Title

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Journal

JTO Clinical and Research Reports, 2(2)

ISSN

2666-3643

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Publication Date

2021-02-01

DOI

10.1016/j.jtocrr.2020.100132

Identification of Novel *CDH1-NRG2α* and *F11R-NRG2α* Fusions in NSCLC Plus Additional Novel *NRG2α* Fusions in Other Solid Tumors by Whole Transcriptome Sequencing

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ABSTRACT

Introduction: A novel *CD74-NRG2α* fusion has recently been identified in NSCLC. We surveyed a large tumor database comprehensively profiled by whole transcriptome sequencing to investigate the incidence and distribution of *NRG2* fusions among various solid tumors.

Methods: Tumor samples submitted for clinical molecular profiling at Caris Life Sciences (Phoenix, AZ) that underwent whole transcriptome sequencing (NovaSeq [Illumina, San Diego, CA]) were retrospectively analyzed for *NRG2* fusion events. All *NRG2* fusions with sufficient reads ($\geq$ three junctional reads spanning $\geq$ seven nucleotides) were identified for manual review, characterization of fusion class, intact functional domains, EGF-like domain isoforms, breakpoints, frame retention, and co-occurring alterations by next-generation sequencing (NextSeq [Illumina, San Diego, CA], 592 genes).

Results: Seven inframe functional (containing the intact EGF-like domain) *NRG2α* fusions were identified, namely, the following: (1) NSCLC (two of 9600, 0.02%: *CDH1-NRG2α* [C11, N2], *F11R-NRG2α* [F1, N4]); (2) endometrial (two of 3060, 0.065%: *CPM-NRG2α* [C2, N2], *OPA3-NRG2α* [O1, N2]); (3) ovarian (one of 5030, 0.02%: *SPON1-NRG2α* [S6, N2]); (4) prostate (one of 1600, 0.063%: *PLPP1-NRG2α* [P1, N2]); and (5) carcinoma of unknown origin (one of 1400, 0.07%: *CYSTM1-NRG2α* [C2, N2]). No *NRG2β* fusions were identified. Both NSCLC samples contained the reciprocal *NRG2* fusions (*NRG2-CDH1*, *NRG2-F11R*). Almost all inframe *NRG2α* fusions have no (N = 6, 85.7%) or low (N = 1, 14.3%) programmed death-ligand 1 expression. No additional known driver mutations were identified in these seven *NRG2α* fusion-positive tumor samples.

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Disclosure: Dr. Ou has stock ownership and was on the scientific advisory board of Turning Point Therapeutics Inc. (until February 28, 2019); is a member of the scientific advisory board of Elevation Oncology and has stock ownership in Elevation Oncology; has received speaker honoraria from Merck, Roche/Genentech, AstraZeneca, Takeda/ARIAD and Pfizer; and has received advisory fees from Roche/Genentech, AstraZeneca, Takeda/ARIAD, Pfizer, Foundation Medicine Inc., Spectrum, Daiichi Sankyo, Janssen/Johnson and Johnson, and X-Covery. Drs. Xiu, Q. Zhang, Swensen, Spetzler, and Korn are employees of Caris Life Science. Dr. Nagasaka has received honoraria from AstraZeneca, Caris Life Sciences, Daiichi Sankyo, Takeda, Novartis, EMD Serono, and Tempus. Dr. Xia has received honoraria from AstraZeneca, Roche/Genentech, and Regeneron Pharmaceuticals. Dr. Zhu has received honoraria from AstraZeneca, Blueprint, Roche-Foundation Medicine, Roche/Genentech, Takeda, and Xcovery; had stock ownership of TP Therapeutics (until May 2020). Dr. Liu has received advisory fees from AstraZeneca, Blueprint, Bristol-Myers Squibb, Celgene, G1 Therapeutics, Genentech/Roche, Guardant Health, Inivata, Janssen, Jazz, Eli Lilly, Merck/Merck Sharp & Dohme, PharmaMar, Pfizer, Regeneron, and Takeda; nonfinancial support from AstraZeneca, Boehringer Ingelheim, Genentech/Roche, and Merck/Merck Sharp & Dohme; and research grant support (to institution) from Alkermes, AstraZeneca, Bayer, Blueprint, Bristol-Myers Squibb, Corvus, Genentech, Janssen, Eli Lilly, Lycera, Merck, Molecular Partners, Pfizer, Rain Therapeutics, RAPT, Spectrum, and Turning Point Therapeutics. Dr. S. Zhang declares no conflict of interest.

