Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH) Analysis for Characterization and Quantification of Histone Post-translational Modifications

Simone Sidoli‡, Shu Lin‡, Lei Xiong§, Natarajan V. Bhanu‡, Kelly R. Karch‡, Eric Johansen§, Christie Hunter§, Sahana Mollah§, and Benjamin A. Garcia‡¶

Histone post-translational modifications (PTMs) have a fundamental function in chromatin biology, as they model chromatin structure and recruit enzymes involved in gene regulation, DNA repair, and chromosome condensation. High throughput characterization of histone PTMs is mostly performed by using nano-liquid chromatography coupled to mass spectrometry. However, limitations in speed and stochastic sampling of data dependent acquisition methods in MS lead to incomplete discrimination of isobaric peptides and loss of low abundant species. In this work, we analyzed histone PTMs with a data-independent acquisition method, namely SWATH™ analysis. This approach allows for MS/MS-based quantification of all analytes without upfront assay development and no issues of biased and incomplete sampling. We purified histone proteins from human embryonic stem cells and mouse trophoblast stem cells before and after differentiation, and prepared them for MS analysis using the propionic anhydride protocol. Results on histone H3 peptides verified that sequential window acquisition of all theoretical mass spectra could accurately quantify peptides (<9% average coefficient of variation, CV) over four orders of magnitude, and we could discriminate isobaric and co-eluting peptides (e.g. H3K18ac and H3K23ac) using MS/MS-based quantification. This method provided high sensitivity and precision, supported by the fact that we could find significant differences for remarkably low abundance PTMs such as H3K9me2S10ph (relative abundance <0.02%). We performed relative quantification for few sample peptides using different fragment ions and observed high consistency (CV <15%) between the fragments. This indicated that different fragment ions can be used independently to achieve the same peptide relative quantification. Taken together, sequential window acquisition of all theoretical mass spectra proved to be an easy-to-use MS acquisition method to perform high quality MS/MS-based quantification of histone-modified peptides. Molecular & Cellular Proteomics 14: 10.1074/mcp.O114.046102, 2420–2428, 2015.

Chromatin is a highly organized and dynamic entity in cell nuclei, mostly composed of DNA and histone proteins. Its structure directly influences gene expression, DNA repair, and cell duplication events such as mitosis and meiosis (1). Histones are assembled in octamers named nucleosomes, wrapped by DNA every ~200 base pairs. Histones are heavily modified by dynamic post-translational modifications (PTMs)1, which affect chromatin structure because of their chemical properties and their ability to recruit chromatin modifier enzymes and binding proteins (2). Moreover, histone PTMs can be inherited through cell division and thus are crucial components of epigenetic memory (3). The function of histone PTMs has been extensively studied in the last 15–20 years, and several links have been found between aberrations of histone PTM levels and development of diseases (4, 5). Such discoveries revealed the importance of histone PTMs in fine-tuning cell phenotype. Because of this, technology has been rapidly evolving to investigate histone PTM relative abundance with higher accuracy and throughput.

Mass spectrometry (MS)-based strategies have continuously evolved toward higher throughput and flexibility, allowing not only identification and quantification of single histone PTMs, but also their combinatorial patterns and even characterization of the intact proteins (reviewed in (6, 7)). For histone analysis, a widely adopted workflow for nano-liquid chromatography–tandem mass spectrometry (nLC-MS/MS)

1 The abbreviations used are: PTM, post-translational modification; Ab, antibody; ac, acetylation; DDA, data dependent acquisition; hESCs, human embryonic stem cells; me1/me2/me3, mono/di/tri-methylation; SRM, selected reaction monitoring; SWATH™-MS, sequential window acquisition of all theoretical mass spectra; mTSCs, mouse trophoblast stem cells.
SWATH™-MS is a data independent workflow that uses a sequential window acquisition of all theoretical mass spectra that recently gained popularity in proteomics (14, 15). Se- it far from ideal.

