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Inactivation of *Itf2* promotes intestinal tumorigenesis in $Apc^{Min/+}$ mice

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ABSTRACT

Deregulation of Wnt/β-catenin signaling following inactivation of the adenomatous polyposis coli (*APC*) tumor suppressor gene is frequently found in colorectal cancer. We have previously shown that levels of ITF-2B, encoded by the β-catenin target gene *ITF2* that is located on the tumor suppressor gene locus 18q21, are increased in colonic adenomas with deregulated β-catenin activity. However, during tumor progression ITF-2B levels are reduced, suggesting that ITF-2B interferes with tumor development. To investigate the role of *ITF2* in intestinal tumorigenesis, we specifically inactivated *Itf2* in the intestinal epithelium of $Apc^{Min/+}$ mice. We found that genetic disruption of *Itf2* on the $Apc^{Min/+}$ background results in earlier death and a significant increase in tumor number and size in the small intestine. Based on these data *Itf2* acts as a tumor suppressor gene of the intestinal tract that inhibits tumor initiation and growth.

1. Introduction

Colorectal cancer is a genetic disease that develops from adenomatous precursor lesions by the accumulation of multiple independent somatic alterations in oncogenes and tumor suppressor genes [1]. Inactivating mutations of the *APC* tumor suppressor gene are viewed as an early event in up to 80% of human sporadic colorectal cancers and have been implicated in the development of hundreds of adenomas mainly in the colon in hereditary familial adenomatous polyposis (FAP) [2–4]. Loss of APC function leads to deregulation of the Wnt/β-catenin signaling cascade, resulting in stabilization and nuclear translocation of the protein β-catenin (reviewed in Ref. [1]). Subsequently, β-catenin modulates the transcription of its target genes. The gene *ITF2* alias *TCF4* encoding the basic helix-loop-helix protein (bHLH) and transcription factor ITF-2B has been identified as a β-catenin target gene [5]. Previously, we have demonstrated that deregulated activity of the protein β-catenin induces the transcription of the *ITF2* gene in colonic adenomas. However, during tumor progression, ITF-2B protein levels are frequently reduced due to loss of heterozygosity on chromosome 18q as well as deacetylation of the *ITF2* promoter, suggesting that loss of *ITF2* function is necessary for tumor development [6].

The $Apc^{Min/+}$ mouse is a widely used mouse model for the study of colorectal carcinogenesis. $Apc^{Min/+}$ mice carry an inactivating mutation in one allele of the *Apc* tumor suppressor gene [7]. Somatic loss of the remaining *Apc* wild-type allele leads to deregulation of the Wnt signaling pathway, resulting in the development of a multitude of tumors throughout the whole intestinal tract [8]. The majority of these neoplastic lesions is distributed to the small intestine and only few occur in the colon [9–11]. In $Apc^{Min/+}$ mice all intestinal tumors are benign adenomas. Progression to adenocarcinoma is very rare and may occasionally be observed in older animals. $Apc^{Min/+}$ mice die as a consequence of secondary effects of tumor growth, mainly intestinal bleeding and obstruction caused by tumors [9,11].

The aim of the present study was to investigate the role of *Itf2* in intestinal tumorigenesis *in vivo*. Therefore, we inactivated *Itf2*

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specifically in the intestinal epithelium of $Apc^{Min/+}$ mice and analyzed consequences for tumor formation.

2. Material and methods

2.1. Animals

$Itf2^{fl/fl}$ mice [12] were crossed with *vil-Cre*$^{+}$ mice [13]. $Itf2^{fl/fl}$;*vil-Cre*$^{+}$ mice heterozygous for the *vil-Cre* transgene were bred into the $Apc^{Min/+}$ background to obtain $Itf2^{fl/fl}$;$Apc^{Min/+}$ and $Itf2^{fl/fl}$;*vil-Cre*$^{+}$;$Apc^{Min/+}$ mice, respectively. In all experiments, mice on the $Itf2^{fl/fl}$ background were used as controls. $Apc^{Min/+}$ mice were purchased from the Jackson Laboratory. All mouse strains were maintained on a C57BL/6 background.