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Cite this article as: Ou S-HI, et al. Identification of Novel *CDH1-NRG2α* and *F11R-NRG2α* Fusions in NSCLC Plus Additional Novel *NRG2α* Fusions in Other Solid Tumors by Whole Transcriptome Sequencing. JTO Clin Res Rep 2:100132

ISSN: 2666-3643

https://doi.org/10.1016/j.jtocrr.2020.100132

Conclusions: Similar to *NRG1* fusions, *NRG2α* fusions are recurrent and rare ligand-fusions in NSCLC and other multiple tumor types, especially gynecologic malignancies.

Keywords: *NRG2* fusion; *CDH1-NRG2α*; *F11R-NRG2α*; Whole transcriptome sequencing; ligand-fusion- positive malignancies

Introduction

Recently, a novel *CD74-NRG2α* fusion was identified in a Japanese NSCLC patient by whole transcriptome sequencing (WTS).[1] Neuregulin-2 (NRG2) belongs to a family of six closely related members (NRG1-6) of signaling ligands to the HER receptor tyrosine kinase family members through the frequently shared EGF-like domain.[2] The EGF-like domain is encoded by a core EGF-like domain exon and either an α- or β-specific EGF domain exon among each *NRG* gene.[2] *NRG1* fusions have been identified in multiple tumor types, albeit at a very low frequency, with anecdotal evidence of *NRG1* fusions responding to HER2/HER3 or pan-HER blockade approach.[3–5] We undertook this study to assess the incidence of *NRG2* fusions in solid tumors that had undergone profiling by WTS.

Materials and Methods

This study was conducted in accordance with guidelines of the Declaration of Helsinki, Belmont report, and U.S. Common rule. In keeping with 45 CFR 46.101(b)(4), this study was performed utilizing retrospective, deidentified clinical data. Therefore this study is considered IRB exempt and no patient consent was necessary from the subject. All unique cases submitted to a Clinical Laboratory Improvement Amendments–certified laboratory (Caris Life Sciences, Phoenix, AZ) for comprehensive genomic profiling, that underwent successful fusion testing by WTS were identified. All histologic characteristics were reviewed by board-certified pathologists. An American Board of Medical Genetics and Genomics–certified geneticist (Dr. Swensen) determined whether the *NRG2* fusions were inframe or out-of-frame, including occasionally midexonic breakpoints, which can sometimes be indicators of artifactual fusion events (that arise through misalignment, mispriming, etc.).

Gene Fusions Detection by WTS

Gene fusion detection was performed on mRNA isolated from formalin-fixed, paraffin-embedded (FFPE) tumor samples using the Illumina NovaSeq platform (Illumina, Inc., San Diego, CA) and Agilent SureSelect Human All Exon V7 bait panel (Agilent Technologies, Santa Clara, CA). FFPE specimens underwent pathology review to diagnose percent tumor content and tumor size; a minimum of 10% tumor content in the area for microdissection was required to enable enrichment and extraction of tumor-specific RNA. Qiagen RNA FFPE tissue extraction kit (Qiagen, Hilden, Germany) was used for extraction, and RNA quality and quantity were determined using the Agilent TapeStation (Agilent Technologies). Biotinylated RNA baits were hybridized to the synthesized and purified complementary DNA targets, and the bait-target complexes were amplified by postcapture polymerase chain reaction. The resultant libraries were quantified and normalized. The pooled libraries were denatured, diluted, and sequenced; the reference genome used was GRCh37/hg19, and analytical validation of this test revealed greater than or equal to 97% positive percent agreement, greater than or equal to 99% negative percent agreement, and greater than or equal to 99% overall percent agreement with a validated comparator method.

