

Recurrent *YAP1-MAML2* and *YAP1-NUTM1* fusions in poroma and porocarcinoma

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Poroma is a benign skin tumor exhibiting terminal sweat gland duct differentiation. The present study aimed to explore the potential role of gene fusions in the tumorigenesis of poromas. RNA sequencing and reverse transcription PCR identified highly recurrent *YAP1-MAML2* and *YAP1-NUTM1* fusions in poromas (92/104 lesions, 88.5%) and their rare malignant counterpart, porocarcinomas (7/11 lesions, 63.6%). A *WWTR1-NUTM1* fusion was identified in a single lesion of poroma. Fluorescent in-situ hybridization confirmed genomic rearrangements involving these genetic loci. Immunohistochemical staining could readily identify the YAP1 fusion products as nuclear expression of the N-terminal portion of YAP1 with a lack of the C-terminal portion. *YAP1* and *WWTR1*, also known as *YAP* and *TAZ*, respectively, encode paralogous transcriptional activators of TEAD, which are negatively regulated by the Hippo signaling pathway. The YAP1 and WWTR1 fusions strongly transactivated a TEAD reporter and promoted anchorage-independent growth, confirming their tumorigenic roles. Our results demonstrate the frequent presence of transforming YAP1 fusions in poromas and porocarcinomas and suggest YAP1/TEAD-dependent transcription as a candidate therapeutic target against porocarcinoma.

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Recurrent *YAP1-MAML2* and *YAP1-NUTM1* fusions in poroma and porocarcinoma

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Abstract

Poroma is a benign skin tumor exhibiting terminal sweat gland duct differentiation. The present study aimed to explore the potential role of gene fusions in the tumorigenesis of poromas. RNA sequencing and reverse transcription PCR identified highly recurrent *YAP1-MAML2* and *YAP1-NUTM1* fusions in poromas (92/104 lesions, 88.5%) and their rare malignant counterpart, porocarcinomas (7/11 lesions, 63.6%). A *WWTR1-NUTM1* fusion was identified in a single lesion of poroma. Fluorescent *in-situ* hybridization confirmed genomic rearrangements involving these genetic loci. Immunohistochemical staining could readily identify the YAP1 fusion products as nuclear expression of the N-terminal portion of YAP1 with a lack of the C-terminal portion. *YAP1* and *WWTR1*, also known as *YAP* and *TAZ*, respectively, encode paralogous transcriptional activators of TEAD, which are negatively regulated by the Hippo signaling pathway. The YAP1 and WWTR1 fusions strongly transactivated a TEAD reporter and promoted anchorage-independent growth, confirming their tumorigenic roles. Our results demonstrate the frequent presence of transforming YAP1 fusions in poromas and porocarcinomas and suggest YAP1/TEAD-dependent transcription as a candidate therapeutic target against porocarcinoma.

Introduction

Poroma is a skin adnexal tumor exhibiting features of the terminal sweat gland duct (1, 2). It typically presents as a solitary sessile nodule on the soles and palms but may occur in any area where sweat glands are present. Histologically, poroma consists of monomorphic small basaloid cells and contains occasional ductal structures. Most commonly, the tumor is broadly connected to the epidermis and grows down into the dermis forming broad anastomosing bands. Some lesions exhibit a spectrum of architectural variations that allows them to be classified into histological subtypes (Supplemental Figure 1) (2-6). Hidroacanthoma simplex represents an intraepidermal variant whereas dermal duct tumor is primarily confined to the dermis. Poroid hidradenoma forms a discrete nodule centered in the dermis and shows characteristic solid and cystic components. Although poromas are relatively common, their genetic background has only been analyzed in a few studies. A recent targeted next-generation sequencing analysis of six poromas identified a number of mutations, including *HRAS* mutations as the sole recurrently mutated gene (7). However, the reported prevalence of *HRAS* mutations was limited to 17%, suggesting that poromas are a rather heterogeneous group of tumors in terms of genetic alterations (7, 8).

Porocarcinoma represents the rare malignant counterpart of poroma predominantly affecting elderly individuals (9-11). In contrast to poromas, porocarcinomas exhibit invasive growth, are prone to local recurrence, and may develop distant metastasis. Because of their rarity and lack of distinct clinical features, the diagnosis of porocarcinoma is often challenging. A subset of porocarcinomas is associated with benign poromas, a finding implying malignant transformation (2, 9, 11). Furthermore, porocarcinomas arising from longstanding poromas have been reported (9, 12). Apart from these, many of porocarcinomas do not have a benign precursor component and thus may develop *de novo* (11, 13). A recent sequencing analysis of five

porocarcinomas identified recurrent mutations in *TP53*, *RB1*, *EGFR*, and *HRAS* but simultaneously, revealed the molecular diversity of this rare type of tumor (8).

