EPHB4 and Survival of Colorectal Cancer Patients

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Abstract
The family of receptor tyrosine kinases EPH and their Ephrin ligands regulate cell proliferation, migration, and attachment. An important role in colorectal carcinogenesis is emerging for some of its members. In this study, we evaluate the role of EPHB4 in colorectal cancer and its value as a prognostic marker. EPHB4 levels were assessed using immunohistochemical staining of tissue microarrays of 137 colorectal tumors and aberrant hypermethylation of the EPHB4 promoter was investigated using methylation-specific PCR. We found that EPHB4 expression is frequently reduced in colorectal tumors. Patients with low EPHB4 tumor levels had significantly shorter survival than patients in the high EPHB4 group (median survival, 1.8 and >9 years, respectively; P < 0.01, log-rank test), and this finding was validated using an independent set of 125 tumor samples. In addition, we show that EPHB4 promoter hypermethylation is a common mechanism of EPHB4 inactivation. Moreover, reintroduction of EPHB4 resulted in a significant reduction in the clonogenic potential of EPHB4-deficient cells, whereas abrogation of EPHB4 in cells with high levels of this receptor lead to a significant increase in clonogenicity. In summary, we identified EPHB4 as a useful prognostic marker for colorectal cancer. In addition, we provide mechanistic evidence showing that promoter methylation regulates EPHB4 transcription and functional evidence that EPHB4 can regulate the long-term clonogenic potential of colorectal tumor cells, revealing EPHB4 as a potential new tumor suppressor gene in colorectal cancer.

(Cancer Res 2006; 66(18): 8943-8)

Introduction
EPHB4, a member of the largest family of receptor tyrosine kinases (RTK), is overexpressed in several tumor types, including prostate, breast, and bladder tumors and down-regulation of EPHB4 in tumor cell lines of these origins results in reduced cell viability, migration, and invasion (1–3). EPH receptors and their Ephrin (EFN) ligands regulate numerous developmental processes, particularly in the vasculature and nervous system (4, 5). The EPH family also has an important physiologic role in the intestinal epithelium. EPHB2 and EPHB3, together with their ligand Ephrin-B1, regulate proliferation and cell positioning within the intestinal crypts (6, 7). Mutations in key genes, such as adenomatous polyposis coli, which result in increased transcriptional activity of TCF/β-catenin, are the initiating event in most colorectal sporadic tumors. However, although EPHB4 is a direct transcriptional target of the TCF/β-catenin complex, the expression of EPHB4 is frequently lost in colorectal tumors (8), suggesting that inhibition of EPHB4 signaling may contribute to tumor progression; therefore, EPHB4 levels may be of prognostic significance in colorectal cancer patients. Colorectal cancer is the second most common type of cancer in the western world. Over 50% of the patients with locally advanced colorectal cancer are cured by the standard treatment options, which may include surgery and chemotherapy. However, a significant proportion of these patients will relapse and die of their disease. Currently, it is not possible to accurately differentiate between good and poor prognosis patients following surgical removal of the primary tumor; therefore, there is an acute need for markers capable of distinguishing these two groups.

Here, we show that low levels of expression of EPHB4 can identify a subset of colorectal cancer patients that are at high risk of recurrence. Moreover, we provide evidence that EPHB4 is frequently down-regulated in colorectal tumors through the aberrant hypermethylation of its promoter. In addition, we found that blocking EPHB4 signaling with a dominant-negative form in cells expressing high EPHB4 levels results in increased clonogenicity, whereas reintroduction of EPHB4 into cells expressing low levels of this receptor results in a significant reduction in their long-term clonogenic potential, indicating that EPHB4 has tumor suppressor activity in colorectal cancer cells.