Immunohistochemistry was performed on full FFPE sections of glass slides. Slides were stained using automated staining techniques per manufacturer's instructions and were optimized and validated per Clinical Laboratory Improvement Amendments and College of American Pathologists and International Organization for Standardization requirements. For NSCLC, the primary programmed death-ligand 1 (PD-L1) antibody clone was 22c3 (Dako, Agilent Technologies). The tumor proportion score, or the percentage of viable tumor cells exhibiting partial or complete membrane staining at any intensity, was measured. For non-NSCLC tumors, the primary PD-L1 antibody used was SP142 (Spring Biosciences, Pleasanton, CA). The staining was regarded as positive if the intensity on the membrane of the tumor cells was greater than or equal to plus 2 (on a semiquantitative scale of 0–3: 0 for no staining, 1+ for weak staining, 2+ for moderate staining, or 3+ for strong staining) and the percentage of positively stained cells was greater than 5%. The determination of tumor mutational burden (TMB) microsatellite status has also been described.[6,7] The α-isoforms and β-isoforms of *NRG2* fusions are determined by manually reviewing the junction reads of exon 4 and 5 of the EGF domains.

Table 1. List of Inframe *NRG2a* Fusions in NSCLC and Other Solid Malignancies (Endometrial, Ovarian, Prostate, and Unknown Primary) and Their Associated Molecular and Pathologic Characteristics

Number	Age, Sex, Tumor source	Fusion Partner	Chromosomal Location	Exons Breakpoints[a]	Junctional Read	TMB (muts/MB)	PD-L1 (TPS, %)	MSI/MMR	Associated Genetic Alterations	Chromosomal Breakpoints	RNA TPM EGFR	RNA TPM ERBB2	RNA TPM ERBB3	RNA TPM ERBB4
NSCLC														
1	81, M, primary lung (adenocarcinoma)	CDH1[b]	16q22.1	(C11, N2)	88	7	0	Stable	ARID1A, CTNNB1, GNAS, SMAD4	chr16:68853328:+/ chr5:139267096:-	34.3865	35.4288	150.347	NA
2	63, M, primary lung (adenocarcinoma)	F11R[b]	1q23.3	(F1, N4)	87	7	10	Stable	FH, HOXB13, TP53, CNA: NOTCH2, CDK6	chr1:160990800:-/ chr5:139251426:-	13.0594	25.289	52.5874	2.0615
Endometrial carcinoma														
3	65, F, lung metastasis (adenocarcinoma)	CPM	12q15	(C2, N2)	43	9	0	Stable	ARIAD1A; PIK3CA; SPOP; TP53	chr12:69326458:-/ chr5:139267096:-	20.3438	179.383	155.371	82.87067
4	64, F, lymph node metastasis (carcinosarcoma)	OPA3	19q13.32	(O1, N2)	11	10	0	Stable	TP53 CNA: RARA, NSD2, ERBB3, FGFR3	chr19:46087881:-/ chr5:139267096:-	30.7567	92.45705	419.4975	75.2951
Ovarian adenocarcinoma														
5	61, F, liver metastasis (serous adenocarcinoma)	SPON1	11p15.2	(S6, N2)	14	5	0	Stable	TP53, MAP3K1, CNA: MCL1, CCNE1, ATP1A1, TRIM33	chr11:14101567:+/ chr5:139267096:-	2.9828	35.3005	24.6104	26.926
Prostate adenocarcinoma														
6	81, M, supra-clavicular lymph node metastasis (adenocarcinoma)	PLPP1	5q11.2	(P1, N2)	4	9	0	Stable	TMPRSS2-ERG ARv7 variant	chr5:54830400:-/ chr5:139267096:-	34.1716	39.888	59.7252	12.5726
Carcinoma of unknown primary														
7	71, M, liver metastasis (adenocarcinoma)	CYSTM1	5q31.3	(C2, N2)	9	3	0	Stable	CHEK2, TP53	chr5:139574237:+/ chr5:139267096:-	26.7383	26.2464	62.0065	0.58000

[a]The nomenclature of fusion breakpoint is first alphabet of the fusion partner, followed by the exon number in which the fusion occurs. Similarly, the same nomenclature for *NRG2* fusion in which the exon number in which the fusion occurred is listed after N.
[b]Reciprocal fusions were identified.
CAN, copy number alterations; F, female; M, male; MMR, mismatch repair; MSI, microsatellite instability; Muts/MB, mutations per megabase; NA, not applicable; *NFR2*, neuregulin-2; PD-L1, programmed death-ligand 1; TMB, tumor mutation burden; TPM, transcript per millions; TPS, tumor proportion score.

