) MJS and SK28 amelanotic melanoma cells immunostained for DCT and Cav1 and analyzed by confocal fluorescence microscopy demonstrate DCT and Cav1 in cytoplasmic and PM common structures; in DCT downregulated cells, the morphologies of Cav1 positive structures are severely altered. The fourth and the sixth panels represent the enlarged details of the indicated insets; (B) the DCT-high clones in MJS having downregulated Cav1 expression analyzed by tissue FAXS. In the upper part of quadrant are shown the cells with high DCT expression; (C) the crosstalk between DCT and Cav1. The impact of si-DCT on Cav1 and of si-Cav1 on DCT is indicated. Possible processes mediated by either DCT or Cav1 are indicated in dotted boxes; (D) DCT, unlike TYR or TRP1 is overexpressed during transition from subconfluent (48 h) to semi-confluent (72 h) and confluent (96 h). Medium was not replenished for 96 h (MR−) or replenished every 24 h (MR+). Autocrine/paracrine stimulation (starvation, secreted factors by proliferative MJS tumor cells within 48 h) decrease Cav1, increase DCT expressions, and change the cell morphology. The cells at 48 h are polygonal with visible contacts between adjacent cells, whereas cells at 96 h are elongated with no cell-cell contacts and form large clusters.

the upregulation of DCT in si-Cav1 cells, we consider that Cav1 acts as a tumor suppressor gene, at least in this early malignant phenotype. The exact mechanism of how Cav1 controls DCT gene expression and how this intersects DCT-mediated processes (**Figure 10C**) needs to be deciphered and validated in one or more melanoma cell line(s) in addition to MJS.

5.4. DCT and melanoma phenotype switching

The oncogenic epithelial-mesenchymal transition (EMT) is a multistep process by which epithelial cells acquire invasive mesenchymal phenotype characteristics essential in metastatic spread [121]. EMT is regulated and characterized by molecular mechanisms involving specific transcription factors, signaling pathways, and biomarkers. In melanoma cells which do not have epithelial origin, there is a phenotype switching, with similitudes between the EMT program from development, and this EMT-like switch is a major determinant in tumor metastasis [122]. The role of Cav1 in the oncogenic EMT process is significant but controversial and depends on the type of cancer. In bladder cancer cells, Cav1 promotes invasive phenotypes by inducing EMT [123] in A431 human epidermoid carcinoma cells, the Cav1 downregulation by EGF (an EMT inducer) results in E-cadherin loss, and increased tumor cell invasion [124], whereas in primary tumors of head and neck, squamous cell carcinoma increases EMT and prometastatic properties [125]. During transition from subconfluent (48 h) to confluent (96 h) cultures in MJS, SK28, or MNT-1 cell lines, there is an increase in DCT expression, not observed for either TYR or TRP1 and more abrupt in MJS (VGP) than in MNT or SK28 (metastatic) cells (**Figure 10D**). Oppositely, in the same MJS culture, Cav1 was severely downregulated, in the same cells highly expressing DCT. The most stimulating agent for DCT overexpression is the culture medium exhausted in nutrients but rich in cytokines and growth factors secreted by the tumor cells during 96 h proliferation, whereas changing medium every 24 h has a lower impact on DCT increase (**Figure 10D**). EMT can result from multiple extracellular stimuli; for instance, a synergistic effect on EMT has been observed with combined stimulation of EGF and TGF-β [126]. Interestingly, the cell morphology of MJS, but not SK28 cells was dramatically changed during transition from subconfluent to confluent stage from a polygonal, low-expressing DCT/high-Cav1 to an elongated phenotype high-DCT/low- or negative Cav1 (**Figure 10D**). The same phenotype switching has been observed in si-Cav1 cells highly expressing cytoplasmic DCT. Oppositely, si-DCT cells adopt a wider morphology. We consider that, in MJS phenotype, the DCT and Cav1 crosstalk is a possible part of the EMT program. In subconfluent MJS culture (48 h), groups of 2–4 polygonal cells are interconnected via fine filaments and express low DCT and high Cav1. In confluent culture (96 h), the environmental signals trigger probably, independently, the DCT increase and Cav1 decrease. Furthermore, Cav1 downregulation itself sustains even more the DCT increase. The dynamic analysis of tumor cell populations with Tissue FAXS system demonstrates the perpetuation of a subset of DCT-high/Cav1-low, elongated fibroblast-like cells with long extensions, and forming large clusters (**Figure 10D**). This metamorphosis is an *in vitro* recapitulation of an *in vivo* situation encountered during our analysis of the molecular signature of the DCT+ cells in tumor components of human specimens [38]. The tumor cells in subepidermal layer are DCT+/Cav1+, whereas the ones in deep dermis, a more hostile environment, are DCT+/Cav1- (**Figure 8**). In DCT-phenotype, TYR was always in cells from superficial tumor components,