Materials and Methods
Cell Lines and Clinical Samples
The cell lines used in this study were obtained and maintained as described previously (9, 10). Primary colorectal tumor samples were collected at collaborating medical institutions in Spain and Finland. Informed consent for genetic analysis of the tumor sample was obtained from each patient according to research proposals approved by the Human Investigations and Ethical Committee in the corresponding institution. Three different series of samples were used in this study. (a) Tissue microarray (TMA)-1: 137 formalin-fixed, paraffin-embedded tumor samples from Finnish patients (11) that were used for immunohistochemical assessment of EPHB4 (mean follow-up, 8.7 years; range, 6.8–11.2 years). (b) TMA-2: 125 formalin-fixed, paraffin-embedded tumor samples from Spanish patients that were independently used for immunohistochemical assessment of EPHB4 (mean follow-up, 5.8 years; range, 5.4–6.3 years). Disease recurrence information was not available for these patients. (c) Fresh frozen colorectal tumor samples from 112 patients from Spain and Finland. The patients in the sample series (a) and (c) partially overlapped (30 patients).
The clinicopathologic features of the patients in these three sample sets are shown in Supplementary Table S1 to S3.

Assessment of EPHB4 Protein Levels

Immunohistochemistry. A monoclonal antibody raised against the COOH terminus of the human EPHB4 receptor was used (clone 3D7G8; Zymed Laboratories, San Francisco, CA). The specificity of this antibody has been tested previously in formalin-fixed, paraffin-embedded samples (12). For immunohistochemical analysis, the commercial PowerVision Poly-HRP IHC detection kit was used (ImmuNoVision Technologies, Brisbane, CA) as reported previously (11, 13). EPHB4 expression was evaluated blinded from the clinical data. A semiquantitative scale from 0 (no staining) to 4 (highest staining) was used to assess the intensity of the staining. EPHB4-immunostained sections of the TMA-1 and TMA-2 were independently stained and scored in different institutions. Kaplan-Meier plots were used to compare survival differences and statistical significance was assessed with the log-rank test ( Prism 4.0, GraphPad Software, San Diego, CA). The optimal EPHB4 intensity threshold to allocate patients to the high or low EPHB4 groups was determined as described in Supplementary Materials. In addition, the Cox regression model was used to assess associations between survival and EPHB4 levels when considered as a continuous variable as well as the simultaneous contribution of the following covariates: EPHB4 tumor levels, histologic grade, sex, age, and tumor location (colon/rectum).

Western blot analysis. One hundred microgram aliquots of protein extracts from the indicated cell lines were fractionated in a 7% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and probed with an anti-EPHB4 antibody as described previously (1:200 dilution; clone 3D7G8; ref. 10). The blot was then stripped and probed with an anti-β-actin antibody (clone AC74; 1:1,000; Sigma, St. Louis, MO; ref. 10). The signal from the β-actin probe was used as a loading control.

Methylation-Specific PCR

DNA methylation status of an EPHB4 promoter-associated CpG island (from −793 to −34) was determined in 112 primary colorectal tumors (Supplementary Table S3) and 8 cell lines. DNA was extracted from fresh frozen tumor samples by phenol/chloroform extraction (14). EPHB4 promoter hypermethylation was studied by bisulfite conversion of unmethylated, but not methylated, cytosines to uracil as described previously (15, 16). PCRs using primers specific for either the methylated or the modified unmethylated DNA [methylation-specific PCR (MSP)] were carried out to determine the methylation profile of each sample. Primers were designed using MethPrimer1.1 software (17). EPHB4 primers sequences for the methylated sequence were 5′-GTTTATTAGGGTTTGGGCGG-3′ (sense) and 5′-TATCCAAAACCGAACATATCGA-3′ (antisense) and 5′-TTTTATAGGGGTGGTTGCTT-3′ (sense) and 5′-CTATCCAAAACCAACATATCAGA-3′ (antisense) for the unmethylated sequence. PCR amplification was done using EcoStar DNA polymerase (EcoGen, Barcelona, Spain) under the following conditions: 95°C for 3 minutes of final extension at 72°C for 30 seconds at 95°C, 35 cycles of 30 seconds at 95°C, 30 seconds at 61°C, and 30 seconds at 72°C, and 4 minutes of final extension at 72°C. In vitro–methylated DNA (CpG Genome Universal Methylated DNA, Chemicon International, Temecula, CA) was used as a positive control for methylated alleles, whereas DNA from normal lymphocytes was used as a negative control.

Quantitative Real-time Reverse Transcription-PCR

SW620 cultures were treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-Aza-dC; Sigma) for 72 hours (0, 5, or 10 μmol/L). RNA was isolated using Totally RNA (Ambion, Austin, TX) according to the manufacturer’s recommendations. RNA aliquots were reverse transcribed and EPHB4 mRNA levels were quantified using real-time reverse transcription-PCR with the Taqman Gene Expression Assay Hs00174752_m1 (Applied Biosystems, Branchburg, NJ). 18S RNA was used as endogenous control. Relative levels of EPHB4 were quantified using the ΔΔCt method as described previously (10).

