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Review

p63 in tooth development

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ABSTRACT

Recent findings have shown that the development of teeth involves a complex sequence of molecular events in which the p53 family member p63 is involved. Indeed, mice lacking p63 do not have teeth and humans bearing mutations in p63 suffer developmental syndromes that affect tooth morphology and number. Several isoforms of p63 have been described: the use of two different promoters produces longer TAp63 isoforms, or shorter, 5′ truncated isoforms known as ΔNp63. The 3′ end of primary transcripts is then subject to alternative splicing resulting in three additional isoforms: alpha (α), beta (β) and gamma (γ). Tooth development relies mainly on the activity of the N-terminally truncated ΔNp63 isoforms. Here we review the experimental evidence for the involvement of ΔNp63 in tooth development through its ability to sustain the molecular signalling that orchestrates epithelial–mesenchymal interaction.

Contents

1. Tooth development

Teeth develop after a series of tightly regulated, sequential steps. At embryonic day 11.5 (E11.5) thickening of the dental epithelium starts to form the dental lamina or dental placode, the first stage in tooth development. Subsequently, the lamina invaginates into the underlining neural crest derived mesenchyme. At E13.5 this invagination assumes a "bud" conformation, surrounded by condensed mesenchyme. During the following 24 h, the developing tooth matures into the cap stage, with the dental epithelium folding to embrace the mesenchyme. In this structure, the epithelium above the mesenchyme assumes a button-like morphology known as the enamel knot. The further shaping of the tooth germ results from asymmetric proliferation: cells outside the enamel knot actively proliferate, while inner cells cease to proliferate and thus act like an anchor to enforce moulding of the cap stage. This asymmetric proliferation also splits the enamel knot into the inner and outer enamel knots: the mesenchyme adjacent to the inner knot will form the dental papilla, whereas that on the outside will develop into the dental follicle. Incisor teeth assume a conical shape at this stage and lose their enamel knot at the end of the cap stage. Molars have a more complex destiny: after loss of the first enamel knot, they develop secondary enamel knots, which shape the tooth into a multicuspid "bell" stage (E16.5). Finally, deposition of enamel by the epithelial-derived ameloblasts and dentin by the mesenchyme-derived odontoblasts lead to the final mature tooth (E20); for details see Fig. 1A.

Like other epithelial appendages, dental development relies on a tight molecular cross-talk between the mesenchyme and the

Abbreviations: BMP, bone morphogenetic protein; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; SHH, sonic hedgehog; TNF, tumor necrosis factor; Apc, adenomatous polyposis coli; SAM, sterile alpha motif; TID, transactivation inhibitory domain; ABBP1, apobec-1-binding protein-1.

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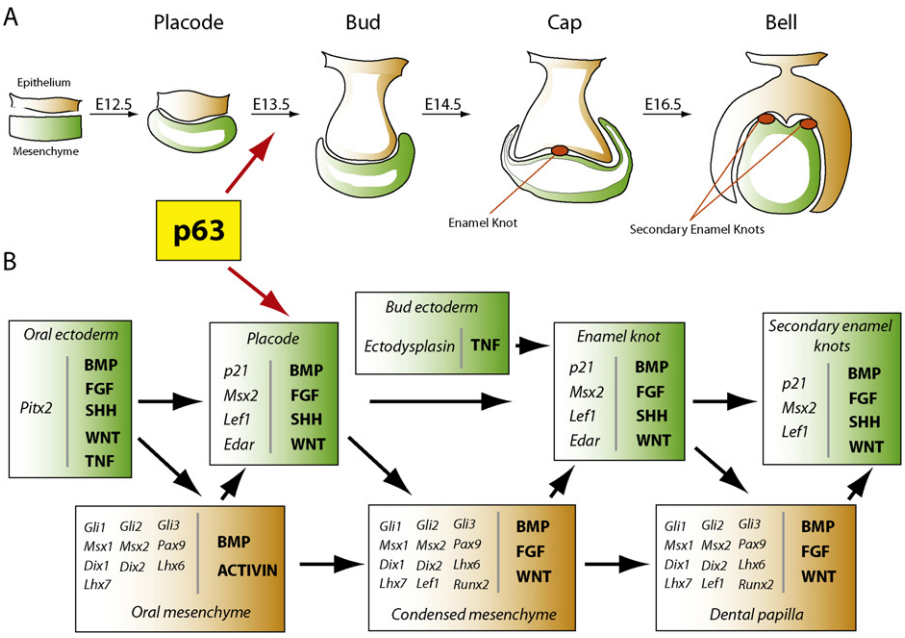

