The transmembrane channel-like protein family and human papillomaviruses
Insights into epidermodysplasia verruciformis and progression to squamous cell carcinoma

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Abbreviations: AEV, acquired epidermodysplasia verruciformis; CMI, cell-mediated immunity; CSCC, cutaneous squamous cell carcinoma; ERCC3, excision repair cross-complementing rodent repair deficiency, complementation group 3; ER, endoplasmic reticulum; EV, epidermodysplasia verruciformis; FADD, Fas-associated protein with death domain; FasL, Fas ligand; HPV, human papillomavirus; MST1, macrophage stimulating 1; MT, mecanotransducer; MTF-1, metal-responsive transcription factor 1; NF-κB, nuclear factor kappa B; NK, natural killer; NMSC, non-melanoma skin cancer; ORF, open reading frame; pRb: retinoblastoma protein; PBMC, peripheral blood mononuclear cell; RHOH, ras homolog gene family member H; RIPK1, receptor-interacting serine/threonine protein kinase 1; STK4, serine/threonine kinase 4; TGFβ, transforming growth factor beta; TMC6/EVER1, transmembrane channel-like 6; TMC8/EVER2, transmembrane channel-like 8; TNFα, tumor necrosis factor alpha; TRADD, TNF receptor type 1-associated death domain; TRAIL, TNF-related apoptosis-inducing ligand; UVR, ultraviolet radiation; XPB, xeroderma pigmentosum type B; XRCC1, x-ray repair cross-complementing protein 1; YAP, yes-associated protein; Zn2+, zinc; ZnT-1: zinc transporter 1

Epidermodysplasia verruciformis (EV) is a rare genodermatosis characterized by increased sensitivity to infection by the β-subtype of human papillomaviruses (β-HPVs), causing persistent, tinea versicolor-like dermal lesions. In a majority of affected individuals, these macular lesions progress to invasive cutaneous squamous cell carcinoma (CSCC) in sun-exposed areas. While mutations in transmembrane channel-like 6 (TMC6/EVER1) and 8 (TMC8/EVER2) have been causally linked to EV, their molecular functions are unclear. It is likely that their protective effects involve regulation of the β-HPV life cycle, host keratinocyte apoptosis vs. survival balance and/or T-cell interaction with infected host cells.

Introduction

TMC6 and TMC8 proteins have emerged as the causal link between epidermodysplasia verruciformis (EV), the β-genus of human papillomaviruses (β-HPVs) and squamous cell carcinoma (SCC). These proteins, as yet without a well-defined molecular function, appear to critically control the interplay between viral homeostasis, humoral immune response and UV damage. This review summarizes what is known to date of these key components and explores our current understanding of this complex disease process.

Human Papillomaviruses

Papillomaviruses are small, non-enveloped DNA viruses with tropism for cutaneous or mucosal stratified squamous epithelium. Human papillomaviruses have 5 phylogenetic genera encompassing at least 120 genotypes.1 While genital HPV genotypes are grouped exclusively in the α-papillomavirus (α-HPV) genus, cutaneous HPVs demonstrate greater heterogeneity, belonging to alpha, beta, gamma, mu, and nu genera.2 HPV genotypes associated with epidermodysplasia verruciformis (EV-HPVs) constitute the β genus that is further distributed.
into 5 species, of which genotypes associated with epidermodysplasia verruciformis (EV-HPVs) mainly belong to the β1 (genotypes 5, 8, 14, 20, and 47) and β2 (genotype 17) species. HPV5 and HPV8 are the genotypes most frequently associated with the development of non-melanoma skin cancer (NMSC) and account for more than 90% of EV-associated cancers.

**HPV Life Cycle**

HPV genomes constitute less than 8000 base pairs, and can be separated into 2 coding regions, early (E) and late (L), as well as a non-coding regulatory region, also referred to as long control region or upstream regulatory region. The L1-protein is the major capsid protein and is relatively well conserved between HPV types. This ubiquitous HPV protein is the basis of prophylactic vaccines and is used for classification and phylogenetic analysis.