Clonogenic Assays

Two colon cancer cell lines expressing high levels of EPHB4 (HT29 and LIM2405; see Fig. 3) were transfected (Lipofectamine 2000, Invitrogen, Carlsbad, CA) with a vector expressing the extracellular and transmembrane domains of EPHB4 (amino acids 1-584) and enhanced green fluorescent protein (EGFP) in place of the cytoplasmic domain (pEPHB4-ΔC-EGFP) or with the corresponding control empty vector (pEGFP-N2; Clontech, Mountain View, CA). The resulting chimeric kinase-deficient protein has been shown previously to function as a dominant-negative form of EPHB4 (18, 19). After 24 hours, cells were trypsinized and plated at a 1:15 dilution in 100-mm plates. Two additional cell lines showing low levels of EPHB4 expression (SW837 and KM12; see Fig. 3) were transfected with a vector conferring neomycin resistance and expressing full-length EPHB4 (pECP-EPHB4) or the corresponding empty vector (pECPDA3.1, Invitrogen). After 24 hours, cells were trypsinized and plated at a 1:10 dilution in six-well plates. All cells were cultured for 14 days in the presence of 500 μg/mL neomycin (Invitrogen). All macroscopically visible colonies were scored after fixation with 4% paraformaldehyde and crystal violet staining. These experiments were done thrice in triplicate, and equal transfection efficiency of pEPHB4-ΔC-EGFP or pECP-EPHB4 compared with the corresponding empty control vectors was confirmed (P < 0.3, χ² test) through direct fluorescence-activated cell sorting analysis of the number of EGFP-positive cells or in parallel cotransfection experiments with pEGFP-C3 (Clontech), respectively.

Results and Discussion

Low-EPHB4 tumor levels identify a group of colorectal cancer patients with poor prognosis. The EPH/EFN family plays an important role in the maintenance of the normal intestinal epithelium and defects in the signaling cascades originated in some of these receptors are common in colorectal tumors (7, 8, 20, 21). Although EPHB4 is frequently lost in colorectal tumors (8), the clinical implications, the mechanism of inactivation, and the functional relevance of these losses have not been investigated.

In this study, we evaluate the potential value of EPHB4 as a prognostic marker in colorectal cancer by immunohistochemically assessing the level of EPHB4 tumor expression. In good agreement with EPHB4 being a direct transcriptional target of Wnt signaling (8), levels of EPHB4 expression were maximal in the lower crypt, where TCF/β-catenin transcriptional activity is highest, and a gradient of expression was observed with lower EPHB4 levels in the colonic flat mucosa (Fig. 1A, I and 2). The majority of tumors studied showed a membrane-associated pattern of expression (Fig. 1A, 3). The level of expression of EPHB4 was evaluated in sections of a TMA containing triplicate tumor samples from 137 Dukes C colorectal cancer patients from Finland (TMA-1). EPHB4 expression showed great variability in this tumor series from complete lack of immunoreactivity (Fig. 1A, 4) to very high levels of expression (Fig. 1A, 8). The level of expression of EPHB4 was assessed blinded from the clinical data using a semiquantitative scale from 0 (no staining) to 4 (strong staining). A significant association was observed between low EPHB4 levels and poor prognosis (Fig. 1B). The median disease-free survival (time to disease recurrence in 50% of the patients) in the low EPHB4 group was 1.8 years, whereas patients in the high EPHB4 group had a median survival >9 years (Fig. 1B).

When the EPHB4 staining intensity was considered as a continuous variable, a significant association with overall and disease-free survival was also observed (P < 0.05, Cox regression). Moreover, low EPHB4 tumor levels remained a strong independent marker of poor disease-free and overall survival (P = 0.009 and 0.005, respectively, Cox regression) on multivariate analyses using sex, age, tumor location (colon/rectum), histologic grade, and EPHB4 tumor levels as covariates. In addition, tumor samples from 16 regional lymph node metastases were included on the TMA used.
in these experiments. The expression of EPHB4 was significantly lower in the lymph node metastases compared with primary tumors \((P = 0.027, t\) test), further indicating that low EPHB4 levels are associated with disseminated disease. No associations were found between EPHB4 levels and other clinicopathologic variables (Supplementary Table S1).