Given that mutations in known oncogenes and tumor suppressor genes unlikely underlie the tumorigenesis of a majority of poromas, we sought to explore the possible roles of gene fusions using RNA sequencing. Our analysis identified highly recurrent *YAP1* fusions in poromas and porocarcinomas and demonstrated their transforming activity.

Results and Discussion

To probe the possible tumorigenic roles of gene fusions in poroma, we subjected 12 formalin-fixed paraffin-embedded specimens of poromas to RNA sequencing. The analysis identified *YAP1-MAML2* and the reciprocal *MAML2-YAP1* fusions in four lesions, *YAP1-NUTM1* fusions in three lesions, and a *WWTR1-NUTM1* fusion in one lesion (Supplemental Table 1). To expand on this finding, we examined 104 poromas, including those analyzed by RNA sequencing, for the presence of the fusions detected by next-generation sequencing and possible fusion transcripts predicted from the exon structures of *YAP1*, *MAML2*, and *NUTM1* (Supplemental Table 2). RT-PCR followed by Sanger sequencing detected *YAP1-MAML2*, *MAML2-YAP1*, *YAP1-NUTM1*, and *WWTR1-NUTM1* fusions in 71, 48, 21, and 1 poromas, respectively (Figure 1A, B, Supplemental Figure 2). Furthermore, an examination of 11 porocarcinomas identified *YAP1-MAML2* and *YAP1-NUTM1* fusions in one and six lesions, respectively. All the lesions with *MAML2-YAP1* fusions concurrently expressed the reciprocal *YAP1-MAML2* fusions; conversely, 48 of 71 (67.6%) *YAP1-MAML2*-positive lesions expressed *MAML2-YAP1* fusions. The *YAP1-NUTM1* and *WWTR1-NUTM1* fusions were mutually exclusive with others. Multiple variant transcripts were detected for *YAP1-MAML2*, *MAML2-YAP1*, and *YAP1-NUTM1*, often in single tumors. All the fusion transcripts were in-frame. Collectively, *YAP1* fusions were expressed in 92 poromas (88.5%) and 7 porocarcinomas (63.6%), and one poroma was associated with a *WWTR1* fusion (1.0%). The common presence of *YAP1* fusions in poromas and porocarcinomas supports their histogenetic relationship, which is consistent with the idea that a significant proportion of porocarcinomas develop through the malignant transformation of preexisting poromas (2, 9, 11). To our knowledge, among the identified *YAP1* and *WWTR1* fusions, only a *YAP1-MAML2* fusion was previously reported in a single case of nasopharyngeal carcinoma (14), whereas the other fusion transcripts have not been previously

described.

Interestingly, there appear to be associations between specific *YAP1* fusion types and clinicopathological features. Hidroacanthoma simplex predominantly expressed the *YAP1* exon 1-*MAML2* fusion (4/5 lesions). *YAP1-NUTM1* fusions were enriched in dermal duct tumors (2/2 lesions), poroid hidradenomas (4/6 lesions), porocarcinomas (6/11 lesions), poromas in the head and neck region (15/27 lesions), and poromas with necrotic foci (10/15 lesions). Because the number of each histological variant is rather limited in this study due to their rarity, further investigation based on a larger case series is desirable. However, it is notable that *YAP1-NUTM1* fusions are enriched in porocarcinomas and poromas with primarily dermal localization, implying the link between the specific fusions and growth patterns. Examination of other skin tumors, including 24 squamous cell carcinomas, 32 basal cell carcinomas, 5 cutaneous adenocarcinomas, 9 Merkel cell carcinomas, and 27 seborrheic keratoses, did not identify any of the recurrent *YAP1* fusions. These observations indicate that *YAP1* fusions are highly recurrent and specific to poromas and porocarcinomas among skin neoplasms.