Mice were inspected on a daily basis and sacrificed when moribund. Animals were housed under specific pathogen free conditions in a closed barrier system. Experiments were carried out in accordance with the German Animal Welfare Act and with permission of the Government of Upper Bavaria.

2.2. Tissue processing, tumor scoring and histology

Mice were sacrificed by cervical dislocation, the intestine was excised and rinsed with PBS to remove fecal material. The small intestine was cut into 3 equal segments and each intestinal section was placed on a piece of filter paper, opened longitudinally, laid open and fixed in 4% buffered formaldehyde solution. Tumor number and their maximum diameter were determined under a dissecting microscope at 10x magnification. The colon and rectum were scored as “colon”. A quantity of small intestinal lesions was resected including adjacent normal tissue. In case no polyps were found, the small intestine and colon were processed as “Swiss rolls” [14]. The material was dehydrated and embedded in paraffin. 4 μm tissue sections were cut in parallel with the mucosal surface and stained with H&E or Periodic acid-Schiff reagent (PAS) according to standard protocols. Histopathological analysis of neoplastic lesions was performed in a blinded manner using standard criteria [15].

The numbers of PAS-positive cells and enteroendocrine cells, respectively, were counted in 20 crypts each in the small intestine

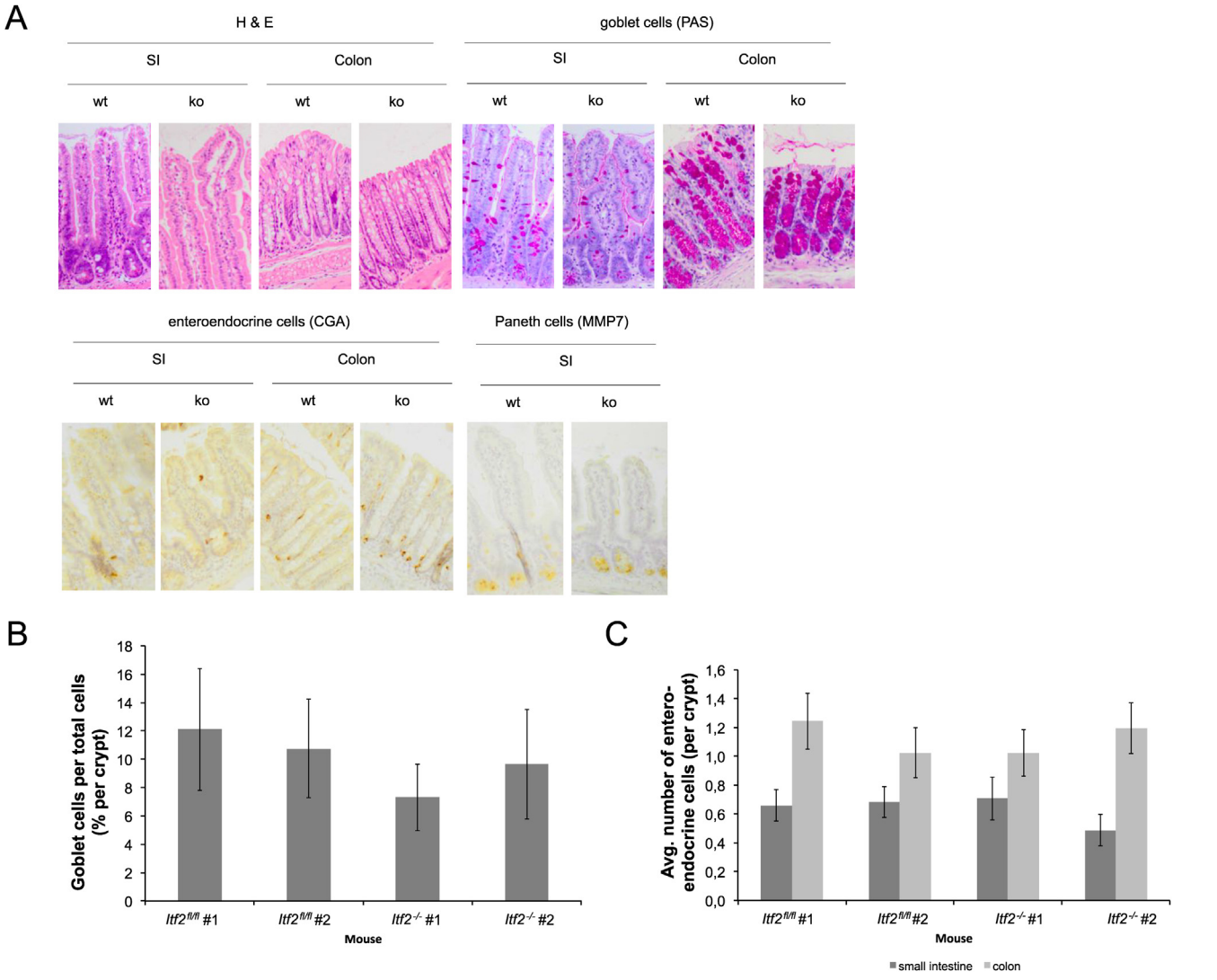

Fig. 1. Genetic disruption of *Itf2* has no effect on the maintenance of the intestinal epithelium. (A) H&E-staining of small intestine and colon of an $Itf2^{fl/fl}$ and an $Itf2^{-/-}$ mouse. PAS staining (goblet cells) in the small intestine and colon of an $Itf2^{fl/fl}$ and an $Itf2^{-/-}$ mouse. Immunohistochemical staining for chromogranin A (enteroendocrine cells) in the small intestine and colon of an $Itf2^{fl/fl}$ and an $Itf2^{-/-}$ mouse. Immunohistochemical staining for MMP7 (Paneth cells) in the small intestine of an $Itf2^{fl/fl}$ and an $Itf2^{-/-}$ mouse. (B) The number of PAS-positive cells was counted in 20 crypts in the small intestine of $Itf2^{fl/fl}$ and $Itf2^{-/-}$ mice. Error bars indicate standard deviations. (C) The number of enteroendocrine cells was counted in 20 crypts in the small intestine of $Itf2^{fl/fl}$ and $Itf2^{-/-}$ mice. Error bars indicate standard deviations.

