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Gene expression patterns in adenoid cystic carcinoma with and without diffuse NOTCH1 intracellular domain (NICD1) immunohistochemistry staining

Introduction

Adenoid cystic carcinoma (ACC) is a rare salivary gland cancer with diverse tumour growth kinetics. Studies have applied proteo-genomic approaches to identify poorer prognostic subgroups [1–5]. Most recently, Ferrarotto *et al.* described two groups: ACC-I characterised by *NOTCH1* activating mutations, MYC and NOTCH1 intracellular domain (NICD1) protein expression and reduced survival; and ACC-II differentiated by TP63 and EGFR protein overexpression and better outcomes [5].

In our previous study of 120 ACC patients, we demonstrated adverse clinical outcomes with *NOTCH1* activating mutations and/or NICD1 diffuse immunohistochemical (IHC) staining [6]. We have now used comprehensive transcriptome analysis to determine the differential gene expression patterns in tumour samples with and without NICD1 diffuse staining from a cohort of the above patients. To examine whether these patterns allow for stratification in an independent dataset, we used the differentially expressed gene-sets identified from these tumours to analyse the ACC transcriptomic dataset reported by Ferrarotto *et al.* [5].

Results

Of 92 ACC tumour samples successfully evaluated for NICD1 protein expression on IHC, 8 (9%) were positive for diffuse NICD1 staining (see Supplementary data for methods). The median overall survival (OS) from diagnosis in the NICD1-positive (n = 8) and NICD1-negative (n = 84) tumours was 3.5 and 14.3 years, respectively ($p < 0.0001$) (Figure 1A); whilst median OS from recurrence/metastatic disease was 1.1 years for the NICD1-positive group and 6 years for the NICD1-negative cohort ($p < 0.0001$) (Figure 1B). Median recurrence-free survival (RFS) was 1.6 years for NICD1-positive ACC compared with 4.5 years for NICD1-negative tumours ($p = 0.08$).

Bulk RNA sequencing was successful in 5/8 NICD1-positive tumours, which were selected for further analysis. *NOTCH1* genomic alterations were detected in 4/5 NICD1-positive tumours (three tumours having gain-of-function mutations; one with copy number gain) using targeted DNA-based next generation sequencing (NGS). However, certain *NOTCH1* mutations (e.g., gene rearrangements, intronic insertions) are missed by targeted DNA NGS [7]. These samples were analysed alongside six NICD1-negative ACC tumour samples (see Supplementary data) and a tumour sample with a *NOTCH1* loss-of-function mutation.

Multi-dimensional scaling (MDS) analysis of the RNA gene expression data showed that the samples separate into two groups reflecting NICD1 status (Figure 2A). More heterogeneity was observed in the NICD1-negative tumours, two of which (p-6 and p-8) clustered with the NICD1-positive samples. Notably, p-6 harboured two likely loss-of-function mutations in *SPEN,* a negative regulator of *NOTCH* target genes [8], R1418* and Q1250fs*27; whereas p-8 possessed a *TP53* loss-of-function mutation, which has also been associated with reduced survival [9]. One NICD1-positive sample (p-2) possessing a *NOTCH1* copy number gain rather than a *NOTCH1*-activating mutation placed proximally to the NICD1-negative samples.

Differential gene expression analysis identified 340 significantly differentially expressed genes (false discovery adjusted p-value < 0.05) between NICD1-positive and NICD1-negative tumours. *MYB* was significantly more expressed ($p = 0.003$) in NICD1-positive ACC compared to NICD1-negative disease. *MYC, BCL2* and *HEY1*, also characteristic of ACC-I, showed higher expression in the NICD1-positive group but this was not statistically significant.

Unsupervised hierarchical clustering of significantly differentially expressed genes based on NICD1 status revealed two distinct RNA expression patterns. We applied these acquired gene sets to the data of Ferrarotto *et al.* [5], including only genes evaluated in both our cohort and that of Ferrarotto *et al.* This divided the tumours within Ferrarotto *et al.*'s dataset into two subgroups which closely correlated with ACC-I and -II subtypes. However, these subgroups showed less concordance with NICD1 status (Figure 2B). Using a Support Vector machine model trained on the gene sets to separate NICD1 status, we found that the classification accuracy was higher in predicting ACC type than NICD1 status in the Ferrarotto *et al.* dataset (Figure 2C). However, our differentially expressed gene sets performed similarly to several random gene sets (13/100) in predicting ACC or NICD1 status in the Ferrarotto *et al.* dataset. This could be indicative of more widespread gene expression changes that distinguish ACC subtype and NICD1 status, respectively, and warrants further analysis.