Considering that *YAP1* and *MAML2* are on the opposite strands of adjacent genetic loci on chromosome 11p, the frequent co-expression of *YAP1-MAML2* and *MAML2-YAP1* fusion transcripts is thought to be the consequence of intrachromosomal inversions (Supplemental Figure 3). Since *NUTM1* and *WWTR1* are on chromosome 15q14 and 3q25, respectively, the *YAP1-NUTM1* and *WWTR1-NUTM1* fusions are expected to result from interchromosomal translocations. Fluorescence *in-situ* hybridization analysis revealed the *YAP1* locus rearrangement in 22 of 23 lesions with a *YAP1* fusion and the *NUTM1* locus rearrangement in 14 of 15 lesions with a *NUTM1* fusion (Figure 1C, Supplemental

Table 3). Most lesions with reciprocal *YAP1-MAML2* and *MAML2-YAP1* transcripts showed narrow split signals in the assays using *YAP1*-5' and *YAP1*-3' probes, consistent with intrachromosomal rearrangement. The *YAP1-MAML2* and *YAP1-NUTM1* fusion signals were also detected in the tumors with their respective fusion transcripts. Rearrangements of the *WWTR1* and *NUTM1* loci were confirmed in the lesion with the *WWTR1-NUTM1* fusion.

Although there were some differences in the prevalence of respective fusion types, *YAP1* fusions were frequently detected in both poromas and porocarcinomas. Therefore, we wanted to find out if any genetic alterations were involved in the malignant transformation of poroid neoplasms. To address this issue, we analyzed 23 poromas and 9 porocarcinomas using the NCC oncopanel test: a next-generation sequencing panel targeting 114 cancer-associated genes (15). The analysis identified a total of 49 protein-altering mutations (Supplemental Table 4, 5). Porocarcinomas tended to harbor a larger number of mutations than poromas (average, 3.0 vs. 0.96 mutations/lesion). However, only three genes were recurrently mutated in porocarcinomas: *KRAS*, *SETD2*, and *TP53*. Each of these genes was mutated in only two of the lesions and *SETD2* and *TP53* mutations were also detected in poromas. These results indicate that mutations in major cancer-related genes cannot be used to distinguish benign and malignant poroid neoplasms.

To examine the expression of fusion protein products in clinical specimens, we performed immunohistochemical staining using two antibodies against *YAP1*. The analysis revealed the diffuse nuclear expression of the N-terminal portion and the absence of C-terminal portion expression in 100 poromas (96.2%) and 7 porocarcinomas (63.6%), including all the lesions with the *YAP1* fusions (Figure 2 and 3). The discrepant expression of the N- and C-terminal portions of *YAP1* is consistent

with the presence of YAP1-MAML2 or YAP1-NUTM1 fusion products; at the same time, it suggests the absence of MAML2-YAP1 fusion products. Seven poromas showed the discordant expression of N- and C-terminal regions of YAP1 in the absence of *YAP1* fusion transcripts. We expect that these lesions might also have different types of *YAP1* fusions, but the limitation in sample quality, due to the use of formalin-fixed paraffin-embedded specimens, precluded further investigation of other fusions in these lesions. The lesion with the *WWTR1-NUTM1* fusion was also diffusely positive for the YAP1 N-terminus antibody, likely reflecting the known cross-reactivity of the antibody with the N-terminal portion of WWTR1 (16). Expression of NUTM1, also known as NUT, was observed in 25 poromas (24.0%) and 6 porocarcinomas (54.5%), including all lesions with the *YAP1-NUTM1* fusions.

YAP1 immunohistochemistry on other skin tumors, including 23 squamous cell carcinomas, 24 basal cell carcinomas, 5 cutaneous adenocarcinomas, 9 Merkel cell carcinomas, and 26 seborrheic keratoses, showed common expression of YAP1, in agreement with previous reports (17, 18). However, in contrast to poromas and porocarcinomas, the expression patterns of the N- and C-terminal regions of YAP1 were consistently identical in the respective tumors, consistent with the wild-type protein expression. Also, they exhibited cytoplasmic, in addition to nuclear, expression of YAP1 unlike poromas and porocarcinomas. Thus, our immunohistochemical analysis could readily distinguish YAP1-MAML2 and YAP1-NUTM1 fusion products from the wild-type YAP1 based on the discordant expression of the N- and C-terminal regions of YAP1. Additionally, NUTM1 expression was never observed in skin tumors other than poromas and porocarcinomas, indicating its specificity for *NUTM1* fusion-positive poromas and porocarcinomas among skin tumors. Taken together, *YAP1* and *WWTR1* fusions were exclusive to poromas and porocarcinomas among skin tumors and thus, the detection of these fusion transcripts or protein products

would have diagnostic applications.

