Asparagine Hydroxylation is a Reversible Post-translational Modification

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In Brief
Amino acid hydroxylation is a common post-translation modification that regulates signaling networks through the creation or destruction of protein-protein interactions. Although the effects of hydroxylation on dynamic networks has been known for some time it was generally assumed that this modification is static and irreversible. By using pulse labeling and quantitative mass spectrometry Rodriguez et al. demonstrate that asparagine hydroxylation is indeed reversible, allowing for a more dynamic regulation of this modification.

Highlights
- Quantitative mass spectrometric method to monitor PTM stability.
- Pulse labeling reveals dehydroxylation of several asparagine hydroxylation sites.
- Reversal of TNKS2, TRPV3 and HIF1a asparagine hydroxylation sites.
- Protein dehydroxylation is an additional level of control for cellular signaling networks.

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Amino acid hydroxylation is a common post-translational modification, which generally regulates protein interactions or adds a functional group that can be further modified. Such hydroxylation is currently considered irreversible, necessitating the degradation and re-synthesis of the entire protein to reset the modification. Here we present evidence that the cellular machinery can reverse FIH-mediated asparagine hydroxylation on intact proteins. These data suggest that asparagine hydroxylation is a flexible and dynamic post-translational modification akin to modifications involved in regulating signaling networks, such as phosphorylation, methylation and ubiquitylation.

Post-translational modifications (PTMs) are chemical alterations of amino acids or proteins that increase the complexity of the proteome and allow the cell to modify protein function in a dynamic or sustained manner (1–4). Some of these modifications are irreversible, such as the cleavage of a polypeptide chain resulting in altered activity of the protein. Nevertheless, most PTMs are reversible and comprise the dynamic addition or elimination of functional groups that range from large oligosaccharide chains to a few atoms, such as glycosylation, phosphorylation, acetylation, methylation, or carboxylation. The reversibility of these PTMs is frequently achieved by the action of pairs of enzyme classes opposing functions, one of which catalyzes the forward reaction and another the reverse reaction (5–7). In cases where one of the reactions is thermodynamically unfavorable, the reaction may not be straightforwardly reversible and therefore includes intermediate products. An example would be the formation and dissolution of cysteine bonds, where the formation of the bond can include the oxidation of the sulfur of cysteine to sulfenic acid, which then forms a disulfide bond by reacting with another cysteine (8, 9).

Oxidation or more precisely hydroxylation of proteins on residues other than cysteine was recognized as a PTM in the 1960s, when the enzymatic hydroxylation of proline and lysine was identified as taking place during collagen synthesis (10, 11). Subsequently, the molecular mechanism of hydroxylation catalyzed by the evolutionarily conserved family of the 2-Oxoglutarate dependent dioxygenases (2OG-ox) was described (12, 13). The 2OG-ox are a family of proteins composed of over 60 enzymes of which the so-called HIF-hydroxylases form a subgroup consisting of four hydroxylases: 3 prolyl hydroxylases (PHD1, 2 and 3) and an asparaginyl hydroxylase “factor inhibiting HIF” (FIH). HIF-hydroxylases act as sensors of the oxygen levels within the cells. Under normoxic conditions prolyl hydroxylases (PHD1, 2 and 3) catalyze the specific hydroxylation of two proline residues in the alpha subunit of HIF1, the master regulator of the hypoxic response. Once hydroxylated, HIF1α is bound by the von Hippel-Lindau ubiquitin ligase (VHL) complex, which promotes the ubiquitination and rapid proteasomal degradation of HIF1α, resulting in the ablation of the protein under normoxic conditions (14–18). FIH, the other component of the subfamily, catalyzes an asparagine hydroxylation in the C terminus of HIF1α. Upon hydroxylation of this residue, the interaction with co-factors required for the formation of the active transcription factor complex is impeded, resulting in the down-regulation of HIF-driven transcription in the presence of oxygen (19, 20).

Over the past few years, it has been argued that HIF1α is not the only protein that is hydroxylated by HIF-hydroxylases and several additional substrates, particularly of FIH, have been postulated and validated (21). In a similar manner to that observed in the context of HIFα, hydroxylation of these other substrates by FIH alters the physicochemical properties of the hydroxylated domains. These changes can induce or destroy protein-protein interactions that ultimately control substrate activity, folding or localization (22–26).

Presently, it has been suggested that the hydroxylation mediated by HIF-hydroxylases is an irreversible process, and that the hydroxylation can only be reset by the degradation and new synthesis of a nonhydroxylated protein (27–

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Asparagine Hydroxylation is Reversible

A mass spectrometric study monitoring two FIH-mediated hydroxylation sites of Rabankyrin-5 substantiated this, as no evidence of dehydroxylation was found under the investigated conditions (30). Nevertheless, some authors have proposed the existence of a dehydroxylation mechanism (25, 31), but this notion is only founded on the assumption that PTMs should be generally reversible. Some data have emerged that implicitly suggest that asparagine hydroxylation may be a reversible PTM after all. First, in contrast to proline hydroxylation of HIF, FIH-mediated asparagine hydroxylation does not lead to the rapid decrease of protein half-life. This suggests that FIH-substrates are longer-lived proteins and that the cell would therefore benefit from a mechanism of resetting the level of hydroxylation in a more dynamic, nondestructive manner (21). One such example is transient receptor potential vanilloid 3 (TRPV3), an ion channel that is hydroxylated by FIH. Hydroxylation on an asparagine residue reduces TRPV3-mediated current, whereas hypoxia, FIH inhibition or mutation of the asparagine residue potentiates it without affecting protein stability. Intriguingly, the increases in current through the channel are observable in less than one hour of hypoxia or FIH inhibition. This rapid response indicates that there has to be a very rapid turnover of the protein or that the hydroxylation can be reversed without destruction and re-synthesis of TRPV3 (25). Moreover, indirect evidence from mathematical modeling indicates that signaling networks require reversibility of asparagine hydroxylation. Nguyen et al. published a comprehensive ODE-based mathematical model of the immediate HIF network (32). Intriguingly, the mathematical model assumes irreversibility of both proline hydroxylations but includes an undefined reaction that leads to the dehydroxylation of the N-terminal asparagine residue (Fig. 1A). The reversibility of the asparagine hydroxylation was required for the model to reproduce the experimental data, which showed the transient induction of HIF protein levels and transcriptional output. Upon removal of the unspecified dehydroxylation reaction, the model predicts that HIF levels and activity would increase in a sustained manner, which is at odds with the experimental observations (Fig. 1B–1E).