Fig. 1. A: Scheme of tooth development: the epithelium (orange) thickens and invaginates the underlying, condensing mesenchyme (green); this results in the formation of a dental placode at E12.5, which further evolves into the bud stage (E13.5). Subsequently, during the cap stage, the primary enamel knot appears as a "bulge-like" derived epithelium. Finally, by E16.5, the epithelium has further deepened into the mesenchyme assuming a "bell" shape. Secondary enamel knots substitute the primary knot and dictate the future distribution of the cuspids in the mature tooth. p63 affects tooth morphogenesis in its early stages, as mice depleted of p63 do not progress beyond the dental placode or rudimentary bud stage. B: The main signalling pathways (bold characters) and single genes orchestrating epithelial–mesenchymal interaction are shown during the different tooth developmental stages. In addition, the intraepidermal signalling between Ectodysplasin and Edar (TNF) involved in the formation of the enamel knot is shown. p63 expression in the oral epithelium sustains the FGF and BMP signalling necessary for tooth morphogenesis.
Modified from Thesleff and Tummers "Tooth organogenesis and regeneration" (Stembook; available online at http://www.stembook.org/node/551).

epithelium. In other words, molecular signals arising from the mesenchyme instruct the morphological and functional changes in tooth epithelium and vice versa. Several signalling pathways mediate epithelial–mesenchymal interaction, including BMP, FGF, WNT, SHH, Notch and TNF [1–3]. For example, inactivation of the FGF receptor FRGR2b arrests tooth development at the bud stage [4], while depletion of FGF8 results in arrest at the lamina stage [5]. Similarly, mesenchyme-derived BMP4 plays a critical role in the formation of the enamel knot. Conditional depletion of the receptor bmpr1a in epithelial tissue abolishes BMP signalling and tooth development does not proceed beyond the bud stage [6]. BMP4 also induces the synthesis of its own antagonist, *ectodin*, that dampens its activity [7]. This negative feedback loop is finely tuned, and ectodin null animals, which experience hyperactive BMP signalling, present enlarged enamel knots, cuspal defects and supernumerary teeth [8]. Manipulation of WNT signalling has a similar impact on tooth morphogenesis. Adenomatous polyposis coli (APC) negatively regulates WNT signalling, promoting degradation of β-catenin, and its conditional depletion in the oral epithelium triggers supernumerary teeth [9], a phenotype resembling mice over-expressing β-catenin [10]. Recently, alterations in the enamel knot, additional cuspids in molars and defective enamel deposition have been described in mice carrying functional inactivation of the Notch ligand Jagged-2 (Jag2) [11]. Although most signals mediate interaction between mesenchyme and epithelium, sometimes they also act within different compartments of the same tissue. For example, Edar (a receptor of the TNF superfamily) is expressed in the enamel knot and its ligand Ectodysplasin, which supports the formation of mature enamel knot through Edar signalling, is secreted by the surrounding epithelium [12–14], outlined in Fig. 1B.

2. The p63 gene

p63 belongs to the p53 family of tetrameric transcription factors, together with p53 and p73 [15–17]. All three genes produce multiple isoforms, due to alternative promoter usage and differential C-terminal splicing. In the case of p63, two promoters are present at the 5′-end of the gene. Transcription from the P1 promoter produces longer TAp63 isoforms, whereas transcription started from the downstream P2 promoter results in shorter, 5′ truncated isoforms known as ΔNp63. The 3′ end of primary transcripts is then subject to alternative splicing giving rise to three additional isoforms: alpha (α), beta (β) and gamma (γ). TA isoforms contain a fully functional transactivation domain, a core DNA binding domain and an oligomerization domain [15]. N-terminal truncated proteins lack the transactivation domain. Initially, this finding fostered the notion that N-terminal truncated isoforms were transcriptionally inactive and acted as a dominant negatives, either forming heterotetramers or via competition for DNA binding. This view has been challenged by the identification of a second transactivation domain (TA2) located downstream of the DNA binding domain [18] and by the description of putative transactivation activity in the first 26 amino acids of ΔNp63 [19]. Indeed, several studies have shown the ability of ΔNp63 to transactivate target genes [19–21]. The C-terminal alpha isoforms, which are the most abundantly expressed, bear a SAM domain that mediates protein–protein interactions, together with a C-terminal Transcriptional Inhibitory Domain (TID), which mediates an intramolecular inhibition of the N-terminal transcriptional activity. Indeed, the C-terminal region of TAp63α folds over and binds to the transactivation domain, resulting in a closed conformation that dampens TAp63 transcriptional activity [22,23]; see Fig. 2 for details.