targeted sequences in the method reduce the duty cycle and or dedicated software to deconvolute such complex raw data. eluting isobaric species, and tedious manual peak integration previous knowledge about retention time and mass of co- sition within the same MS method (13). This method requires monly performed by integrating shotgun and targeted acqui- Therefore, LC-MS/MS analysis of histone peptides is com- such method cannot be used for any classical PTM discovery. Histone proteins are an excellent target sample to test SWATH™, as the peptides are heavily modified by PTMs and often have isobaric proteoforms present. We analyzed with both DDA and SWATH™ two model systems: (1) extracted histones from untreated (pluripotent) and retinoic acid (RA) treated (differentiated) human embryonic stem cells (hESCs, strain H9), and (2) extracted histones from undifferentiated and differentiated mouse trophoblast stem cells (mTSCs). The results from the DDA experiment were used to evaluate the reproducibility of peptide retention time and the variety of species identified. For the SWATH™ analysis we focused on histone H3, as it is the histone with the highest variety of modified peptides (6). Results highlighted that such acquisition method provides sensitive and precise MS/MS-based quantification of both isobaric and nonisobaric peptides. Our data demonstrate that quantification at the MS/MS level is highly reproducible, and identification of the peptide elution profile is assisted by the high mass accuracy and the large number of overlapping elution profiles of the fragment ions. Moreover, we show that by using different fragment ions for MS/MS quantification we achieved similar quantification re- results. Thus, we used all unique fragment ions for a given species to provide a robust quantification method, where by unique is intended fragment ions that belong to only one of the possible isobaric peptide proteoforms. Taken together, we prove that SWATH™-MS is a reliable and simple-to-use acquisition method to perform epigenetic histone PTM analysis.

**EXPERIMENTAL PROCEDURES**

*Human Embryonic Stem Cells Tissue Culture*—hESCs strain WA09 (or H9) were purchased from WiCell, (Madison, WI). hESCs were grown at 37 °C on matrigel (BD Biosciences) in mTeSR™1 Complete medium (Stem Cell Technologies, Vancouver, BC, Canada). Once cells reached 80% confluency, they were detached using Accumax (Millipore) and washed in mTeSR. The cells were replated 1:4 and treated with 1 μM retinoic acid. On day 5, cells were collected, washed in PBS, and snap-frozen in liquid nitrogen until histone extraction.
**Mouse Trophoblast Stem Cells Tissue Culture**—mTSCs were cultured as previously described (20). Briefly, mTSCs were plated onto feeder mouse embryonic fibroblast cells (inactivated by mitomycin C) supplemented with 0.1% 25 μg/ml FGF4 (Product No. 235-F4, R&D Systems, Minneapolis, MN) and 0.1% 1.5 mg/ml heparin (Product No. H3393, Sigma) in TSC media. Media included 20% FBS, 1% penicillin (500 U/ml) and streptomycin (5 mg/ml), 1% 200 mM l-glutamine, 1% 100 mM sodium pyruvate, and 100 μM β-mercaptoethanol in RPMI 1640 (pH 7.2). mTSCs were separated from embryonic fibroblasts by replating them for 45 min after trypsinization. Afterward, the supernatant was transferred to a new plate for another 45 min. The new supernatant, embryonic fibroblast-free, was used for protein analysis. mTSCs were differentiated by culturing them in TSC media without FGF4 and heparin for 5 to 6 days. Finally, cells were harvested and snap-frozen in liquid nitrogen until histone extraction.

**Histone Extraction and Digestion**—Histones were acid-extracted from both cell lines and processed with two cycles of chemical derivatization, trypsin digestion, and desalting as previously described (19). Briefly, histones were acid-extracted from nuclei with 0.2 μm H2SO4 for 2 h and precipitated with 33% trichloroacetic acid (TCA) overnight. Protein concentration was calculated using the Bradford assay. Purified histones were then dissolved in 30 μl of 50 mM NH4HCO3, pH 8.0. Derivatization reagent was prepared by mixing propionic anhydride with 2-propanol in the ratio 1:3 (v/v) and added to the histone sample in the ratio of 1:2 (v/v) for 15 min at 37 °C. This reaction was performed twice to obtain complete labeling. Histones were then digested with trypsin (enzyme/sample μg ratio of 1:20, 6 h, 37 °C) in 50 mM NH4HCO3. After digestion, the derivatization reaction was performed again twice to derivatize peptide N termini. Samples were then desalted by using C18 Stage-tip (21).