To further validate our results, we used an independent set of 125 colorectal tumors from Spanish patients (TMA-2). Sections of this TMA were independently stained and scored for EPHB4 levels at a different institution blinded from the clinical data. Patients with low EPHB4 tumor levels had significantly shorter survival than patients with high EPHB4 tumor levels \((P = 0.02, t\) test; Supplementary Fig. S1). No associations were found between EPHB4 levels and other clinicopathologic variables (Supplementary Table S2). This independent validation further shows that low EPHB4 tumor levels can identify a subset of colorectal cancer patients with poor prognosis.

The clinical management of patients with locally advanced colorectal cancer is currently limited by the impossibility to accurately predict the probability of recurrence following surgical resection of the primary tumor. Patients with tumors that are indistinguishable at the histopathologic level frequently show great difference in the course of their disease. Approximately 50% of these patients are cured by the standard treatment, whereas the remaining 50% have disease recurrence and die of their disease within 5 years of initial treatment. We show here that low EPHB4 tumor levels can identify a subset of patients that have higher risk of disease recurrence and these patients could therefore benefit from a more aggressive treatment of their disease.

**Figure 1.** EPHB4 as a prognostic marker in colorectal cancer. **A**, 1 and 2, immunohistochemical staining revealed a gradient of EPHB4 expression in the normal colonic epithelium, with maximal levels in the lower crypt; 3, EPHB4 expression was localized to the cellular membrane in the majority of the colorectal tumors investigated; 4 to 8, there was great variability in EPHB4 expression in the 137 tumors in this study. **B**, Kaplan-Meier plots of overall and disease-free survival in 137 colorectal cancer patients as a function of EPHB4 tumor levels. Patients with low EPHB4 tumor levels (immunostaining <2.2) had significantly shorter survival than patients with high EPHB4 tumor expression \((P < 0.01, \text{log-rank test})\).

**Frequent hypermethylation of the EPHB4 promoter in colorectal tumors.** Loss of EPHB4 expression is a common event in colorectal tumors, but the mechanisms of EPHB4 inactivation have not been investigated. Hypermethylation of CpG islands within the promoter sequences of genes with tumor suppressor activity is emerging as an important mechanism capable of silencing these genes, and we have reported recently the presence of aberrant methylation of the promoter of EPHB2, a related EPH subfamily member, in >50% of the 101 colorectal tumors studied (15). The proximal promoter of EPHB4 contains a CpG island spanning 759 bp starting 34 bp upstream of the transcription start site, which could be methylated and thus regulate the expression of this gene. We used MSP with primers complementary to the methylated or unmethylated bisulfite-treated sequence to investigate the presence of promoter hypermethylation in 112 primary tumors.

We found evidence of EPHB4 promoter hypermethylation in 54 of the 112 tumors tested \((48.2%; \text{Fig. 2A})\). There was a trend for rectal cases to have higher incidence of promoter hypermethylation compared with tumors in the colon \((P = 0.06, \chi^2\) test), and a higher proportion of tumors from older patients showed EPHB4 promoter methylation \((P = 0.014, \text{Mann-Whitney test}; \text{Supplementary Table S3})\). Information about both EPHB4 protein levels and promoter hypermethylation was available from our immunostainings for 30 of these cases. The mean level of EPHB4 in the 14 tumors with promoter hypermethylation was 2-fold lower than in the 16 cases with no evidence of promoter methylation (mean EPHB4 IHC intensity, 0.8 and 1.6, respectively; \(P = 0.01, t\) test; Supplementary Table S3), suggesting that methylation of the EPHB4 promoter in
the region studied could regulate EPHB4 transcriptional activity. To further investigate this possibility, we identified a colon cancer cell line displaying EPHB4 promoter methylation (SW620) and exposed it to increasing concentrations of the DNA methyltransferase inhibitor 5-Aza-dC for 72 hours. This treatment resulted in demethylation of the EPHB4 promoter and a significant up-regulation of EPHB4 mRNA levels in SW620 cells (5-fold; Fig. 2B). Collectively, these results show that aberrant methylation of the EPHB4 promoter occurs frequently in colorectal tumors and that this mechanism can regulate EPHB4 expression. Increased TCF/β-catenin signaling is one of the hallmarks of colorectal cancer, and EPHB4 is a direct transcriptional target of this transcriptional complex (8). The finding that EPHB4 is frequently lost or reduced in colorectal tumors compared with normal colonic epithelium and premalignant lesions suggests that inactivation of EPHB4 is necessary for the oncogenic potential of the elevated TCF/β-catenin signaling to be fully realized. Here, we provide mechanistic evidence showing that hypermethylation of the regulatory sequences of EPHB4 is a common event that can result in reduced EPHB4 levels in colorectal tumors.