The first knockout mouse models engineered to study the function of p63 *in vivo* revealed a fundamental role for p63 in epidermal development [24,25]. p63 null mice die shortly after birth and show severe developmental defects, including lack of skin, limbs and epidermal appendages. The lack of skin has been interpreted either as a lack of proper stratification and commitment of epidermal embryonic precursors [24], or as a failure in the

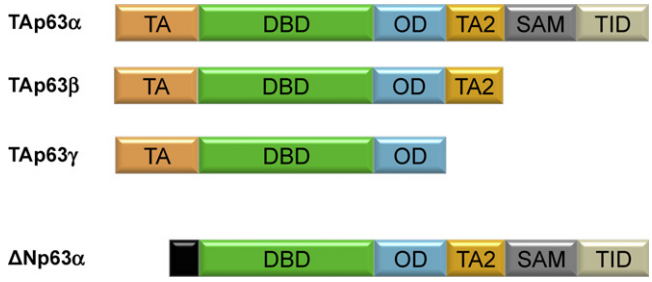

Fig. 2. p63, shown here, and its siblings p53 and p73 (not shown) are expressed as full length (TAp63) or N-terminal truncated (ΔNp63) isoforms, with (TAp63) or without (ΔNp63) the transactivation domain (TA), and therefore with distinct transcriptional properties. Differential splicing affecting the C-terminus creates three main p63 variants: alpha (α), beta (β) and gamma (γ). This occurs with both TAp63 and ΔNp63 isoforms, even though the figure reports only the ΔNp63α isoform for simplicity. The p63 isoforms share a common modular structure, with a transactivation domain (TA), replaced in ΔNp63 by a region of 26 amino acids, a DNA binding domain (DBD), an oligomerization domain (OD) and a second transactivation domain (TA2). In addition, the longest α isoforms contain a sterile alpha motif (SAM) domain, involved in as yet uncharacterized protein–protein interactions, and a transactivation inhibitory domain (TID), able to inhibit *in*-cis the transcriptional activity of TAp63. The SAM domain and the TID are absent in the β and γ isoforms.

maintenance of the full repertoire of stem cell function, despite normal commitment and differentiation capabilities [25]. However, data from several sources favour the hypothesis that p63 expression in epithelia is a determinant of the homeostasis of the stem cell niche and of the proper proliferation of committed precursors, although there remains an ongoing conflict of opinion [26–28]. This stem cell maintenance property has been attributed to the ΔNp63α, which is the most abundantly expressed isoform in proliferating compartments of epidermal tissues. On the other hand, the role of TAp63 isoforms in specifying epidermal development has been controversial. Initially, TAp63 was postulated to promote skin stratification [29] and/or to trigger differentiation of suprabasal committed keratinocytes [20,30], but data from TAp63 selective knockout models reveal a more complex picture. A mouse model developed in McKeon's lab did not show any evident morphological defects and lacked the severe developmental abnormalities triggered by concomitant depletion of ΔNp63 [31]. Strikingly, a selective TAp63 null mouse developed independently in Flores's lab showed a significant phenotype: following normal development, TAp63 null mice age prematurely and develop blisters, skin ulcerations, senescence of hair follicle-associated dermal and epidermal cells, and alopecia. These disorders are due to defects in dermal and epidermal precursors, which show defective proliferation, senescence, and widespread genomic instability. Thus, according to these data, TAp63 may also serve to maintain skin stem cells [32].