Discussion

NOTCH1 activating mutations occur in approximately 18% of recurrent/metastatic ACC; whilst alterations of other components of the NOTCH pathway are present in up to 40% of cases [4]. Diffuse NICD1 staining has been shown to be a sensitive marker of *NOTCH1* activating mutations and solid histopathology [1,3,5]. In our study, 9% of tumours exhibited diffuse NICD1 staining; similar to the prevalence described by Sajed *et al.* [3] but fewer than reported by Ferrarotto *et al.* [5]. This may be partly explained by the different thresholds used to define NICD1 positive staining by IHC. Ferrarotto *et al.* defined NICD1 positivity as nuclear staining in ≥70% tumour cells; whilst a cut-off of >90% was

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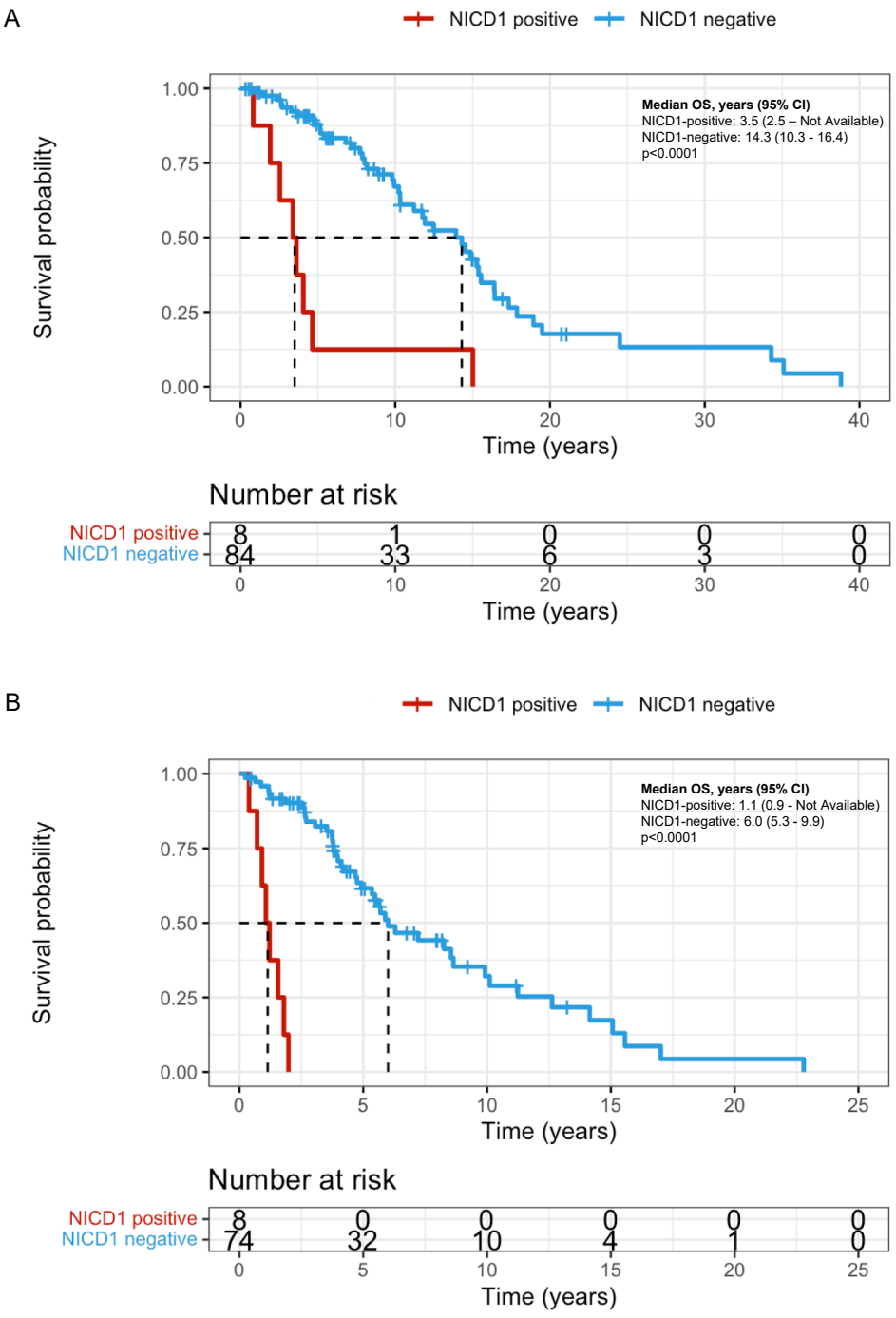