Modulation of EPHB4 levels affects the long-term clonogenicity of colon cancer cells. We next measured the levels of expression of EPHB4 protein in a panel of eight colorectal cancer cell lines. As observed in colorectal primary tumors, we found considerable variability in the level of EPHB4 protein (Fig. 3). Although four of these cell lines had low relative levels of EPHB4, only SW620 cells showed evidence of EPHB4 promoter hypermethylation, suggesting the existence of additional mechanisms of regulation of EPHB4 expression. Alternative mechanisms leading to the loss of EPHB4 expression remain to be elucidated but could include promoter methylation in other regulatory regions, EPHB4 mutations, transcriptional regulation, or increased protein degradation, and we are currently investigating these possibilities.

We then wanted to investigate the functional effects of reintroducing EPHB4 in colorectal cancer cells that have low levels

![Figure 2. EPHB4 promoter hypermethylation in colorectal tumors. A, primers specific for the methylated or unmethylated bisulfite-treated sequence were used to PCR amplify DNA samples from a series of 112 colorectal tumors. Representative results of tumor samples with and without EPHB4 promoter hypermethylation. U, unmethylated; M, methylated; NL, DNA from normal lymphocytes; IVD, in vitro–methylated DNA; arrows, tumors with EPHB4 promoter methylation. B, bottom, treatment of SW620 cells showing EPHB4 promoter hypermethylation with the DNA methyltransferase inhibitor 5-Aza-dC for 72 hours resulted in a substantial reduction in the level of EPHB4 promoter methylation as revealed by PCR amplification with primers specific for the methylated bisulfite-treated sequence (see Materials and Methods); top, measurement of relative EPHB4 mRNA levels by real-time RT-PCR showed that demethylation of the EPHB4 promoter was associated with a significant increase in EPHB4 levels in 5-Aza-dC-treated cells.](#)

![Figure 3. EPHB4 expression in colorectal cancer cell lines. Western blotting with a monoclonal EPHB4 antibody revealed substantial variability in EPHB4 expression in colorectal cancer cell lines. β-Actin was used as a loading control.](#)
of this tyrosine receptor kinase on tumor cell survival. For this purpose, we transfected two colon cancer lines expressing low EPHB4 levels (SW837 and KM12) with an EPHB4 expression vector. The number of colonies observed following 14 days of selection with neomycin was scored in three independent experiments in triplicate. Forced expression of EPHB4 in SW837 and KM12 cells resulted in a significant reduction in the number of neomycin-resistant colonies (3-fold reduction; \( P < 0.0008, t \) test; Fig. 4A and B), showing that restoring EPHB4 expression can reduce the clonogenic potential of colorectal cancer cells. In addition, we transfected two cell lines expressing high EPHB4 levels (HT29 and LIM2405) with a vector expressing a dominant-negative form of EPHB4 resulted in a significant increase in the number of neomycin-resistant colonies. Bottom, representative examples of crystal violet–stained colonies; top, results of quantifying the number of neomycin-resistant colonies in one of three independent experiments run in triplicate. Columns, mean; bars, SD; \( P \)s are from Student’s \( t \) test.