and the colon per mouse. All experiments were carried out in a blinded manner.

2.3. Immunohistochemistry

Immunohistochemistry was performed on 4 μm paraffin slides. Antigen retrieval was performed by cooking for 30 min in 10 mM sodium citrate buffer (pH 6.0) for MMP7 or for 20 min in Epitope Retrieval Solution (pH 8.0; Novocastra Laboratories) for chromogranin A. Primary antibodies used were rabbit anti-chromogranin A (1: 2000; Cat.No. 20085, clone SP-1, Immuno Star) and goat anti-MMP7 (1: 100; Cat.No. 3801, clone D4H5, R&D Systems). For detection the HRP conjugated donkey anti-goat IgG (Cat.No. sc-2033, clone, SCBT) was used for MMP7 and the Imm-PRESS Reagent Kit Anti-Rabbit Ig (Vector Laboratories) for chromogranin A. Diaminobenzidine (Sigma) was used as chromogen and slides were finally counterstained with hematoxylin (Vector Laboratories).

2.4. Statistical analyses

Kaplan–Meier analysis was employed to display the time to tumor mortality and to estimate cancer specific survival. Significance of the Kaplan–Meier statistic was tested applying the log-rank test. To analyze significance of differences, two-tailed Student's t-test or two-tailed Mann Whitney U test were performed. P values ≤ 0.05 were considered statistically significant. Statistics were performed using SPSS statistical software (version 15.0; SPSS Inc., Chicago, IL).

3. Results

To study the effect of inactivation of the *Itf2* gene on intestinal tumor formation, we crossed $Itf2^{fl/fl}$ mice with mice expressing Cre recombinase under the control of the intestinal epithelium specific *Villin* promoter ([7,12,13,16]). $Itf2^{fl/fl}$;*vil-Cre*$^{+}$ ($Itf2^{-/-}$) mice were viable, fertile and showed no increased morbidity and mortality. Detailed histological and immunohistochemical analysis of small intestine and colon revealed no changes in gut anatomy or in the number and distribution of the epithelial cell lineages, i.e. absorptive enterocytes, goblet cells, Paneth cells, and enteroendocrine cells (Fig. 1). Thus, *Itf2* expression appears to be dispensable for the maintenance of the intestinal epithelium. Moreover, loss of *Itf2* function did not result in spontaneous intestinal tumor formation.