Figure 1. A. Kaplan-Meier survival curve demonstrating overall survival from diagnosis, defined as time from diagnosis of any stage ACC to death from any cause, dashes indicate censored events. Red colour indicative of patients with NICD1-positive tumours; blue colour indicative of patients with NICD1-negative tumours. Upper limit of the confidence interval for the median survival time of NICD-positive tumour group could not be estimated due to insufficient numbers. **B.** Kaplan-Meier survival curve demonstrating overall survival from recurrence/metastatic disease, defined as time from first recurrence or metastatic disease to date of death from any cause, dashes indicate censored events. Red colour indicative of patients with NICD1-positive tumours; blue colour indicative of patients with NICD1-negative tumours. Upper limit of the confidence interval for the median survival time of NICD-positive tumour group could not be estimated due to insufficient numbers.

used in this study and by Sajed *et al.*

Our results show reduced survival rates for NICD1-positive ACC compared with NICD1-negative tumours, corroborating the results of previous studies [3,6]. NICD1-positive and -negative ACC tumours clustered into two groups following MDS analysis of RNA data. Outlying samples may result from genetic alterations outside of the *NOTCH* genes. For example, p-6 – an NICD1-negative sample found adjacent to NICD1-positive tumour – harboured biallelic *SPEN* loss-of-function mutations, thus potentially resulting in the upregulation of *NOTCH* target genes, independent of NICD1 activity.

The gene expression patterns identified through RNA-sequencing in our study more accurately predicted ACC subtype than NICD1 status in the Ferrarotto *et al.* dataset [5]. One possible explanation is that diffuse NICD1 staining is not wholly representative of the ACC-I subtype, which may include tumours with "off-*NOTCH1*" mutations that influence Notch target gene expression, such as *SPEN* mutations. Indeed, in Ferrarotto's study, 3/19 samples in the ACC-I subgroup were NICD1-negative. A possible confounding factor is the different definition of NICD1 positivity used in Ferrarotto *et al.*'s study, which may explain the higher number of ACC-II tumours (9/31) deemed NICD1-positive and why OS differences between NICD1-positive and -negative ACC were non-statistically significant ($p = 0.07$).

In summary, our data suggest that while NICD1-positive ACC has distinct biological characteristics and is predictive of worse outcomes, it