3. p63 is required for tooth development

Most of the work linking p63 to dental development has been carried out in mice. Indeed, the fundamental role of p63 in dental development was established in 1999, when the phenotypical characterization of p63 null animals revealed the absence of teeth [24,25]. Later, more detailed studies described p63 expression at different dental stages and started to characterize the p63 regulators and the p63 downstream targets [33,34] (Fig. 3). During mouse embryonic development, p63 is expressed at E10 in the epithelium of the dental lamina, and its epidermal expression remains robust throughout the bud and cap stages. During the bell stage of molar development p63 is mainly detectable in the outer enamel epithelium, compared to weak expression in the inner knot. Importantly, similarly to the basal layer of the skin, only ΔNp63α has been reported in the developing tooth, while no expression of TAp63 was detected [34]. On the other hand, p63 positive epithelial cells stained also for the proliferative marker Ki67, indicating that the proliferative function of ΔNp63 is conserved in dental epithelium. In E18.5 incisors, weak p63 positivity has been reported in mature differentiated ameloblasts, but its function remains unknown [33]. As aforementioned, analysis of tooth development in p63 null mice revealed absence of teeth [24,25,33,34]. E11 p63 null embryos form a dental lamina, which never progresses to a cap stage, but eventually regresses during embryonic development [34]. Only rare, rudimentary buds have been described in mutant animals [34], indicating that p63

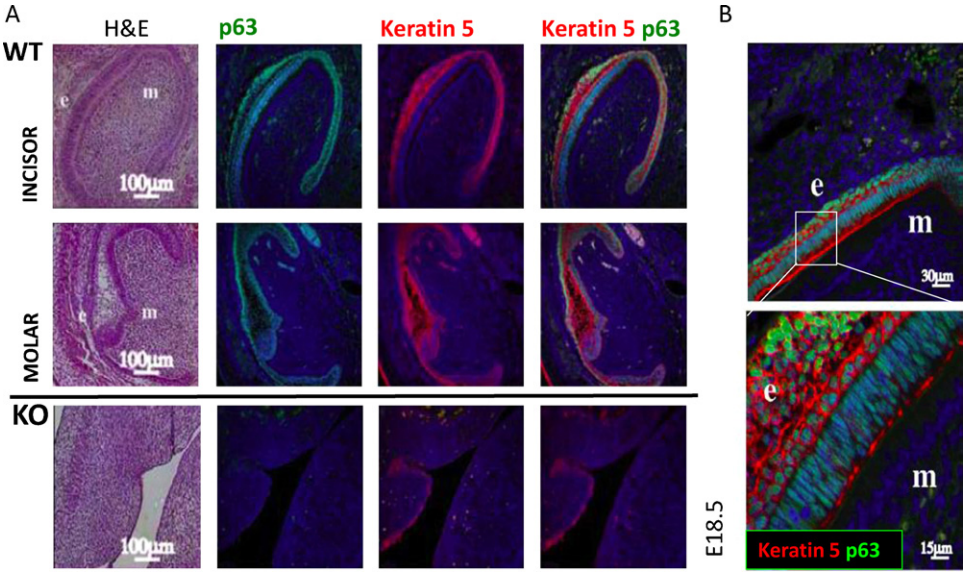

Fig. 3. p63 is essential for tooth development. (A) Immunofluorescence of p63 (green) expression in the epithelium of wild-type (WT) incisor and molar (upper rows) at E18.5 (Bell stage). The epithelium is stained with the epithelial marker keratin-5 (red). The absence of tooth development in p63 null mice (KO) is evident in the lower row. (B) A more detailed picture shows p63 and keratin-5 colocalization in the incisor epithelium. At higher magnification the lower levels of p63 can be seen in the more mature ameloblasts, recognized by their elongated shape. e = epithelium and m = mesenchyme.
Modified from Rufini et al. [33].

expression is necessary at the very first stages of tooth development; see Fig. 3.

The molecular mechanism(s) underlining p63 function in tooth development is still poorly defined. Nonetheless, studies performed on mouse models with regards to epithelial, thymus or dental development have unveiled some p63 regulators and targets and may allow some speculation on how p63 affects tooth morphogenesis.