The identification of a *WWTR1* fusion in a single lesion of poroma, in addition to frequent *YAP1* fusions, is remarkable because both *YAP1* and *WWTR1*, also known as *YAP* and *TAZ*, respectively, encode paralogous transcriptional regulators of TEAD (19, 20). Activation of *YAP1/WWTR1* is widely observed in various tumorigenic processes and plays roles in stemness, proliferation, invasion, and chemoresistance (19, 21). Physiologically, the activity of *YAP1/WWTR1* is negatively regulated by the Hippo signaling pathway. Inactivating mutations of Hippo pathway components, leading to aberrant activation of *YAP1/WWTR1*, have been reported in various cancers, supporting the tumor suppressive function of this pathway (19, 20). Recurrent genetic alterations of *YAP1* and *WWTR1* themselves have only been reported in epithelioid hemangioendothelioma (22-24). More than 90% of epithelioid hemangioendotheliomas have *WWTR1-CAMTA1* fusions and most of the remaining cases express a *YAP1-TFE3* fusion. The *WWTR1-CAMTA1* fusion protein shows constitutive nuclear localization, dysregulated TEAD activation, and transforming activity (25). Notably, similar to these previously reported *WWTR1* and *YAP1* fusions, the putative *YAP1-MAML2*, *YAP1-NUTM1*, and *WWTR1-NUTM1* fusion products retain TEAD-binding domains derived from *YAP1* or *WWTR1* (Figure 4A).

MAML2 constitutes the 3'-part of *CRTC1-MAML2* fusions, which is common in mucoepidermoid carcinoma of the salivary gland, and the interaction between p300 and the *MAML2*-derived transactivation domain is critical for their transforming activity (26, 27). The *NUTM1*-derived portion of *YAP1-NUTM1* fusions also constitutes other tumor-related fusion proteins, including *BRD4-NUTM1* in NUT midline carcinoma and *CIC-NUTM1* in central nervous system Ewing sarcoma family tumor (28-30), and the recruitment of *CBP/p300* is required for the oncogenic property of the *BRD4-NUTM1*

fusion (29). Thus, all the YAP1 and WWTR1 fusions detected in poromas and porocarcinomas harbor the N-terminal TEAD-binding domain of YAP1 or WWTR1 (31), and the MAML2- or NUTM1-derived regions that interact with transcriptional coactivators, CBP/p300 (27, 29). Based on the common presence of domains to interact with transcription factors and transcriptional co-factors across these fusions, we postulated that these fusions function as a potent transcriptional activator of TEAD. In contrast, MAML2-YAP1 fusions lack these domains.

To test the functional significance of the YAP1 fusions, we first examined the intracellular localization of the YAP1 fusion products in HEK293T cells. Normally, active Hippo pathway signaling leads to the cytoplasmic retention and degradation of YAP1, inhibiting the formation of a complex with TEAD transcription factors (19, 20). Wild-type YAP1 and a constitutively active mutant, YAP1^{S127A}, were distributed in both the nucleus and cytoplasm, with a predominant localization in the cytoplasm (Figure 4B). In contrast, YAP1-MAML2 and YAP1-NUTM1 fusions exhibited exclusively nuclear localization with speckled and homogeneous staining patterns, respectively, suggesting that the 3'-fusion partners contribute to the nuclear localization of these fusions through distinct mechanisms. Next, we performed a reporter assay to test the role of these fusions in TEAD-dependent transcription. As expected, YAP1-MAML2, YAP1-NUTM1, and WWTR1-NUTM1 fusions strongly upregulated the expression of a TEAD reporter, and the reporter expression levels were even higher than those with two YAP1 mutants that are resistant to negative regulation by the Hippo pathway. In contrast, the MAML2-YAP1 fusion, MAML2, and NUTM1 did not significantly induce the TEAD reporter expression (Figure 4C).

We then established a series of doxycycline-dependent inducible NIH3T3 cell lines. Despite repeated attempts, we could not obtain sublines with high expression levels of

YAP1-MAML2 fusions (Supplemental Figure 4). However, in a soft agar colony formation assay, the induction of *YAP1-MAML2*, *YAP1-NUTM1*, and *WWTR1-NUTM1* expression resulted in anchorage-independent growth, similar to that seen in constitutively active *YAP1* mutants (Figure 4D) (32). In contrast, the wild-type *YAP1* and the *MAML2-YAP1* fusion did not promote anchorage-independent growth. These observations indicate the role of YAP1 and WWTR1 fusions in the activation of TEAD transcription factors and their transforming activity. Although two-thirds of *YAP1-MAML2* fusions were associated with the reciprocal *MAML2-YAP1* fusions, functional assays showed the lack of their transforming activity and immunohistochemical study failed to detect their protein products. Thus, *MAML2-YAP1* fusions are likely non-functional by-products of *YAP1-MAML2* fusions, resulting from intrachromosomal inversion.