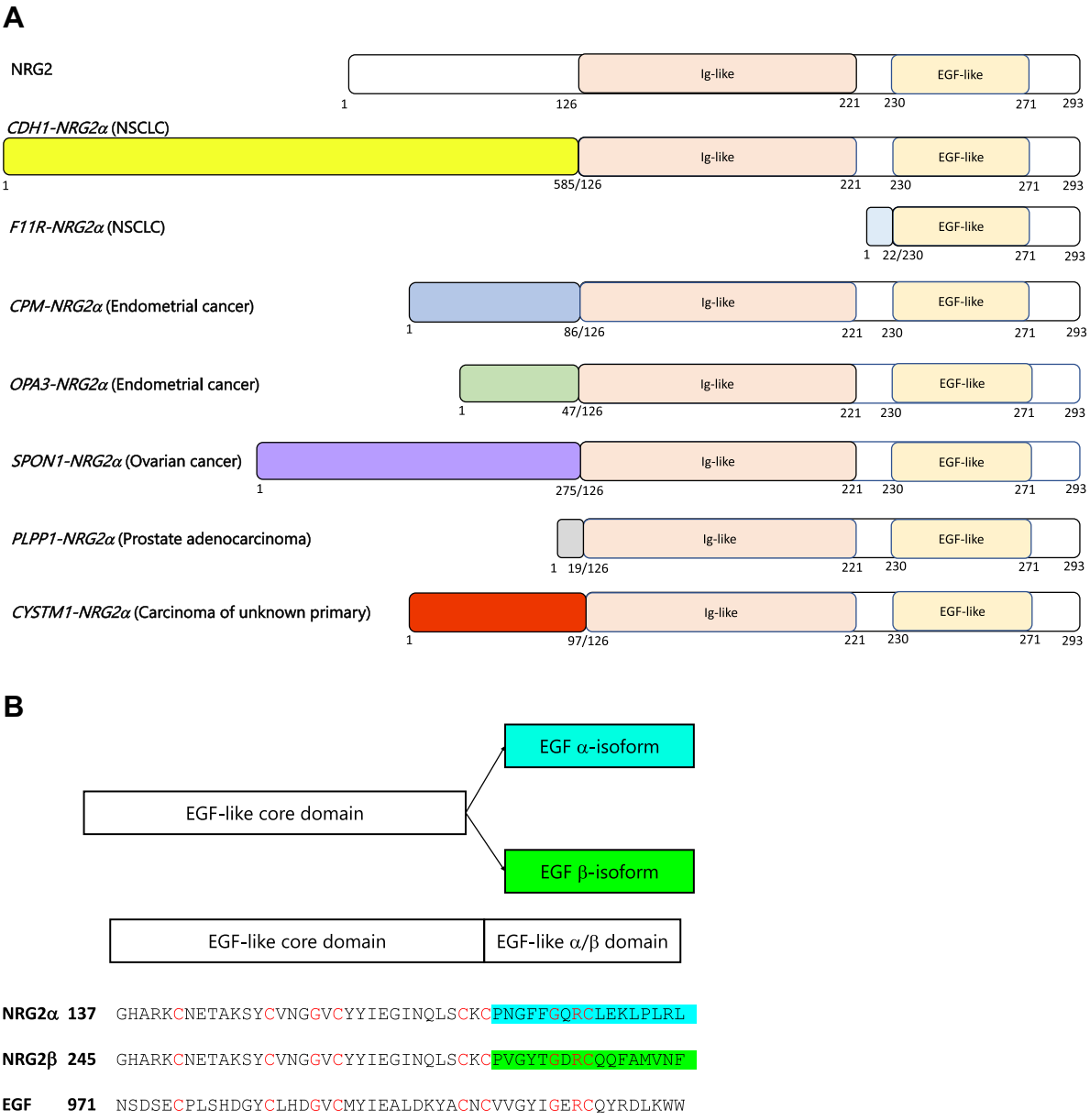

Figure 1. *(A)* A schematic of the seven inframe *NRG2α* fusions. *(B)* A schematic of the generation of the full EGF-like domain from the differential splicing of the EGF-like core domain and the α or β isoform. The alignment of amino acid sequences of EGF-like domain between NRG2α and NRG2β with reference to the EGF domain of EGFR are shown with the aqua color highlighted region representing the α-isoform and the green color highlighted representing the β-isoform. Modified from Jones et al.[15] Ig-like, immunoglobulin-like; *NFR2*, neuregulin-2.

Results

The following number of samples by selected tumor types underwent WTS at Caris Life Sciences: (1) 9600 cases of NSCLC; (2) 6400 cases of colon cancer; (3) 5400 cases of breast cancer; (4) 5030 cases of ovarian cancer; (5) 3060 cases of endometrial cancer; and (6) 1600 cases of prostate cancer. Seven inframe predicted functional (containing the EGF domain) *NRG2* fusions (all *NRG2α*) were identified. Six of the seven *NRG2α* fusions had breakpoints at exon 2, whereas the other one had its breakpoint at exon 4 (Table 1, Fig. 1*A*). The distribution of junction reads of *NRG2* reported here were not different from other actionable fusions like *ALK* in NSCLC. The average *NRG2* junctional reads were 36.5 copies (SD = 36.9), with average junctional reads from 100 randomly selected *ALK*-positive NSCLC tumors was 16.9 (SD = 25.3, p by t test is 0.2).

The RNA reads from the transcriptome analysis were investigated for unique EGF domain splice junctions (139245209:139251305 for α, and 139244757:139251305 for β) and alternative splicing of exon 5 that forms part of the EGF domain (Fig. 1*B*). The