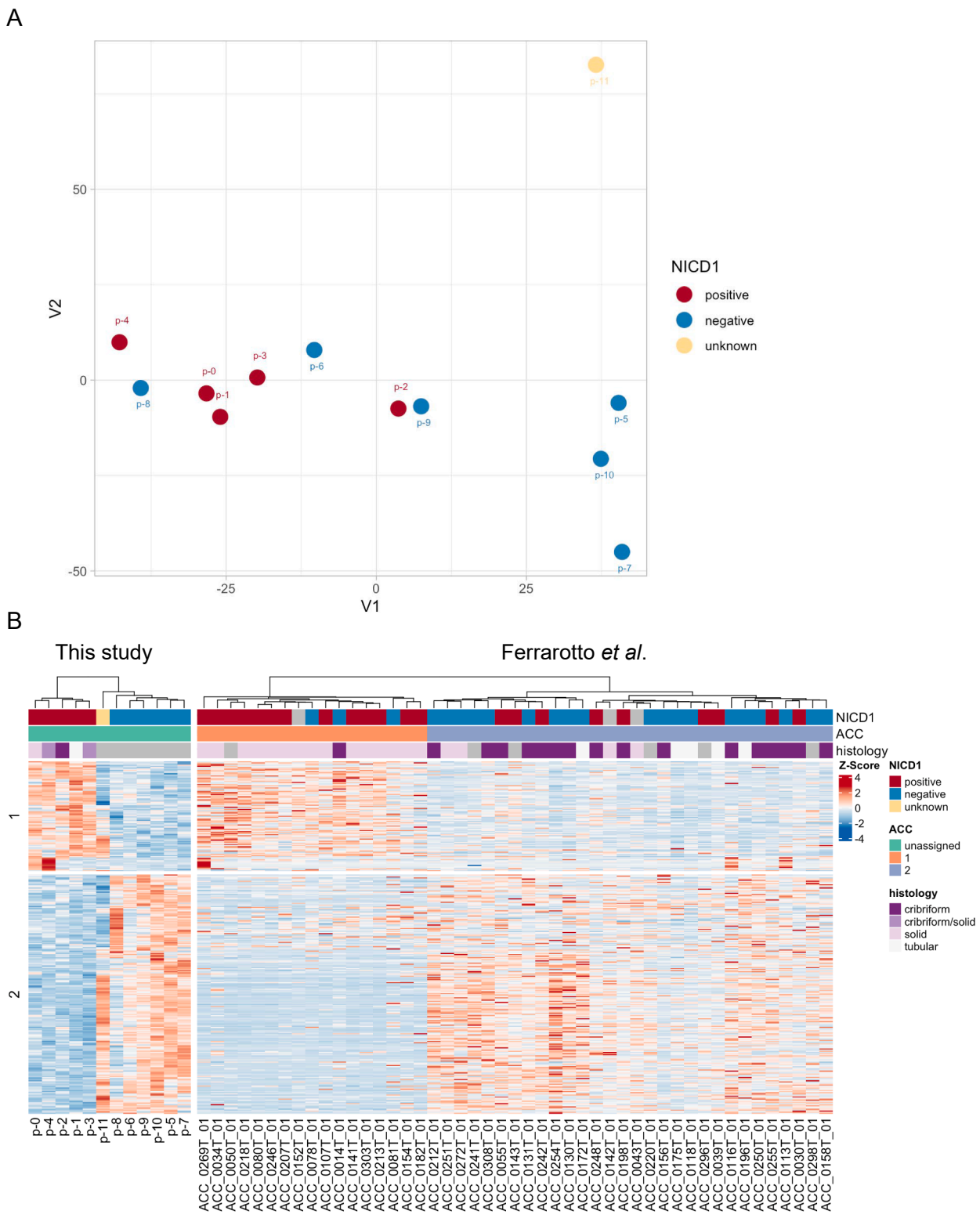

Figure 2. **A**. Multidimensional Scaling of RNA differential gene expression data of NICD1-positive and NICD-1 negative ACC tumour samples. Red colour indicative of NICD1-positive status; blue colour indicative of NICD1-negative status. Yellow colour indicative of unknown NICD1 status; this sample possessed a *NOTCH1* loss-of-function mutation. **B**. Combined Heatmaps of this cohort (left) and Ferrarotto *et al.* (right). Heatmaps of statistically significant differentially expressed genes that are shared between both datasets. NICD1 status as determined by IHC, ACC class and histology subtypes are colour coded. Red colour indicative of NICD1-positive status; blue samples indicative of NICD1-negative status. Yellow colour sample had unknown NICD1 status and possessed a *NOTCH1* loss-of-function mutation. Expression values for each gene (row) are normalized across samples of the respective cohort - red and blue shadings represent higher and lower relative expression levels, respectively. Hierarchical clustering dendrograms are indicated above the samples. **C**. Box-violin plot for observed classification accuracies for ACC subtype or NICD1 staining status predictions. Random or statistically differentially expressed gene sets (green dot) were used to construct SVM model based on this study samples and predict sample class in the Ferrarotto *et al.* samples.

Figure 2. . (*continued*).

may not be representative of all aggressive cases of ACC. Additional work with larger ACC cohorts incorporating clinical, genomic, transcriptomic, and proteomic data is merited to further elucidate the role of various biomarkers in ACC prognostication.