Next, we tested if YAP1 and WWTR1 fusions show transforming activity also in epithelial cells by using an immortalized human dermal keratinocyte (HDK) cell line (33). A reporter assay was performed by using doxycycline-dependent inducible cell lines stably transduced with a TEAD reporter (Supplemental Figure 5A), because of the low transfection efficiency of the HDK cells. As observed with NIH3T3 cells, expression of *YAP1-MAML2*, *YAP1-NUTM1*, and *WWTR1-NUTM1* fusions strongly upregulated the expression of the TEAD reporter (Supplemental Figure 5B). A soft agar colony formation assay showed enhanced anchorage-independent growth upon the induction of *YAP1-MAML2*, *YAP1-NUTM1*, and *WWTR1-NUTM1* (Supplemental Figure 5C). However, these colonies were smaller than those formed by cells expressing constitutively active YAP1 and WWTR1 mutants. In addition, cells overexpressing wild-type YAP1 and WWTR1 also formed small colonies. These findings indicate that YAP1 and WWTR1 fusions activate TEAD transcription factors and promote anchorage-independent growth also in epithelial cells. However, they

may exhibit somewhat different biological effects in different cell types.

The present study identified highly recurrent *YAP1* and *WWTR1* fusions in poromas and porocarcinomas. The *YAP1* and *WWTR1* fusions are potent transcriptional activators of TEAD and have transforming activity. The respective fusion types are likely associated with several clinicopathological features, including the enrichment of *YAP1-NUTM1* fusions in porocarcinomas. Since *YAP1* fusions are specific to poromas and porocarcinomas among skin tumors, the detection of their protein products would have immediate diagnostic applications. Furthermore, the identification of recurrent *YAP1* fusions in porocarcinomas suggests *YAP1/TEAD*-dependent transcription as a potential therapeutic target against this rare malignancy.

Methods

Further information is provided in Supplemental Methods.

Study approval

The study was approved by the Ethical Committee of National Cancer Center, Tokyo, Japan (Approval #2017-078).

Author Contributions

SS, TK, and TM contributed equally to this work. SS, TK, and TM designed the study. SS, ER, RO, HI, TM performed next-generation and Sanger sequencing. SS, WS, and TM performed histological, immunohistochemical, and FISH analyses. TK performed functional assays. KS, SA, KT, MI, YS, and NY provided tissue samples and clinical information. NG provided cDNA clones. SS wrote the manuscript with support from TK and TM.