**Nano-liquid Chromatography and Tandem Mass Spectrometry**—Histones purified and digested from hESCs (2 μg per run) and mTSCs (3 μg per run) were loaded onto a two μm × 15 cm ChromXP™ C-18 chip column (particle diameter 3 μm, pore size 120 Å) in serial mode with a Eksigent 425 cHPLC® system. The run gradient was over 60 min with a gradient from 0% to 30% buffer B (buffer A: 2% acetonitrile, 0.1% formic acid; buffer B: 98% acetonitrile, 0.1% formic acid) at a flow rate of 300 nI/min. The Eksigent 42 cHPLC® system was coupled to a TripleTOF® 6600 (ABSciex, Redwood City, CA) mass spectrometer interfaced to a nano source. The source parameters were set as follows: IS at 2350V, Cur gas at 30, GS1 at 5, and IHT at 150 °C. The acquisition parameters were as follows: one 200 mscs MS scan (at >30K resolution), followed by 85 variable SWATH™ MS/MS windows each at 30 mscs accumulation time for m/z 250–1040, MS/MS SWATH scans (at high sensitivity mode with resolution >15K) were set at 6 amu window for m/z 460–950 and varied on each side of the mass range. (Fig. 1C). The total cycle time was ~2.8 s and each analysis was performed in triplicate. All DDA and SWATH™ files are available at the Chorus database (https://chorusproject.org/). project ID: 701.

**Data Analysis**—DDA spectra processing and database searching was performed with ProteinPilot (v5.0, ABSciex) by using the Paragon algorithm. The search parameters were as follows: sample type, identification; cysteine alkyl, none; digestion, ArgC (cleaves R); instrument, TripleTOF 6600; special factors, Histones pre- and post dig.; Propionylated; and ID focus, biological modifications. The database was downloaded from Uniprot (October 2014), filtering for human histones reviewed only (58 entries). Mouse histones were not downloaded because of the high homology between the two species and to avoid excessive redundancy. The resulting .group file was loaded into Peakview® (v2.1, ABSciex) and peaks from SWATH runs were extracted with a peptide confidence threshold of 99% and a false discovery rate <1%. Label-free quantification was performed by using Skyline (18). A list of commonly searched peptides for canonical histone H3 PTM analysis was generated into Skyline, and SWATH™-MS runs were imported. The selection of the proper peak was performed manually using the automated assistance of Skyline. Only charge state 1+ was considered for product ions, and only product ions unique for a given modified form were considered for quantification. The absolute signal of a peptide was calculated by summing the extracted area of all unique fragment ions. The peptide relative abundance was calculated by considering all peptides that share the same amino acid (aa) sequence as 100%. Statistical analysis was performed using a two-tails homoscedastic f test, and differences were considered significant for calculated p values <5%.

**RESULTS**

We tested SWATH™-MS as acquisition method to evaluate the advantages of the MS/MS-based label-free quantification. Because histone peptides are decorated with multiple variable PTMs, we used SWATH™ to perform MS/MS fragmentation through the entire elution and separately quantify isoformic species. Our results allowed for the characterization of significant differences between stem cell lines at different stages of development. Moreover, we could demonstrate the high reproducibility of MS/MS-based quantification using SWATH™ of heavily modified histone peptides derivatized and digested using the highly used propionylation protocol (13).

**DDA Analysis for Assessment of Reproducibility**—For our study, we selected four samples highly relevant in cellular development; ESCs, strain H9 with and without treatment with retinoic acid to induce differentiation (22), and TSCs before and after differentiation. Such model systems have the ability to differentiate to distinct lineages and are thus excellent cellular model systems for studies on early mammalian development. The samples were analyzed first using a DDA method to perform traditional database searching and identify the modified histone peptides. The analysis was performed using the same nLC gradient as the following SWATH™ analysis. The obtained spectral library was loaded into Peakview® together with the SWATH™-MS runs to map the peptides into all the replicates and conditions analyzed. We identified and mapped into all SWATH™ runs 1097 differentially modified peptides by merging the results of all four conditions, including all histone variants (data not shown). We found several peptides with a large variety of PTMs; for instance, the histone H3 peptide KSAPATGGVKKPR (aa 27–40) was found in 17 possible combinations. There were 615 peptides found without any missed cleavage, whereas only 272 with one internal not cleaved arginine, indicating that trypsin efficiently digested most of the histone peptides at the exposed arginine residues. Considering only methylation (me1/me2/me3), acetylation (ac), and phosphorylation (ph) as biological modifications, we identified 798 peptides as unmodified, whereas only 262, 38, and 2 peptides had one, two, and three PTMs, respectively. This indicated that ProteinPilot, as all other traditional proteomics software (23), is still not ideal to confidently identify all heavily modified histone peptides. However, by using the data of peptides identified and
mapped on the nLC elution profile we could assess the high reproducibility of nLC retention time (average CV: 3.38%). We used the reproducibility of peptide retention time as an additional parameter to identify with high confidence the LC peaks during quantification of the SWATH™-MS runs with Skyline (18). The Skyline template is provided in supplementary material.