HPVs infect cells in the basal layer of stratified squamous epithelia, exposed during microabrasions or wounding. These basal cells are the only proliferating cells in normal epithelia, as differentiated cells in the suprabasal layers have exited the cell cycle. It is likely that cutaneous HPVs target the stem cells residing in the epidermal basal layer and the bulge of hair follicles. HPV genomes are amplified with replication of host cells, followed by capsid protein synthesis, virion assembly, and release. Infection results in the transient proliferation of keratinocytes that normally regress as a consequence of poorly understood cell-mediated innate and acquired immune responses. HPV DNA can persist after lesion regression, with viral latency attributed to low-titer virus infection. A summary of the viral life cycle is shown in Figure 1.

**HPV Malignant Transformation and Early Gene Involvement**

Failure of the immune system to clear persistent HPV infections can lead to the development of cancer over time. The transforming abilities of the high-risk cervical cancer genotypes HPV16 and 18 have been extensively studied. In both α- and β-HPV, the E6 and E7 oncoproteins are crucial for transformation, maintaining proliferative ability, establishing genomic instability and preventing the apoptosis of infected cells undergoing HPV-mediated transformation. E6 binds and mediates the degradation of the tumor suppressor gene p53, while E7 activates telomerase and inactivates retinoblastoma protein (pRb), preventing cell cycle inhibition. The culmination of these effects is cell immortalization, prevention of apoptosis and continuous replication of viral DNA.

Although the E6 and E7 of β-HPVs do not share the overall transforming potential of the high-risk α types, they still display oncogenic activities. HPV5 viral DNA does not integrate into host DNA, nor does its E6 degrade the p53 tumor suppressor
Epidermodysplasia Verruciformis

Epidermodysplasia verruciformis (EV) is a rare, autosomal recessive genodermatosis, first described by Lewandowsky and Lutz in 1922.26 EV is characterized by the increased susceptibility to specific HPV genotypes that are referred to as the EV-HPVs, including HPV3, 5, 8, 9, 10, 12, 14, 15, 17, 19–25, 28, 29, 36, 46, 47, 49, and 50.27 Viral infection in EV leads to tinea versicolor-like macules on the trunk, neck, arms, and face during early childhood. These benign forms are persistent, refractory and disseminated skin lesions resembling flat warts or presenting as macules of various colors. The HPV genotype determines the morphology of EV lesions and the risk of malignant conversion to cutaneous squamous cell carcinoma (CSCC).28 EV patients are typically infected with more than 1, and up to 10, EV-HPV genotypes, with HPV5 and HPV8 classified as the high risk genotypes most frequently associated with skin carcinomas.3

EV-HPVs are prevalent in the normal skin of healthy adults. Considered as commensal viruses of human skin, β-HPVs are ubiquitous viruses causing asymptomatic cutaneous infections in the general population.28,29 However, the quantity of β-HPV DNA found spread over the body is very low; even in SCC, viral load seldom reaches the level of 1 viral copy per cell, indicating that β-HPVs are not involved in the maintenance of the transformed state of tumor cells, unlike genital HPVs.30 Although antibodies to the L1 capsid protein of HPV5 and other EV-HPV genotypes is low in the general population,31 they are detected in most EV patients.32 Approximately 3 quarters of EV patients with CSCC were found to have developed antibodies to HPV5 E5 and/or E7 oncoproteins, suggesting that there is a defect in viral clearance prior to adaptive immune response in these patients.

Genetic Mutations Associated with EV

The familial occurrence of the disease led to the search for a putative EV gene. Homozygosity mapping led to the identification of a major susceptibility locus located in chromosomal region 17q25.3,34 Genetic analysis of the region revealed 2 novel adjacent genes, EVER1 (TM6c) and EVER2 (TM8c), encoding cytoplasmic proteins that colocalize with calnexin, an integral membrane protein in the endoplasmic reticulum (ER).35 Homozygous, invalidating mutations in either gene are found in roughly 75% of EV patients and confer susceptibility to EV.34,35 A second locus was subsequently identified in chromosomal region 2p21-p24,36 suggesting the nonallelic heterogeneity of the disease.