CRediT authorship contribution statement

Karan Patel: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Massimiliano Manzo:** Data curation, Formal analysis, Visualization, Investigation, Writing - review & editing. **Brindley Hapuarachi:** Data curation, Writing – review & editing. **Samuel Rack:** Data curation, Writing - review & editing. **Philip Jermann:** Investigation, Formal analysis, Supervision, Data curation, Visualization. **Laura Feeney:** Writing – review & editing. **Emily Heathcote:** Writing – review & editing. **Guy Betts:** Writing – review & editing. **Jon C. Aster:** Methodology, Investigation, Writing – review & editing. **Maximilien Murone:** Methodology, Writing - review & editing. **Maria Bobadilla:** Methodology, Resources, Writing - review & editing. **Rajwinder Lehal:** Methodology, Resources, Writing - review & editing. **Florian D. Vogl:** Methodology, Resources, Writing - review & editing. **Kevin Harrington:** Writing – review & editing. **Robert Metcalf:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships: Jon C. Aster serves on the Scientific Advisory Board of Cellestia Biotech AG., and is a consultant to Ayala Pharmaceuticals and Remix Therapeutics. Maximilien Murone is a former employee of Cellestia Biotech AG. Florian D. Vogl is a former employee of Cellestia Biotech AG. Maria Bobadilla, Raj Lehal are employees of Cellestia Biotech AG. Kevin Harrington declares honoraria (paid to his institute) from Arch Oncology, AstraZeneca, Boehringer-Ingelheim, Bristol-Myers-Squibb, Codiak, F-Star, Merck-Serono, MSD, Pfizer, Replimune. Robert Metcalf's conflict of interest includes Bristol-Myers Squibb, Merck Sharp & Dohme, Roche, Bayer, Achillies Therapeutics, Aptus Clinical, PCI Biotech, Ayala Pharmaceuticals and OxSonics. All other authors declare no conflict of interest.

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Institutional Review Board Statement

The study was granted research ethics approval under the MCRC Biobank Research Tissue Bank Ethics (NHS NW Research Ethics Committee 18/NW/0092) and was performed in accordance with the Declaration of Helsinki.

Informed Consent Statement

All subjects provided informed consent to collection of demographic, clinical and genomic data included in this study. One additional patient included in the above-mentioned research study provided informed consent to the clinical trial NCT 03422679 that had received Ethics Committees' and Competent Authorities' approval, permitting analysis of patients' tumour molecular profile.

Appendix A. Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2023.106542.

References

[1] Ferrarotto R, Mitani Y, Diao L, Guijarro I, Wang J, Zweidler-McKay P, et al. Activating NOTCH1 mutations define a distinct subgroup of patients with adenoid cystic carcinoma who have poor prognosis, propensity to bone and liver metastasis, and potential responsiveness to notch1 inhibitors. J Clin Oncol 2017;35(3):352–60.

[2] Frerich CA, Brayer KJ, Painter BM, Kang H, Mitani Y, El-Naggar AK, et al. Transcriptomes define distinct subgroups of salivary gland adenoid cystic carcinoma with different driver mutations and outcomes. Oncotarget 2018;9(7):7341–58.

[3] Sajed DP, Faquin WC, Carey C, Severson EA, H. Afrogheh A, A. Johnson C, et al. Diffuse staining for activated NOTCH1 correlates with NOTCH1 mutation status and is associated with worse outcome in adenoid cystic carcinoma. Am J Surg Pathol 2017;41(11):1473–82.

[4] Ho AS, Ochoa A, Jayakumaran G, Zehir A, Valero Mayor C, Tepe J, et al. Genetic hallmarks of recurrent/metastatic adenoid cystic carcinoma. J Clin Invest 2019;129:4276–89. https://doi.org/10.1172/JCI128227.

[5] Ferrarotto R, Mitani Y, McGrail DJ, Li K, Karpinets TV, Bell D, et al. Proteogenomic analysis of salivary adenoid cystic carcinomas defines molecular subtypes and identifies therapeutic targets. Clin Cancer Res 2021;27:852–64. https://doi.org/10.1158/1078-0432.CCR-20-1192.

[6] Feeney L, Hapuarachi B, Adderley H, Rack S, Morgan D, Walker R, et al. Clinical disease course and survival outcomes following disease recurrence in adenoid cystic carcinoma with and without NOTCH signaling pathway activation. Oral Oncol 2022;133. https://doi.org/10.1016/j.oraloncology.2022.106028.

[7] Ferrarotto R, Mishra V, Herz E, Yaacov A, Solomon Oz, Rauch R, et al. AL101, a gamma-secretase inhibitor, has potent antitumor activity against adenoid cystic carcinoma with activated NOTCH signaling. Cell Death Dis 2022;13(8). https://doi.org/10.1038/s41419-022-05133-9.

[8] Oswald F, Kostezka U, Astrahantseff K, Bourteele S, Dillinger K, Zechner U, et al. SHARP is a novel component of the Notch/RBP-Jkappa signalling pathway. EMBO J 2002;21:5417–26. https://doi.org/10.1093/emboj/cdf549.

[9] Adderley H, Rack S, Hapuarachi B, Feeney L, Morgan D, Hussell T, et al. The utility of TP53 and PIK3CA mutations as prognostic biomarkers in salivary adenoid cystic carcinoma. Oral Oncol 2021;113:105095.

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