Reproducibility of the nLC-MS Runs and SWATH™ Quantification—SWATH™-MS runs were performed in triplicate for each of the four samples analyzed. The replicates showed high reproducibility even visually by just overlapping the elution profiles of the nLC-MS chromatograms (Fig. 1A). Most of the peptides eluted within the time range 15–70 min, which approximately corresponded to the programmed 60 min gradient. The start and the end of the sample elution was highly conserved also for different sample conditions (Fig. 1B comparison mTSCs before and after differentiation), even though the individual peptides had different intensities. The SWATH™ acquisition method was programmed to perform repeated cycles of 86 scans, built as one full MS scan (200 msec) followed by 85 MS/MS events (30 msec each) (Fig. 1C). This led to a total cycle time of ~2.8 s. Such duty cycle allowed accurate profiling of fragment ion chromatograms for each analyte with ~15 data points, considering that our chromatography generates peaks with a peak width of at least 25–30 s (Fig. 2A). The m/z window for MS/MS fragmentation was set as variable; specifically, the extremes of the analyzed m/z range (<460 and >820) were set with a wider isolation window, because fewer peptides are present in such range. The mass dense region of m/z 460–820 was set at an isolation window of 6 amu as a large number of modified histones are 14 amu apart (one additional methyl group is 14.016 Da).
Using an isolation window smaller than the $^{2+}$ charge state of the delta mass ($7 \ m/z$) would increase the specificity of the MS/MS events. The collision energy was also adapted to the $m/z$ value of the peptides, using a higher voltage for higher peptide masses (Fig. 1C).

SWATH™-MS runs were imported in Skyline (18) to perform label-free quantification. We prepared a template including all commonly analyzed histone peptides for canonical histone H3 (Uniprot ID: P68431) (9). Quantification was performed by integrating the extracted ion chromatogram of all unique ions for a given peptide, where for unique ions is intended ions that belong to only one of the possible isobaric species. For instance, for the quantification of the two histone H3 peptides KacQLATKAAR and KQLATKacAAR (aa 18–26), we used only the fragments b1–5 and y4–8 (Fig. 2A). Fragments b6–9 and y1–3 have the same mass for the two different isobaric peptides. The use of multiple fragment ions allowed for more confident identification of the correct nLC peak for quantification, together with the high mass accuracy (<10 ppm).

To confirm that the use of different fragment ions does not affect the relative quantification of a peptide, we selected the usually unmodified histone H3 peptide EIAQDFKTDLR (aa 73–83) and the peptide KQLATKAAR (aa 18–26) in all of its modified forms to perform such comparison. First, we analyzed the y series of the unmodified peptide EIAQDFKTDLR, and we verified that the relative intensity of the fragments between each other was conserved in all four samples analyzed. Results highlighted that the relative intensity of the fragments was consistent with an average CV of ~4.1% (Fig. 2B). By using the same fragment ions, we calculated the relative quantification of such peptide between the different stages of development for both hESCs and mTSCs (Fig. 2C).

The fragment ion comparisons were performed by using the ion absolute intensities, not normalized by the total protein.