Few upstream regulators of p63 are known. Intriguingly, during tooth development, p63 is expressed independently of mesenchyme. Indeed, isolated dental epithelium maintains detectable levels of p63, despite the absence of mesenchymal signalling. p63 expression in these *in vitro* explants is boosted by administration of BMP2 and 7, suggesting a positive role for BMP signalling in promoting p63 expression in dental epithelium [34,35], shown in Fig. 4.

Functionally, p63 regulates expression of several targets involved in dental morphogenesis (Fig. 4). Firstly, p63 influences FGF signalling through regulation of FGF3 and FGF10 FGFR2 [34,36–39]. FGFR2 is subject to alternative splicing, producing epithelial (b isoforms) and mesenchymal (c isoforms) variants. Indeed, epithelial cells preferentially support splicing of the K-SAMIIIα exon leading to FGFR-2-SAM (FGFR2b) expression, whereas in mesenchymal cells splicing of the BEK IIIβ exon leads to expression of the FGFR-2-BEK isoform (FGFR2c). It is noteworthy, that some FGF proteins involved in tooth development, such as FGF3, FGF7 and FGF10 signal exclusively through FGFR2b, highlighting the importance of proper compartmentalized expression of the receptor. The control of the alternative splicing of FGFR2 depends on the activity of apobec-1-binding protein-1 (ABBP1) and p63. Indeed, in the absence of p63, ABBP1 sustains the splicing of the mesenchymal isoform, but its activity is inhibited by direct binding with p63α, thus shifting the balance towards the alternative splice form FGFR2b [36]. As a consequence, whereas developing dental epithelium of WT animals expresses robust levels of FGFR2b, p63 null embryos fail to do so, leading to a severe impairment of FGF signalling in the absence of p63 [34]. Importantly, functional inactivation of FGFR2b arrests tooth formation at the bud stage [4], leading to the possibility that

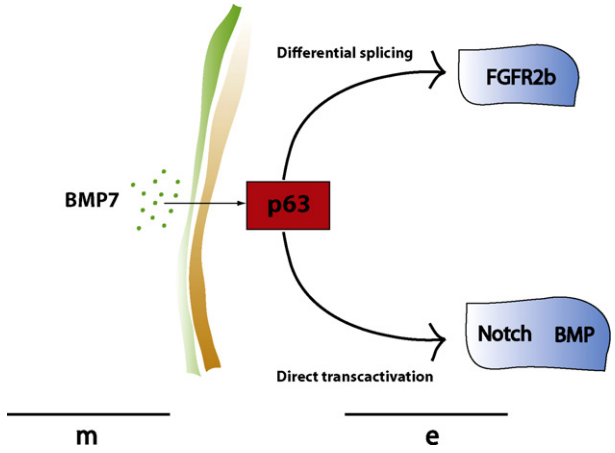

Fig. 4. The scheme shows the hypothetical signalling network that dictates the role of p63 in tooth morphogenesis. Although p63 is expressed independently of mesenchyme signalling, mesenchymal-derived BMP7 is able to increase p63 expression, which could be responsible for sustaining p63 levels in the dental epithelium. In turn, p63 is a positive regulator of fundamental signalling pathways such FGF, BMP and Notch. Indeed, p63 directly upregulates the mRNA of BMP7, Notch1 and Jagged1. In addition, it is able to direct the alternative splicing of FGFR2 towards the epithelial isoform (FGFR2b). Thus, given the fundamental role of these pathways in tooth development, it is not far-fetched to speculate that they function as downstream effectors of p63. Future studies are needed to fully address this possibility. e = epithelium and m = mesenchyme.

p63 mediated regulation of FGFR2 may be pivotal for tooth development.

Epithelia of p63 null mice also show an absence of Notch1 and reduced levels of its ligand, Jag1 [33]. p63 has been consistently reported to cross-talk with Notch1 signalling in keratinocytes and thymic epithelium [37,40,41]. This interaction is complex, as Notch is known to repress p63 signalling and p63 has been reported to either activate or dampen Notch signalling [41]. It is unclear if Notch signals to p63 during tooth development, but it has been shown that ΔNp63 does bind a p53 responsive element in the Notch1 promoter and Notch transcripts are lost in p63-depleted epithelial cells [34]. These data suggest that ΔNp63 could be a pivotal, positive regulator of Notch1 expression during tooth development.