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Figures

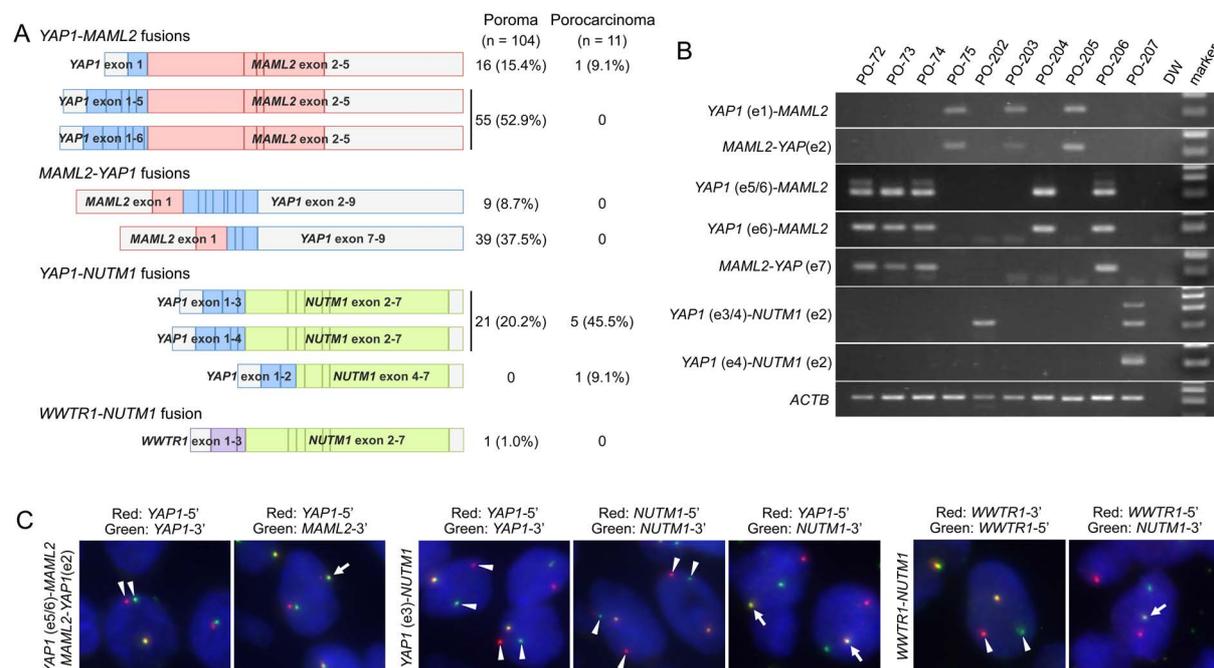


Figure 1: Detection of *YAP1* and *WWTR1* fusions in poromas and porocarcinomas

A: Fusion gene transcripts detected in poromas and porocarcinomas. All of the *MAML2*-*YAP1* fusions were associated with the reciprocal *YAP1*-*MAML2* fusions. **B:** Representative gel images of RT-PCR products. *MAML2*-*YAP1* fusion transcripts were consistently associated with the reciprocal *YAP1*-*MAML2* fusions. Many of the tumors expressed multiple transcriptional variants. *ACTB* served as a positive control. **C:** Representative fluorescent *in-situ* hybridization images. The detected fusion transcripts are indicated on the left of the respective images. Arrowheads, split signals. Arrows, fused signals.

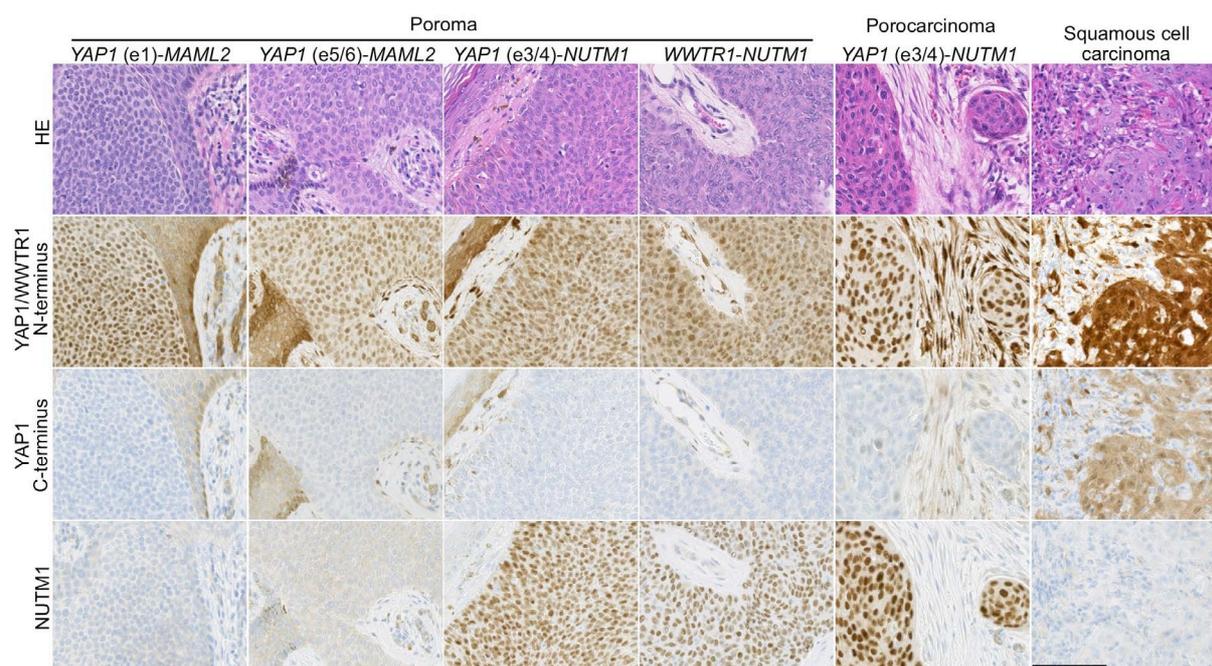


Figure 2: Expression of YAP1 and NUTM1 in poromas, a porocarcinoma, and a squamous cell carcinoma. Tumors with *YAP1* fusions showed diffuse nuclear expression of the N- terminal region of YAP1 but lacked the expression of the C-terminal region. Squamous cell carcinoma showed nuclear and cytoplasmic staining with both N- and C-terminus antibodies. The diffuse nuclear expression of NUTM1 was exclusively observed in tumors with the *YAP1-NUTM1* fusions. The anti-YAP1 N-terminus antibody, but not the C-terminus antibody, cross-reacts with the WWTR1. Scale bar, 100 μm .