Several other EV-associated mutations have been identified and can be generally grouped into (1) mutations affecting the adaptive immune system, and (2) those that affect the host cell, specifically DNA repair/ excision systems. A variant of the xeroderma pigmentosum type B (XPB) gene (ERCC3) on 2q21 was found in an EV patient without mutations in TMC6 or TMC8.37 This gene encodes the excision repair cross-complementing rodent repair deficiency, complementation group 3 protein (ERCC3), a DNA helicase in nucleotide excision repair that is also a subunit of a class II transcription factor.38 MST1 (macrophage stimulating 1/hepatocyte growth factor-like, or STK4, serine/threonine kinase 4) deficiency was also found to underlie susceptibility to EV-HPV infections.39 Located on 20q11.2-q13.2, mutations in this gene lead to T-cell deficiencies, particularly naïve CD4+ and CD8+ T-cell lymphopenia.39 MST1 also negatively regulates proliferation and promotes differentiation through inactivation of the yes-associated protein (YAP), which normally inhibits the antiproliferative Notch transcriptional pathway40 that is negatively regulated by HPV8 and HPV17 E6.41 Another mutation in 4p13, encoding the ras homolog gene family member H (RHOH), was also recently shown to cause T cell defects and susceptibility to EV-HPV infections.42 RHOH encodes an atypical Rho GTPase expressed predominantly in
hematopoietic cells. A final case in an EV patient lacking TMC6 or TMC8 mutations identified defective apoptotic Fas function and a perforin (PRF1) missense mutation, which may contribute to decreased viral clearance through impaired T cell-mediated cytotoxicity. It is yet to be revealed whether TMC6 and TMC8 are specifically involved immunologically and/or oncogenically in the progression of EV and malignant transformation.

Immunological Aspects of EV

Although EV patients display abnormal susceptibility to a subset of HPV genotypes considered mostly harmless to the general population, they are not abnormally prone to additional bacterial, fungal, or viral infections, and humoral immunity appears to be preserved. This suggests a functional deficiency in the immune response specific to EV-HPVs, and/or to keratinocytes infected with these viruses. In support of this, EV has been classified as a primary immunodeficiency with specific defects in innate immunity. However, the specific cell-mediated immune responses toward EV HPVs or keratinocytes infected with these viruses remain obscure. Reduced peripheral blood mononuclear cell (PBMC)-mediated host cell lysis and attenuated natural cell-mediated cytotoxicity against HPV-infected keratinocytes have been previously reported to occur in these patients. T lymphocytes have also been shown to be unresponsive to autologous HPV-infected keratinocytes. In general, reports of impaired cell-mediated immunity (CMI) in several EV patients include reduced T-cell responsiveness to mitogens, and anergy to common skin antigens. Findings in regards to T-cell counts have been mixed, with some reporting decreased T-lymphocyte counts and CD4/CD8 ratios, while others find normal CD4+ and CD8+ counts and proliferative capacity in response to anti-CD3 stimulation. Mild T-cell abnormalities have been observed in TMC8-deficient EV, including an increase in memory, effector memory and skin-homing T-cell subsets, and a bias of TCR Vαβ and Vγδ repertoires. Controversy also exists over Langerhans cells, the major antigen-presenting cells in the epidermal layer, with several groups finding normal numbers and function, while others have shown a drastic reduction in EV patients. It is difficult, however, to determine whether these functional abnormalities are a primary effect in the pathogenesis of the disease, or secondary to chronic HPV infection.

The Development of EV in Immunosuppressed Patients

While the majority of medical literature concerns congenital EV occurring as a primary hereditary disease associated with HPV, in a minority of cases, EV is found in association with a state of impaired CMI. The term “acquired epidermodysplasia verruciformis” (AEV) has been introduced by Rogers and colleagues to describe this group. EV has been reported multiple times in the HIV-infected population, with lesions appearing clinically and histologically similar to that occurring in EV patients without generalized immunodeficiency and HPV detectable by polymerase chain reaction (PCR) testing. Compared with primary EV, however, EV-HIV patients appear to have lower rates of malignant transformation, possibly due to the rarity of EV in this population. EV and EV-like syndromes have also been described in organ transplant recipients and in systemic lupus erythematosus. Of note, immunosuppression per se is insufficient for the initiation and/or maintenance of EV-HPV pathogenesis or oncogenesis, as there is a lack of persistent cutaneous lesions with high levels of EV-HPV DNA replication among patients with genetic, acquired, or iatrogenic depression of CMI.