Another downstream target of p63 is BMP7, whose expression in mouse oral epithelium is lost upon depletion of p63 [34]. This may have a decisive impact on tooth development, consistent with recent findings showing that genetic depletion of BMP7 in mouse severely affects tooth morphogenesis [42]. BMP7 can also positively regulate p63 expression during dental development, suggesting a possible positive feedback loop, although experimental evidence supporting this possibility is currently missing.

As mentioned, the findings linking p63 to tooth development have largely been obtained from mouse models, but there is evidence for a comparable role for the human gene. Indeed, point mutations of p63 gene cause several developmental disorders in man: ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC), ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC), limb mammary syndrome (LMS), acro-dermato-ungual-lacrimal-tooth syndrome (ADULT), Rapp-Hodgkin syndrome (RHS) and split hand/foot malformation (SHFM) [43,44]. Some of these syndromes impact on tooth development. ADULT syndrome was first described in 1993 [43] and affected individuals suffer a wide array of developmental defects including hypodontia and abnormally conical-shaped teeth [45]. To date only a few families have been diagnosed with ADULT syndrome and patients bear a dominant point mutation in exon 8 of the p63 gene, which changes an arginine residue (R268) into glycine or glutamine. Importantly, R268 substitution is a gain-of-function mutation, which potentiates the otherwise negligible ΔNp63γ transcriptional activity [18]. LMS is very similar to ADULT, with both disorders leading to hypoplasia of mammary glands. In addition, one third of patients affected by LMS show dental defects and hypodontia. The mutations reported in LMS patients fall within the putative ΔNp63 TA2 domain, between the TA2 and DBD domains or are located at the C-terminus of the protein, in which case they affect only the alpha isoforms, causing a frame shift and a premature stop codon [44].

The prototype p63-related syndrome is the EEC syndrome, which is characterized by ectodermal dysplasia affecting mainly skin. Nonetheless cases of hypodontia or even anodontia have been described, together with defective enamel deposition and consequent susceptibility to caries [43].

4. Conclusion and future perspectives

In addition to the already reported regulations and functions of p63 [46–50], the data discussed here propose p63 as a pivotal, master gene in the control of tooth development, particularly since p63-null mice show anodontia. The transcriptional profile of p63 also reveals its ability to regulate several genes that are relevant for the embryonic formation of teeth. However, other targets need to be characterized in the context of tooth morphogenesis. In addition, the cause of the early arrest in dental development upon p63 depletion is still ill defined. The harsh phenotype of p63 null animals shows that p63 expression is necessary at the very

first stage of the dental placode, but leaves open the question whether it is also required at later stages, as suggested by the sustained expression of p63 throughout the dental stages. Interesting insights could come from conditional KO of p63 at different phases of tooth development, as well as from the engineering of mouse models able to mimic human diseases. The latter would be greatly beneficial tools, as little is known about the molecular mechanisms that govern the syndromes triggered by human p63 mutations.

The full identification and characterization of stem cells with odontogenic potential can arguably be considered one of the major challenges awaiting further investigation. The ability of p63 to maintain the stem cell repertoire has been demonstrated in different settings [26,27,28,32], but this stem cell role has not been clearly addressed during tooth morphogenesis. In our opinion, efforts in this direction are necessary, as p63 could be a master regulator of the stem cell properties of dental precursor cells. Identifying and dissecting this role of p63 will turn out to be pivotal for any aspirations towards regenerative medicine in dentistry.

Intimately linked to stem cell maintenance is the ability of p63 to affect symmetry in cell division. This property of p63 was first described in the skin [51]: the proliferative cells of the basal layer of the skin can divide symmetrically, thus generating two proliferating basal daughter cells, or asymmetrically to generate a basal cell and a suprabasal cell that will withdraw from the cell cycle and enter the differentiation stage to become part of the cornified envelope. Strikingly, in the absence of p63 the asymmetric division is lost and skin cells only manage to divide symmetrically. This function of p63 may well be conserved in tooth development and could be important, especially during the late stages of molar formation, to help achieve the final tooth shape. It is noteworthy that many p63-related human syndromes show defective tooth shape.

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