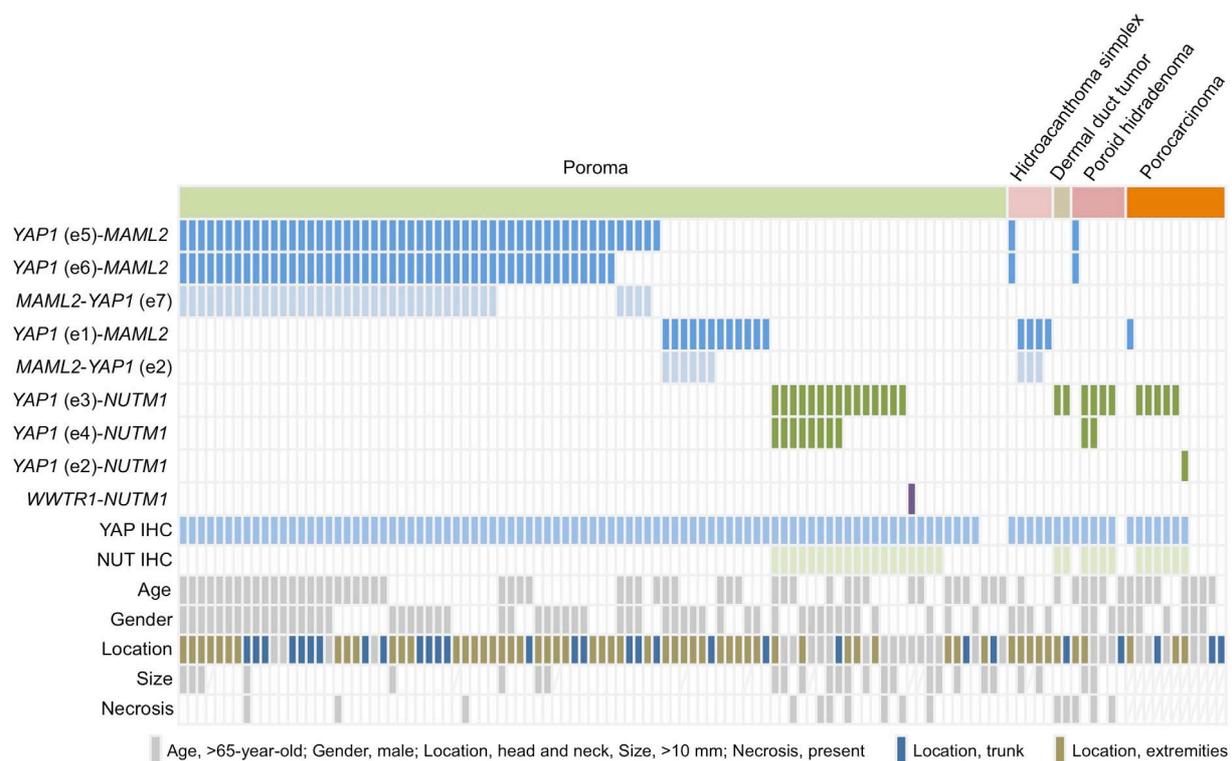


Figure 3: Summary of fusion transcript expression, immunohistochemical results, and clinicopathological features of poroma (n = 104) and porocarcinoma (n = 11)

YAP1 immunohistochemistry (IHC) indicates cases with discordant expression of the N- and C-terminal regions.