To investigate the role of *Itf2* in tumor initiation and progression, we crossed $Itf2^{-/-}$ mice into the $Apc^{Min/+}$ background. Genetic disruption of *Itf2* on the $Apc^{Min/+}$ background resulted in a dramatic reduction of the median survival from 192 days in $Itf2^{-/-}$;$Apc^{Min/+}$ mice to 130.5 days in $Itf2^{fl/fl}$;$Apc^{Min/+}$ controls ($p = 0.004$; Fig. 2A). Earlier death in $Itf2^{-/-}$;$Apc^{Min/+}$ mice was accompanied by a significant increase (~2-fold) in the mean number of tumors in the small intestine ($p < 0.01$; Fig. 2B). Similarly, loss of *Itf2* function in $Apc^{Min/+}$ mice led to an increase in the average tumor diameter in the small intestine ($p < 0.001$; Fig. 2C). H&E staining of tumors revealed tubular adenomas with low and high grade intraepithelial neoplasia/dysplasia in both $Itf2^{fl/fl}$;$Apc^{Min/+}$ controls and $Itf2^{-/-}$;$Apc^{Min/+}$ mice (Fig. 3). There were no differences in the quality of the adenomas in both mouse strains. Immunohistochemical staining of $Itf2^{-/-}$;$Apc^{Min/+}$ and $Itf2^{fl/fl}$;$Apc^{Min/+}$ control adenomas for Ki-67 and cleaved caspase-3 revealed no differences in the rates of proliferation and apoptosis, respectively (Fig. 3).

Our data show that inactivation of the *Itf2* gene promotes tumor development in the small intestine.

4. Discussion

ITF-2B is a widely expressed transcription factor that is involved in differentiation processes [17]. *ITF2* expression is up-regulated in primary endometroid ovarian carcinomas due to deregulation of the Wnt/β-catenin signaling cascade and ITF-2B promotes neoplastic transformation of RK3E cells, an adenovirus E1A-immortalized epithelial cell line [5] In the past constitutive and inducible knockout mouse models revealed a crucial role for *Itf2* in