**Fig. 2. Comparison of quantification precision performed with different fragment ions.** A, nLC-MS/MS elution profile of the histone H3 peptide KQLATKAAR (aa 18–26) modified as K23ac (left) and K18ac (right). Different colored lines represent elution profiles of different fragment ions. Precursor elution profile is marked with the blue line. B, Relative abundance of the y fragments for the histone H3 peptide EIAQDFKTDLR (aa 73–83) in all four samples. The value is the average between the three technical replicates, and the percentage is calculated by considering the total sum as 100%. The peak intensity rank is listed in parenthesis. Ions y1–2 are not present, as their masses are below the scanned range. C, Log2 ratio of the same peptide between the analyzed conditions calculated for each fragment ion. The ratio was calculated for differentiated versus undifferentiated mTSCs (mTSCs diff/undiff) and for hESCs treated and untreated with retinoic acid (hESCs +RA/-RA). D, Log2 ratio of the histone H3 peptide KQLATKAAR (aa 18–26) in differentiated versus undifferentiated mTSCs.
Thus, it is important to remark that Figure 2C and 2D are not valuable for biological insight. We calculated that the unmodified form of the H3 peptide EIAQDFKTDLR (aa 73–83) was almost 53-fold more abundant in hESCs differentiated with retinoic acid as compared with the undifferentiated ones, whereas only 1.2-fold changes more abundant in undifferentiated mTSCs as compared with differentiated ones. Both results showed remarkably low CVs, 2.4% and 4.4%, respectively, when comparing the quantification with different fragment ions. A similar analysis was performed for the histone H3 peptide KQLATKAAR (aa 18–26). Again, the different peptide proteoforms were quantified with high agreement by using different fragment ions of the y series (y4–8) (average CV 14.6%). Taken together, such low CV values highlighted that MS/MS-based quantification can be performed with different fragment ions achieving highly similar final results.

**Comparison of Histone PTMs During Development for the Two Model Systems**—We analyzed the most widely studied histone H3 peptides in the four samples, giving a special care for the peptides that have isobaric proteoforms (Fig. 3 and Supplemental Table S1). The peptides KSTGGKAPR (aa 9–17), KQLATKAAR (aa 18–26), KSAPATGGVKKPHR (aa 27–40), and EIAQDFKTDLR (aa 73–83) can all be present with isobaric variants, for example, KacQLATKAAR and KQLATKacAAR. This made SWATH™-MS an appropriate acquisition method for the analysis, because we could extract the fragment ions unique for a given isobaric form to discriminate what cannot be discriminated at the precursor mass level. We observed an average CV 8% for the three peptides analyzed with isobaric proteoforms, indicating that the method allowed high precision calculation of PTM relative abundance. The peptide KSTGGKAPR (aa 9–17) was detected in all of its known methylated and acetylated forms (Fig. 3A). Moreover, we found significant differ-
ences (<5% two-tails t test) for eight out of nine peptide proteoforms in hESCs with and without retinoic acid treatment, and for five peptides in mTSCs before and after differentiation. For the peptide KQLATKAAR (aa 18–26), we found significant differences for all peptides but K23me1 analyzed in mTSCs, and all but K18me1/K23me1 in hESCs (Fig. 3B). The peptide KSAPATGGVKKPHR (aa 27–40) is probably the most challenging for histone H3, giving the large number of possible PTM combinations. In this experiment, we could not detect K27me2K36me3 regarding known acetylated and methylated forms. The other acetylated and methylated proteoforms were all precisely quantified (CV 6.7%) (Fig. 3C).

Finally, we quantified the peptide EIAQDFKTDLR (aa 73–83) that contains the PTM K79me (Fig. 3D). Once again, we found significant differences for most of the modified forms (K79me1/me2, but not me3) when comparing the two stages of hESCs and mTSCs. The most abundant proteoform of the peptide was found to be the unmodified one, followed by me1 (9.1%–18.5%), me3 (0.5%–1.3%), and me2 (0.1%–1.0%). Taken together, data on histone H3 peptides confirm the high precision of the SWATH™-MS analysis, as we could find significant differences for very small changes in peptide relative abundance.

Lastly, we focused on the combined sensitivity and precision of the nLC-MS method, which we highlighted analyzing the peptides found with the smallest significant differences in relative abundance between the analyzed conditions. We took the top two peptides with the smallest differences in the two stages of development for hESCs and mTSCs separately (Fig. 4A), and we found marks commonly known to be of very low abundance, such as H3K23me1 and H3S10ph (9). The peptides described in Fig. 4A were all found to be statistically different when comparing the developmental stages of the two cell lines. For instance, the peptide K9me2S10ph was found to be <0.05% of the total KSTGGKAPR (aa 9–17) peptide in mTSCs. The SWATH™-MS scan that included the fragmentation of such peptide (m/z 561.29) did not show any remarkable fragment ion representing the given peptide, with the exception for the fragment b7, which was the only peak discriminable from background noise (Fig. 4B). However, we could define a precise elution profile for such peptide, because at least six fragment ions overlapped during nLC-
MS/MS peak extraction (Fig. 4C). Collectively, SWATH™-MS proved to be not only precise, but also highly sensitive for quantification of histone peptides.