splice junction reads for the α isoform revealed an average of 64 (range: 9–267), whereas no β isoform–specific splice junction was detected similar to the observations by Kohsaka et al.[1] All *NRG2* fusions were detected by WTS as we did not bait for *NRG2* gene in DNA next-generation sequencing.

Tumor With Inframe NRG2 *Fusions*

The full list of inframe *NRG2α* fusions with molecular characteristics is listed in Table 1. None of the seven *NRG2α* fusions was mucinous adenocarcinoma. The four quartiles of TMB are 0 to 7, greater than 7 to 10, greater than 10 to 14, greater than 14 mutations per megabase. Hence the TMB of *NRG2α* falls within the two lower quartiles. All seven *NRG2α* fusions were microsatellite stable.

*NSCLC (*CDH1-NRG2α, F11R-NRG2α*)*

The first 585 amino acids of CDH1, which contains five cadherin repeats, were fused to NRG2.[8] Of note, an inframe reciprocal fusion of *NRG2-CDH1* (N5, C12; chr5:139245134:−/chr16:68855904:+) with 205 junction reads were also identified in the same tumor sample. F11R is junctional adhesin molecule A. The first 22 amino acids of F11R were fused to NRG2 (Fig. 1). Again, an inframe complete reciprocal fusion of *NRG2-F11R* (N5, F11; chr5:139245134:−/chr1:160971143:−) with 37 junctional reads was also identified in the same tumor sample. The PD-L1 expression in the *F11R-NRG2α* tumor is 10% (tumor proportion score), and it was the only *NRG2α* fusion with a positive PD-L1 expression. No other known driver mutations were identified in these two samples.