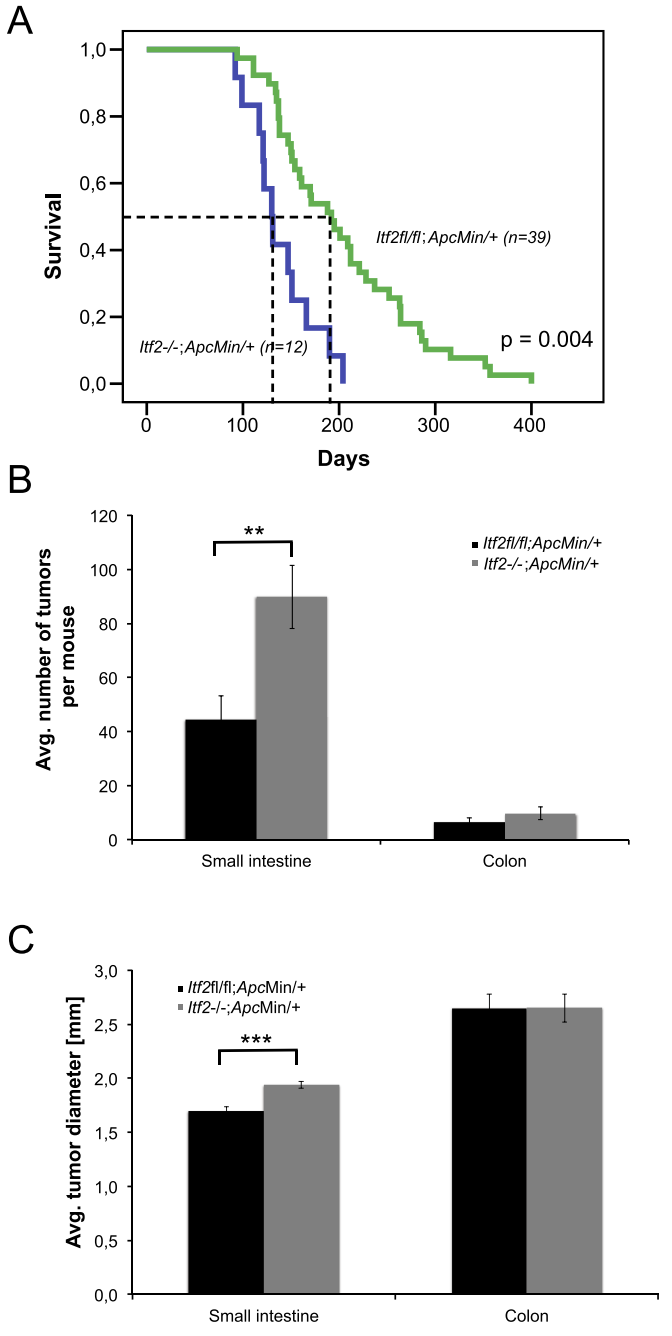

Fig. 2. Inactivation of *Itf2* in $Apc^{Min/+}$ mice reduces median survival and increases adenoma number and size in the small intestine. (A) $Itf2^{-/-}$ mice were crossed into the $Apc^{Min/+}$ background. $Itf2^{fl/fl}$;$Apc^{Min/+}$ (n = 39) and $Itf2^{-/-}$;$Apc^{Min/+}$ (n = 12) mice were monitored for long-term survival. Median survival is indicated. (B) Mice were sacrificed when moribund and the average number of tumors per mouse in small intestine and colon was evaluated. (C) The average tumor size (max. diameter) in small intestine and colon was measured.

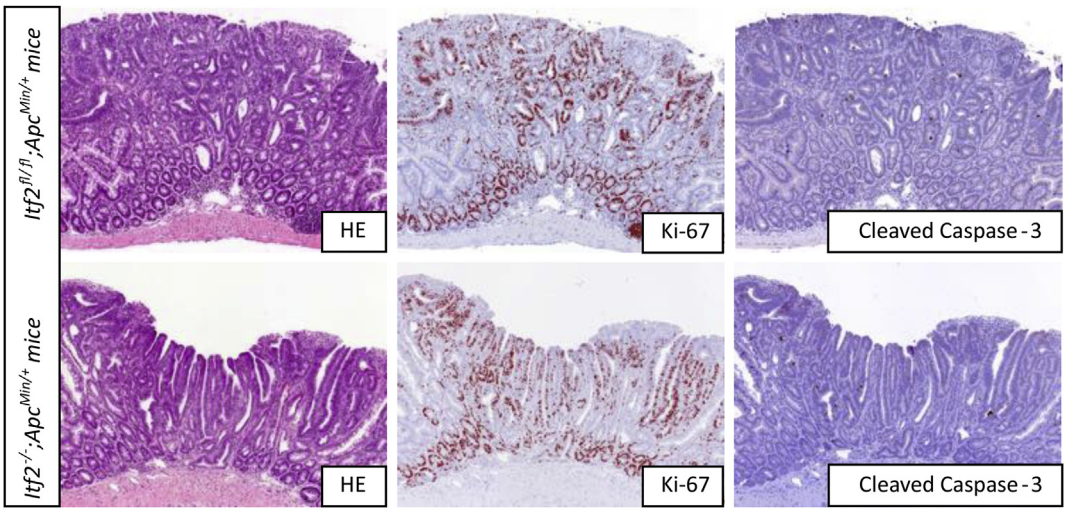

Fig. 3. Disruption of *Itf2* in $Apc^{Min/+}$ mice has no effect on adenoma quality and the rates of proliferation and apoptosis. H&E staining, Ki-67 (proliferation), and cleaved caspase-3 (apoptosis) immunohistochemical staining of tubular adenomas with low grade intraepithelial neoplasia/dysplasia from $Itf2^{fl/fl}$;$Apc^{Min/+}$ and $Itf2^{-/-}$;$Apc^{Min/+}$ mice.

both B and T lymphocyte development [12,18] and plasmacytoid dendritic cell development and function [17]. Mice carrying ubiquitously disrupted *Itf2* alleles are not viable and die shortly after birth [18].