**DISCUSSION**

In this work we highlighted the ease-of-use, high precision, and high sensitivity of SWATH™-MS for histone peptide analysis. We could quantify peptides with relative abundance below 0.1%, confirming that we can monitor histone modified peptides over four orders of magnitude (Supplemental Table S1). A prerequisite of a successful MS analysis is high quality chromatography, which we accomplished with high reproducibility between replicates and samples (Fig. 1A and 1B). Such aspect was crucial to obtain reliable peak area integration with SWATH™, as the retention time of a given peptide could be used as additional feature to confidently identify a nLC-MS/MS peak. The SWATH™ acquisition windows for MS/MS fragmentation were programmed with a small overlap (1 m/z) to prevent occasional peptide fragmentation with only part of its isotopic distribution. We speculate that SWATH™ will provide comparable and equally confident quantification with shorter nLC gradients (e.g. 30 min), because the MS duty cycle (~2.8 s) is sufficiently fast to generate enough data points for nLC peaks of 30 s or less.

The high resolution of the time-of-flight mass analyzer (>20,000) characterized the fragment ions with high mass accuracy (<10 ppm), allowing for confident identification of the peptide elution profile and discrimination between near isobaric PTMs such as acetylation (42.011 Da) and trimetylation (42.047 Da). Such resolution would be sufficient even for middle-down analysis of intact histone N-terminal tails, where quantification is also performed at the MS/MS level (24, 25). Middle-down MS has the interesting advantage to produce data regarding co-existing frequency between PTMs residing on the histone N-terminal tail. However, it is highly recommended to use of electron transfer dissociation for fragmentation of such highly charged longer peptides (>6+ and around 5 kDa), which unfortunately is not installed on every mass spectrometer. However, it should be noted that collisional fragmentation approaches have been used on larger histone peptides or intact protein with some success (26, 27), therefore SWATH™-MS of middle-down size histone peptides should be explored.

The acquired data could also be used for remining newly identified PTMs still performing pseudo-SRM for quantification. This includes an additional value to the analyses we performed, as they are still valuable for future studies of yet undiscovered PTMs. Even though SWATH™-MS is somewhat less sensitive than SRM (28), an SRM experiment of a histone undiscovered PTMs. Even though SWATH™-MS is somewhat performed, as they are still valuable for future studies of yet undiscov-ered PTMs. This includes an additional value to the analyses we identified PTMs still performing pseudo-SRM for quantification.

For instance, considering only the 41 peptide proteoforms we analyzed for histone H3 (supplemental Table S1), we would need 123 transitions to monitor three fragment ions for each peptide proteoform, and 492 transitions considering all fragment ions we used to quantify each peptide proteoform with SWATH™. Such numbers are achievable because current triple quadrupole mass spectrometers can perform scans in <5 msec. However, it becomes prohibitive when more histone variants and proteoforms are included. Our experiment was performed by using variable m/z windows for SWATH™ acquisition, but with fixed accumulation time (30 msec). Further optimization of the cycle time could lead to high benefits in speed and sensitivity, for example, reducing the scan time for SWATH™ analysis of high concentration of ion signals and increasing it for the m/z ranges with lower sensitivity. Moreover, using larger m/z windows (up to 25–30) would reduce the instrument duty cycle, and thus could be used for shorter LC gradients, even though this could complicate confident peak identification.

In conclusion, we proved that SWATH™-MS can be successfully adopted for epigenetic histone PTM analysis. We believe that this methodology will increase popularity in this field, because current quantification methods are mostly based on combinations of DDA and targeted acquisition to discriminate isobaric peptides and shotgun identify rare or novel PTMs. As such methods require prior knowledge about peptide elution and data cannot be further mined in case new PTMs are found on isobaric peptides, SWATH™-MS provides a great balance between these crucial MS parameters.

* BAG acknowledges funding from an NSF CAREER award grant, and NIH grant R01GM110174.

[S] This article contains supplemental Template and Table S1.

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