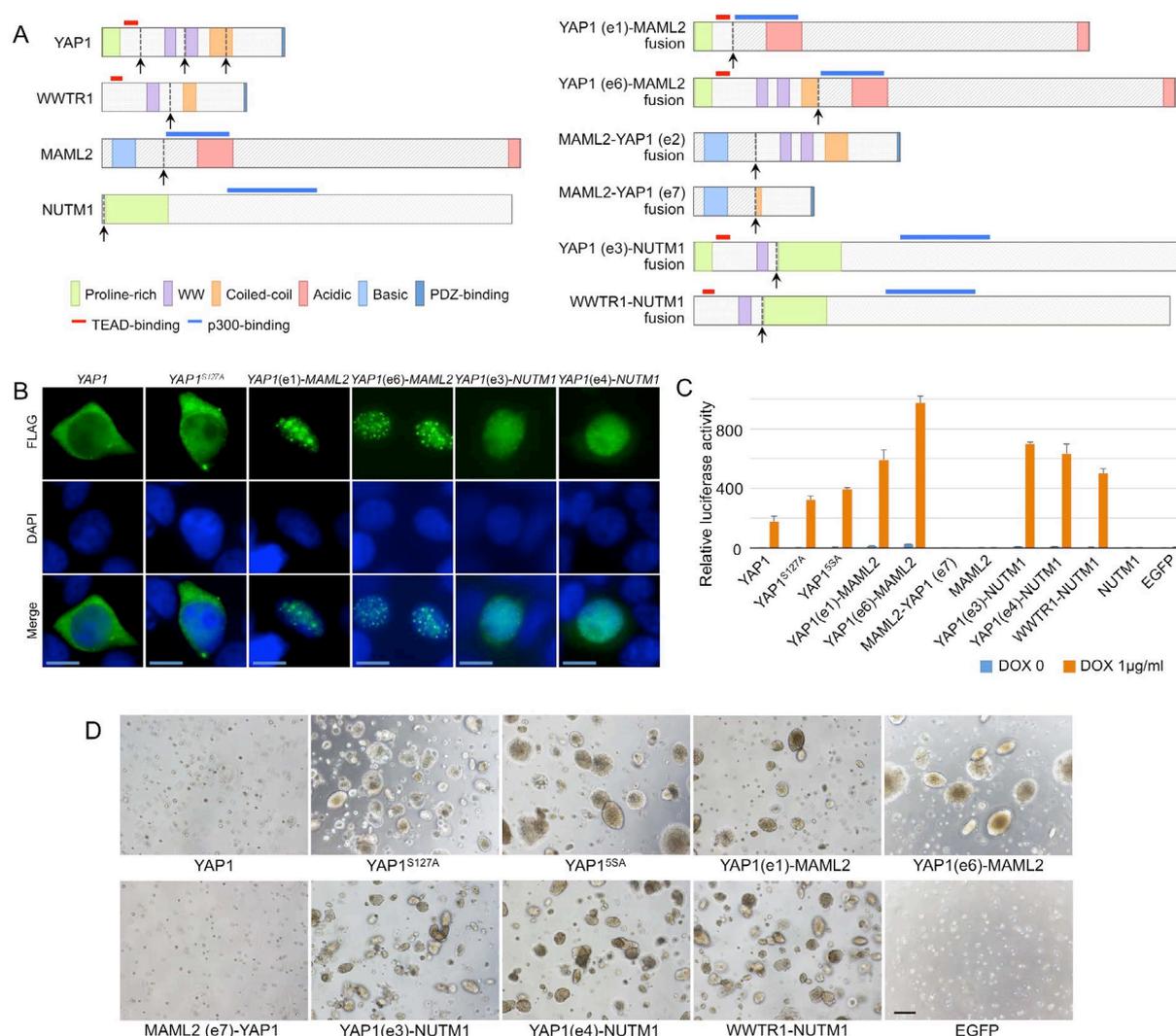


Figure 4: Functional significance of *YAP1* and *WWTR1* fusions

A: Structures of the putative fusion gene products expressed in poromas and porocarcinomas. Arrows indicate fusion breakpoints. Notice that *YAP1-MAML2*, *YAP1-NUTM1*, and *WWTR1-NUTM1* fusion gene products harbor a TEAD-binding domain derived from *YAP1* or *NUTM1* and a p300-binding domain derived from *MAML2* or *NUTM1*. **B:** Intracellular localization of *YAP1* fusion gene products. The FLAG-tagged transgenes were detected by immunofluorescence staining with nuclear staining using DAPI. Scale bars, 10 µm. **C:** TEAD luciferase reporter assays on HEK293T cells transfected with doxycycline-inducible vectors. *YAP1*^{S127A} and *YAP1*^{5SA} represent constitutively active mutants. The luciferase activity of

EGFP-transfected cells in the absence of doxycycline was set at 1 to indicate relative luciferase activities. Data represent mean of triplicate measurements + SD. **D:** Soft agar colony formation assay of NIH3T3 cells expressing the fusion transgenes.

NIH3T3 cells were transduced with doxycycline-inducible retrovirus vectors to express the respective transgenes and subjected to a soft agar colony formation assay in the presence of doxycycline. None of the clones formed colonies in the absence of doxycycline. Scale bar, 200 μm