Cutaneous Squamous Cell carcinoma and EV

In the fourth or fifth decade of life, roughly half of affected EV patients will develop SCC, primarily Bowen’s type carcinoma in situ or invasive SCC, occurring mainly on sun-exposed areas. SCC is 1 of the 2 major types of NMSC, which is more common than lung, breast, prostate, and colon cancers combined, with a rate reported to be increasing by 4–8% yearly. In comparison to benign lesions, malignant lesions can be more closely described as verruca-like papillomatous lesions or seborrheic keratosis-like lesions. β-HPV types are more common than other HPV types in NMSC, but only a subset of β-HPVs is typically associated with malignant conversion, namely, HPV5, as well as occasionally HPV8, 14, 17, 20, or 47.

UV Exposure as a Co-factor in the Development of EV-SCC

UV radiation (UVR), an environmental carcinogen, plays an important role in the induction of SCC in EV patients. Indeed, lymphocytes from EV patients challenged with UVR, but not gamma rays, showed more chromosomal aberrations than controls. Solar UVR, consisting of UVC (190–280 nm), UVB (280–320 nm), and UVA (320–400 nm), is the major risk factor for the development of NMSC in the general population, acting through both immunosuppressive and mutagenic effects. UVB-specific mutations in p53 are seen in the majority of SCCs, with signature UV-induced C→T mutations of the p53 gene and abnormal p53 protein expression also associated with EV tumor progression. Purdie and colleagues first demonstrated UV-induced HPV77 promoter activation in cutaneous lesions from organ transplant recipients, and confirmed the UVB modulation of β-HPV promoter activity for several virus types and p53-response elements. In addition to damaging keratinocyte DNA, UVR also suppresses the skin’s immune system by inhibiting dendritic cells of the skin via the immunosuppressive cytokine interleukin-10 (IL-10). UVR also inhibits apoptosis of UV-damaged cells by downregulating Fas ligand (FasL, CD95L) and TNF-related apoptosis inducing ligand (TRAIL) expression in keratinocytes.
HPV oncoproteins are also capable of preventing the apoptosis of UV-damaged cells in SCCs through proteolysis of the pro-apoptotic regulator BAK, inhibition of apoptosis inducing factor (AIF) release, and homeodomain-interacting protein kinase-2 (HIPK2)-mediated p53 phosphorylation. Furthermore, HPV oncoproteins are also capable of preventing the apoptosis of UV-damaged cells through downregulation of FasL and TRAIL. It also induces immunosuppressive IL-10 expression, which inhibits dendritic cells in the skin, suppressing the skin's immune system. UV-induced proinflammatory cytokines and p53 expression modulate the promoter activity of a number of HPV types. HPV further prevents DNA damage repair by promoting degradation of p300, inhibition of XRCC1 and a reduction in ATR. HPVs also limit apoptosis through proteolytic degradation of pro-apoptotic Bak and upregulation of osteoprotegerin and IL-6. Over time, these interactions result in the accumulation and propagation of somatic mutations, and ultimately, tumorigenesis. FasL: Fas ligand; CD95 ligand, TRAIL: tumor necrosis factor (TNF)-related apoptosis inducing ligand, IL-10: interleukin 10, XRCC1: X-ray repair cross-complementing protein 1, ATR: ataxia telangiectasia-mutated (ATM) and Rad3-related kinase.

**Transmembrane Channel-like (TMC) Family**

The mammalian transmembrane channel-like (TMC) gene family is comprised of 8 genes, TMC1 to 8, with TMC6 equivalent to the locus referred to as EVER1 and TMC8 also known as EVER2. Although none of the mammalian TMC genes share significant sequence similarity to any other genes or motifs, they each encode a conserved 120 amino acid domain termed the TMC domain. The TMC family is grouped into 3 subfamilies, A, B, and C, distinguished on the basis of sequence homology and structural similarities between their respective genes. Subfamily A consists of TMC1, TMC2, and TMC3; subfamily B consists of TMC5 and TMC6, and subfamily C consists of TMC4, TMC7, and TMC8. The TMC genes map to 6 chromosomal locations in both human and mouse, and have also been found in other vertebrates and invertebrates, including zebra fish, nematode, pufferfish, C. elegans, mosquito, and D. melanogaster. So far, no evidence for TMC gene expression has been found among fungi or plants.