EPHB4 is overexpressed in other tumor types, such as prostate and bladder tumors, compared with the corresponding normal tissue of origin (1, 2, 22). Interestingly, down-regulation of EPHB4 in these tumors resulted in a significant reduction in cell viability. There is, however, strong evidence in the literature for a tumor suppressor role of EPHB2 and EPHB3 receptors in colorectal cancer (6, 8), and our results linking EPHB4 levels with long-term clonogenicity, together with the finding that EPHB4 expression is frequently reduced or lost in colorectal tumors and that this is associated with poor patient prognosis, suggest that EPHB4 may be a novel tumor suppressor gene in colorectal cancer. This is in good agreement with an earlier report showing that EPHB4 is lost more frequently in advanced tumors compared with early-stage and premalignant lesions (8) and with our finding that lymph node metastasis have lower levels of EPHB4 than primary tumors.

Epigenetic silencing of tumor suppressor genes through aberrant methylation of their promoter sequence is a common event in cancer cells and is of key importance in the initiation and progression of human tumors. Recently, there has been considerable interest in epigenetic therapeutic strategies with DNA methyltransferase inhibitors, such as 5-azacytidine and 5-Aza-dC,

![Figure 4. EPHB4 regulates the clonogenic potential of colon cancer cells. A and B, transfection of SW837 and KM12, two colon cancer cell lines with low EPHB4 levels, with an EPHB4 expression vector resulted in a significant reduction in the number of neomycin-resistant colonies after 14 days of selection; C and D, transfection of two colon cancer cell lines with high EPHB4 expression levels (HT29 and LIM2405) and with a vector expressing a dominant-negative form of EPHB4 resulted in a significant increase in the number of neomycin-resistant colonies. Bottom, representative examples of crystal violet–stained colonies; top, results of quantifying the number of neomycin-resistant colonies in one of three independent experiments run in triplicate. Columns, mean; bars, SD; \( P \)s are from Student’s \( t \) test.](image)
aimed at the targeted demethylation of methylated regulatory sequences of tumor suppressor genes in cancer cells (23). These nucleoside analogues are currently undergoing clinical testing and showing encouraging results in hematopoietic malignancies (24). Our finding showing that the promoter of EPHB4 (and EPHB2; ref. 15) is methylated in ~50% of colorectal tumors, coupled with the observation that colorectal cancer patients with higher tumor levels of EPHB4 (and EPHB2; ref. 25) have significantly better prognosis than patients with lower EPHB receptor levels and that restoring EPHB4 expression in EPHB4-deficient cells severely compromises the clonogenic potential of tumor cells, may provide the necessary rationale for testing the efficacy of these nucleoside analogues in colorectal cancer patients.

In conclusion, in this study, we show that EPHB4 expression is frequently reduced or lost in colorectal tumors and that reintroduction of EPHB4 into EPHB4-deficient tumor cells significantly reduces the long-term clonogenic potential of these cells, suggesting that EPHB4 has tumor suppressor activities in colorectal cancer cells. Moreover, we show that EPHB4 promoter hypermethylation is a common mechanism of EPHB4 inactivation. Collectively, these results significantly contribute to establish EPHB4 as a new putative tumor suppressor gene and strengthen the emerging view of an important role of the EPH/EFN family of RTKs in colorectal cancer. In addition, we show that low EPHB4 tumor levels identify a subset of colorectal cancer patients with high risk of recurrence. Therefore, assessment of EPHB4 levels in biopsy or resection tumor samples could be used to identify poor prognosis patients that could benefit from more aggressive treatment options.

Acknowledgments


Grant support: Sigrid Juselius Foundation grant, the Spanish Fondo de Investigaciones Sanitarias grant PI051394, and Fundación de Investigación Médica Mutua Madrileña grant (D. Arango); grants from Academy of Finland (48570/Finnish Center of Excellence Program 2000-2005, 76227, 77547, 213183, and 214268), Sigrid Juselius Foundation, Cancer Society of Finland, and Helsinki University Central Hospital (L.A. Aaltonen); National Cancer Institute grant CA10082-01 (J.M. Mariadason); grant AGL2004-07579-04-04/ALI del Plan Nacional 1-D (G. Capella); and Vall d’Hebron Research Institute Predoctoral Fellowship (V. Davalos).

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We thank Drs. Jens Girenke (Research Laboratories of Schering AG, Berlin, Germany) and Elena B. Pasquale (Burnham Institute, La Jolla, CA) for kindly providing the vector pcDNA-EPHB4 and pEPHB4-ΔECFP, respectively, and Laura García Latorre for her technical assistance.

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