Previously, we identified *ITF2* as a candidate tumor suppressor gene of colorectal carcinogenesis [6]. *Itf2/ITF2 is* known to be weakly expressed in normal intestinal epithelium and to be strongly over-expressed in human and $Apc^{Min/+}$ intestinal adenomas with deregulated Wnt/β-catenin signaling [6]. However, with increasing tumor stage, ITF-2B protein levels are frequently reduced or lost in consequence of loss of heterozygosity on chromosome 18q21 and *ITF2* promoter deacetylation [6]. ITF-2B has been shown to inhibit cell growth by increasing $p21^{CIP1}$ protein levels [6], a cell cycle regulator that mediates cell cycle arrest [19,20] and is frequently found to be down-regulated in colon carcinoma [21].

In the present study we show that genetic disruption of *Itf2* specifically in the murine intestinal epithelium does not result in spontaneous tumor formation, indicating that loss of *Itf2* function alone is not sufficient to initiate intestinal tumorigenesis. It is well known that inactivation of a tumor suppressor gene alone is often not sufficient to cause tumorigenesis, and additional mutational events that result in perturbation of other critical signaling pathways are necessary [22].

We further show that disruption of *Itf2* in $Apc^{Min/+}$ mice results in a significantly reduced survival due to an increased number and size of tubular adenomas in the small intestine. These data support the concept that *Itf2* functions as a tumor suppressor by inhibiting tumor development and suggest a protective role of *Itf2* in the adenoma during early tumorigenesis. Consistently, it has been demonstrated that re-expression of *ITF2* in colorectal cancer cell lines leads to cell cycle arrest by p53-independent induction of CDKN1A and increased sensitivity to TRAIL-induced apoptosis [6].

Although we observed an increase in small intestinal adenoma number and size upon inactivation of *Itf2* in $Apc^{Min/+}$ mice, there was no effect on adenocarcinoma formation. This is surprising as *ITF2* is known to be down-regulated with increasing tumor stage [6], suggesting that it interferes with tumor progression. In $Apc^{Min/+}$ mice development of adenocarcinoma is a very rare event [9–11]. It has been suggested that the short life-span of the $Apc^{Min/+}$ mouse might prevent the accumulation of somatic alterations that is necessary for tumor progression. Halberg and coworkers have demonstrated that development of invasive adenocarcinoma can regularly be found in long–lived $Apc^{Min/+}$ hybrids [23]. As survival is even reduced in $Itf2^{-/-}$;$Apc^{Min/+}$ mice due to the accelerated tumor development, it seems very probable that mice die long before disruption of *Itf2* can effect adenocarcinoma formation.

Moreover, we did not observe an effect of *Itf2* inactivation on tumor development in the colon of $Apc^{Min/+}$ mice. The $Apc^{Min/+}$ mouse is the best studied and the most widely used model of FAP. However, in contrast to the human disease, where affected individuals develop hundreds of adenomas mainly in the colon which then progress to invasive cancer early in life [24], $Apc^{Min/+}$ mice commonly develop only few colon adenomas and only rarely colon carcinoma [9–11]. The reason for this discrepancy is not known.

With respect to the low frequency of colonic tumors and the absence of adenocarcinoma, the value of the $Apc^{Min/+}$ mouse for the study of colorectal cancer is limited. However, findings in several mouse models have demonstrated that manifestations of lesions in the small intestine are comparable to human findings in the colon [25,26].

In summary, we show that *Itf2* functions as an important tumor suppressor gene of the intestinal tract. Further studies are needed to examine whether *Itf2* acts also as a tumor suppressor of the colon and to investigate its role during tumor progression.

Conflict of interest

None.

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