The TMC gene family was discovered through positional cloning of the gene underlying both dominant and recessive nonsyndromic sensorineural hearing loss at the DFNA36 and DFNB7/B11 loci, respectively, in chromosomal region 9q13-q21. These allelic disorders are caused by mutation of TMC1, the mouse ortholog also exhibiting dominant and recessive mutant alleles that cause hearing loss in the Beethoven (Bth) and deafness (dn) mutant mouse strains, respectively. Tmc4 through Tmc8 are expressed in most murine organs, while Tmc1 and Tmc3 mRNAs are detectable in most neuronal organs, as well as some non-neuronal organs. Tmc2 transcripts are detected in testis, and Tmc8 mRNA is detected in thymus, spleen and lung.

The TMC genes are predicted to encode proteins with at least 6 conserved transmembrane domains, raising the possibility that TMC proteins may function as ion channels, pumps, or transporters. Recently, TMC proteins were suggested to be evolutionarily related to the anoctamin (ANO) family of calcium-activated chloride channels, sharing conserved amino acid residues in several transmembrane spanning regions. The cochlear electrophysiologic phenotype of dn mutant mice is consistent with an ion channel defect, and tmc1 has been shown to encode a sodium-sensitive channel required for salt chemosensory gene in C. elegans. Furthermore, Tmc1 and Tmc2 were recently suggested to be essential for targeting and interacting with, but not constituting, mechanotransducer (MT) channels in cochlear hair cells. They are thought to serve as protein partners in a transduction complex necessary for MT conductance and calcium permeability.
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TMC Protein Structure

TMC proteins are predicted to contain 6 to 10 membrane-spanning domains, without N-terminal signal peptide sequences or trafficking signals. All of the proteins are predicted to reside in the plasma membrane, with the C-termini of all but TMC6 predicted to be cytoplasmic. Numerous posttranslational modification sites have been predicted in each human and mouse amino acid sequence for amidation, glycosylation, and myriostylation, as well as phosphorylation. There is a high degree of conservation between human and murine TMC proteins (75–96%).

Although predicted to be an ion channel protein, TMC8 is predicted to share structural similarities with subunit 1 of Cytochrome Bα3 Oxidase from *Thermus thermophilus* (2QPE) when using unbiased protein homology modeling. 2QPE belongs to the heme-copper oxidase I superfamily of transmembrane protein complexes located in the respiratory chains of prokaryotes and mitochondria that catalyze the reduction of O₂ and simultaneously pump protons across the membrane. This homology may suggest a similar bimetallic core and/or redox function for TMC8. In support of the latter, TMC8 possesses a CXXC motif, a well-characterized redox-active disulfide sequence that may serve as a reduction state molecular switch.

TMC6 and TMC8 Protection from EV-HPV Infection or Oncogenesis

Although the link between TMC6 and TMC8 mutations and EV has now been well established, the molecular role(s) of TMCs in controlling the progression of the disease remains unclear. Nonetheless, we hypothesize that these TMC proteins work to protect healthy individuals from EV-HPV infection and/or oncogenesis via one or more of the following mechanisms: (1) disruption of the HPV life cycle through host cell cycle regulation, (2) regulation of T-cell-mediated viral clearance, or (3) control of the apoptosis/survival balance in host keratinocytes, especially during UV-induced DNA damage.

TMC6 and 8 Modulation of the HPV Life Cycle

Cellular proliferation is an important part of HPV maintenance, with evidence that viral oncoproteins manipulate host cellular proteins to maintain cell replication and/or for quiescent cells to re-enter the cell cycle. TMC8 may influence cell cycle homeostasis, as keratinocytes with a mutated TMC8 grow at a greater rate than wild-type keratinocytes. TMC6 and TMC8 proteins have been shown to form complexes and interact with zinc transporter 1 (ZnT-1), suppressing the activation of the AP-1, Elk-1, and Fos transcription factors. This may have a pivotal effect on the HPV life cycle, as there are multiple AP-1 binding sites within the LCR of the HPV genome that enhance E6/E7 expression.