whereas DCT was in the deep dermis ones. This is in good correlation with data showing that in MNT-1 cells expressing all TRPs, during autocrine stimulation only DCT expression is increased [23]. The cross talk between DCT and Cav1, DCT as gene target of autocrine/paracrine stimulation as well as the impact of DCT expression on tumor cell-phenotype proliferation and morphology introduce DCT in the complex signaling pathways and networks regulating tumor progression.

6. Conclusions, open questions, and perspectives

TRP2/L-DCT is, undoubtedly, a benefit for the cell expressing it. In melanocytes, the detoxification processes involve the conversion of DCT natural substrate, DHICA into less toxic products. In nonmelanocytic cells, exogenous DCT is able to decrease the effects of oxidative stress acting on substrate analogs. In melanoma, the "preservation" of the expression of certain melanosomal antigens able to ensure tumor cell viability prevails over that of the key-enzymes for pigment production, and TRP2/L-DCT qualifies for this selection. For this prosurvival molecule, the tumor cells reserve complex transcriptional and post-translational mechanisms distinct from the other TRPs. DCT functions as a sensor in case of the autocrine stimulation/stressful conditions when its expression is highly increased, no matter whether the melanogenic pathway is active or not. There is a molecular crosstalk between DCT and Cav1, a master regulator of numerous cellular processes. The members of signaling platforms identified by mass-spectrometry analysis as potential DCT interactors, as well as the impact of DCT expression on cell proliferation, morphology, and cytoskeleton remodeling are strong proofs that DCT is a key player in cellular processes, acting, in our opinion, as a molecular scaffold within one or more signaling hubs. The recent findings about DCT expression pattern in the tumor architecture in correlation with a stable, longlasting/"die-hard" phenotype in benign lesions and with bad prognostic parameters in malignant lesions advocate for considering DCT as a warning indicative of possibly tumor unfavorable outcome.

On the other hand, TRP2/L-DCT has its own vulnerabilities in terms of stability that can be exploited for therapeutic purposes.

In spite of all these information, the role of DCT in melanoma is far from being elucidated or fully exploited and several issues still need clarification: the molecularity behind DCT regulation by Cav1 and DCT impact on Cav1 structural organization; the decipherment of the signaling pathways in which DCT activates, in amelanotic versus pigmented phenotypes in different stages of tumor progression; how are the DCT structural subdomains involved in DCT tumor cell regulatory mechanisms; the DCT role in tumor cell phenotype switching process; the value of DCT phenotype as prognostic indicative; the efficiency of NBDNJ, CQ, as possible adjuvants in melanoma therapeutic strategies; the clarification of DCT expression in nonmelanocytic/nonneuronal cell lines or tumors.

In melanoma, DCT is a double-edged sword, a lethal weapon for cancer cells serving the tumor progression or an exploitable molecular tool for scientists and clinicians to eradicate the malignant cells.

Acknowledgements

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