*Endometrial Adenocarcinoma (*CPM-NRG2α, OPA3-NRG2α*)*

CPM is a membrane-bound arginine/lysine carboxypeptidase M. The first 86 amino acids of CPM were fused to exon 2 of *NRG2*, which retains the two zinc metal-binding sites at amino acids 83 and 86.[9] OPA3 is an outer mitochondrial membrane lipid metabolism regulator. The first 47 amino acids of OPA3 were fused to exon 2 of NRG2. The coiled-coil domain of OPA3 is located between 103 and 163 amino acids of OPA3, thus, not contained in the fusion partner.[10]

*Ovarian Adenocarcinoma (*SPON1-NRG2α*)*

SPON1 is a cell adhesion molecule likely involved in maintaining cell adhesion in both neural and nonneural tissues.[11] The first 275 amino acids of SPON1 containing a reelin domain (heparin-binding domains that can aggregate together to bind calcium) and part of the six type I TSR repeats were fused to NRG2.[11]

*Prostate Adenocarcinoma (*PLPP1-NRG2α*)*

PLPP1 contains six transmembrane regions with the first transmembrane region between seven and 27 amino acids of PLPP1.[12] The first 19 amino acids of PLPP1 were fused to exon 2 of *NRG2*. The tumor also harbored an out-of-frame *PLPP1-NRG2α* fusion (P3, N2; chr5:54763697:−/chr5:139267096:−) with junctional reads of 57. The junctional reads were only four for the in-frame *PLPP1-NRG2α* fusion.

Carcinoma of Unknown Origin

CYSTM1 has a transmembrane region from amino acids 74 to 91. The full length of the 97 amino acids was fused to exon 2 of NRG2. CYSTM1 has a cysteine-rich region from amino acids 88 to 91 and a proline-rich region from 6 to 59.[13]

Discussion

This report confirms the observation of Kohsaka et al.[1] that *NRG2α* fusions are recurrent, albeit rare, ligand fusions present in NSCLC. We further extend that *NRG2α* fusions were identified in other solid malignancies, especially gynecologic malignancies, similarly to NRG1 fusions.[3,4] Importantly, no other known actionable driver mutations were identified in these seven tumor samples. The incidence and distribution of *NRG2α* fusions (0.02%–0.07%) are similar to the rare incidence and broad distribution of *NRG1* fusions.[3] *NRG2* is located on chromosome 5q31.2. Only one of seven fusion partners (CYSTM1 on 5q31.3) is located close to the *NRG2* chromosomal locus. Of note, there were several out-of-frame *NRG2* fusions and one 5′-*NRG2* fusion that did not contain the EGF domain detected by WTS (data not shown); expert bioinformatics and molecular analysis of sequencing data before reporting are of critical importance. Current commercially targeted RNA next-generation sequencing does not bait for *NRG2* fusions.[14] The limitations of this study include the lack of treatment outcome, the unknown phosphorylation status of the ERBB family members, and the lack of smoking status of the two patients with *NRG2α*-positive NSCLC.

The difference in biology and actionability between *NRG1* and *NRG2* ligand-fusions has been recently discussed (reference 2 and references therein). All *NRG2* fusions identified to date have the α-isoform of the EGF domain, which tends to bind less avidly than the β-isoform of EGF to the HER family members of receptor tyrosine kinase.[2,15] In addition, *NRG2α* preferentially binds to HER4, whereas *NRG1α/β* binds to HER3.[15] Kohsaka et al.[1] reported that all four HER family members were phosphorylated in *NRG1* fusion-positive NSCLC samples, but only HER4 was phosphorylated in one *CD74-NRG2α/β* fusion–positive NSCLC tumor.

Hence, to inhibit *NRG2α* fusions, rather than targeting HER3, anti-HER4 (blocking NRG2α binding to HER4 together with disruption of HER4 homodimerization and heterodimerization) is likely needed. Alternatively, a pan-HER approach with a pan-HER tyrosine kinase inhibitor may be able to target both ligand fusions.[2,4,5] Given the novelty and rarity of *NRG2α* fusions, it is hoped that tumor-agnostic clinical trials against *NRG2* fusion-positive solid tumors will answer the many questions regarding the exact role that these *NRG2α* fusions play in the pathogenesis of these tumors and their actionability, as well as the relative contribution of α-isoform to β-isoform of *NRG2* fusions.

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