In keratinocytes, the TMC/ZnT-1 complex also modulated intracellular zinc (Zn²⁺).
distribution, possibly affecting several physiological processes including the host-virus interaction, but none specifically demonstrated. The E5 oncoprotein of HPV16, a high-risk cervical HPV type, has been shown to bind the TMC/ZnT-1 complex and prevent its inhibition of AP-1 transcriptional activity, which may explain how genital HPVs such as HPV16 overcome the protective effects of TMC6 and 8. However, b-, g-, and m-HPVs are reported to lack the E5 ORF that is present in the genomes of genital HPVs, and may account for the asymptomatic natures and limited replication of these HPV types in healthy individuals. It is proposed that in EV patients infected with E5-lacking β-HPVs, mutations in TMC6 or TMC8 prevent ZnT-1 complex formation, making AP-1 and Zn$^{2+}$ levels sufficient for viral transcription. However, it is unclear why EV patients with TMC6 or TMC8 mutations are not more prone to infection with genital HPV types.

### TMC6 and 8 Regulation of Immune Function

The skin is the largest organ in area, containing multiple immunocompetent cells that contribute to cutaneous immunity against environmental microbes and other foreign materials. TMC8 protein expression was found to be much greater in human skin compared with other tissues tested (Fig. 4), suggesting an important yet undefined role in cutaneous immune function. Clinical observations indirectly suggest that the protective role of TMC6 or 8 may not be limited to the epidermis, but may also involve the adaptive immune system. It has been hypothesized that mutations in $TMC6$ or 8 downregulate CMI by decreasing the ability of cells to present EV-HPV antigen-derived peptides to T-lymphocytes triggering the clearance of HPV-infected keratinocytes. $TMC6$ and 8 genes are abundantly expressed in murine and human T cells, and activation of CD4$^+$ and CD8$^+$ T cells via the T-cell receptor (TCR) was found to trigger a rapid decrease in $TMC6$ and $TMC8$ expression, accompanied by an accumulation of free zinc ions. Interestingly, lymphoblastoid and primary T cells from TMC8-deficient patients show similarly elevated Zn$^{2+}$ levels. The same study also showed that an excess of Zn$^{2+}$ blocked T-cell activation and proliferation. However, a more recent study showed that TMC8-deficiency does not seem to significantly impair T-cell development or function, suggesting that the maintenance of Zn$^{2+}$ homeostasis by TMC proteins is not essential for their normal development or activation. Thus, while TMC6 and 8 may be involved in the regulation of cellular zinc ion homeostasis in lymphocytes, this role is not essential for normal T-cell function.

### TMC6 and 8 Control of Host Keratinocyte Apoptosis/Survival Balance

TMC8 was shown to sensitize cells to tumor necrosis factor α (TNFα)-induced apoptosis, through nuclear factor kappa B
(NF-κB) activation and formation of the pro-apoptotic complex II, consisting of receptor-interacting serine/threonine protein kinase 1 (RIPK1), Fas-associated protein with death domain (FADD) and caspase-8.\textsuperscript{108} All such effects were independent of Fas ligand (FasL) stimulation. Importantly, TNFα-induced cell death was mediated by the interaction of the TMC domain of TMC8 with TRADD.\textsuperscript{108} Furthermore, the authors showed that death was mediated by the interaction of the TMC domain of Fas ligand (FasL) stimulation. Importantly, TNFα (NF-κB) induces cell death and the authors showed that TMC8 staining of human skin.

Conclusions

SCC development in EV-HPV patients is a multi-step process, requiring the coordinated targeting of multiple cellular signaling pathways by HPV oncoproteins to maintain infected cells in a proliferative state thereby facilitating viral replication and persistence. It is clear that TMC6 and TMC8 proteins serve as fundamental regulators of HPV-HPV persistence and oncogenicity, yet their specific molecular functions in EV and SCC remain unclear. The recent discoveries of several non-TMC mutations associated with EV have revealed functional deficiencies in base excision repair, T cells, keratinocyte proliferation/differentiation, and apoptosis. It is tempting to speculate that TMC proteins may share some overlapping functionalities or phenotypes since mutations in TMC6 and 8 also result in EV pathology. In addition to investigating these possibilities, characterization of a TMC8 transgenic mouse model, functional analyses of TMC binding partners and electrophysiological analysis should begin to shed further light on the means by which these proteins protect against HPV-EV. Such approaches may facilitate a paradigm shift in our understanding of this primary immunodeficiency and the function of these proteins in response to viral infection.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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