Overall, although these data give initial foundation to the hypothesis that asparagine hydroxylation is reversible, no direct evidence of reversibility has been produced. Notably, amino acid methylation was initially thought to be a static modification (33) but was subsequently demonstrated to be reversible through the action of 2OG-ox. With this in mind, we set out to examine the potential reversibility of asparagine hydroxylation by using quantitative MS.

**EXPERIMENTAL PROCEDURES**

All reagents were purchased from Sigma, Gillingham, UK unless otherwise stated.

**Cell Culture**—HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM glutamine (Invitrogen, Carlsbad, CA) and 10% fetal calf serum (Invitrogen). Plasmids were transfected with LipofectAMINE 2000 (Invitrogen) according to the vendor’s instructions. SILAC media was generated by custom made DMEM medium lacking L-arginine and L-lysine (Thermo Fisher Scientific). This media was supplemented L-Arginine-13C, 15N (R10) and L-Lysine-13C, 15N (K8) (Cambridge Isotope Laboratories, Inc., Leicester, UK) but not with FCS to prevent incorporation of 12C-Lysine or Arginine.

**Materials**—All antibodies were from commercial sources: anti-FLAG M2 peroxidase was obtained from Sigma Aldrich (F4042, 1:1,000 dilution), anti-HIF1α was from BD Biosciences (610958 1:1,000 dilution), anti-GFP and anti-myc were from Cell Signaling (D5.1-9B11, 1:2,000 dilution), anti-tubulin was purchased from Santa Cruz (sc-8035, 1:1,000 dilution) and anti-V5 was obtained from Invitrogen (R96025, 1:5000 dilution). DMOG was obtained from Cayman Chemical (71210), DFO and cycloheximide were purchased from SIGMA (D9533-C4859). For immunoprecipitation anti-Flag-M2 beads (Sigma Aldrich), anti-myc beads (Cell Signaling-9B11) and anti-GFP (GFP-Trap Magnetic Agarose-Chromotek, Munich, Germany) were used.

**Plasmids**—GFP-HIF1A was a gift from Alex Chong (Aston University, FLAG-TNK52 from Addgene (#54691)).

**Cell Lysis and Immunoblotting**—Cells were lysed in ice-cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl), supplemented with protease (5 µg/ml leupeptin, 2,2 µg/ml aprotinin), phosphatase inhibitors (20 mM β-glycerophosphate). Lysates were cleared of debris by centrifugation at 20,000 × g for 10 min in a benchtop centrifuge (4 °C). Total lysates were fractionated by SDS-PAGE and transferred onto nitrocellulose filters. Immunocomplexes were visualized by enhanced chemiluminescence detection (GE Healthcare) with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories).

**In Vitro Dehydroxylation Reaction**—Thioredoxin-6 histidine (Trx-6H) tagged human HIF-2α CAD (774-874) and maltose binding protein (MBP) tagged human FIH were expressed in BL21(DE3) E. coli as previously described (34). Protein expression was induced with 0.5 mM IPTG. CAD was induced for 8 h at 30 °C and purified using a HisTrap HP column (GE Healthcare, Sydney, Australia). FIH was induced for 16 h at 16 °C and purified using a MBPTrap HP column (GE Healthcare). Proteins were exchanged into 150 mM NaCl, 20 mM Tris-HCl pH 8 using PD-10 desalting columns (GE Healthcare). Ten µM CAD was hydroxylated by incubation with 10 µM FIH, 112.5 mM NaCl, 65 mM Tris-HCl, 4 mM sodium ascorbate, 500 µM DTT, 30 µM FeSO4 and 40 µM 2-oxoglutarate for 2.5 h at 37 °C, and repurified using a HisTrap HP column (GE Healthcare). FIH (~2 µg) was incubated with 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, supplemented with HALT EDTA-free protease inhibitors. Protein concentration was ~0.5 mg/ml. 1 µg of hydroxylated CAD was incubated under shaking at 37 °C with 1 ml of FIH (~0.5 µg) for 3 h (or 0 h for control). Precipitated with NiNTA-agarose beads (Qiagen) and digested with Trypsin and GluC and processed as described. Mass spectrometry was done using a Lumos Fusion (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer coupled to an RLS-nano uHPLC (Thermo Fisher Scientific). Peptides were separated by a 40-min linear gradient from 5–30% Acetonitrile, 0.05% Acetic acid. Mass range was 646.5-653.5. XIC were generated with a width of (646.6890-646.7140)/(652.0300-653.0440) for the for nonhydroxylated/hydroxylated peptide. Peptide elution times were calibrated using hydroxylated/nonhydroxylated standards.

**In Cell Dehydroxylation Sample Preparation**—HEK293T cells were transfected with transfected with the different plasmids indicated. The different treatments were done 24 h post-transfection. To prevent re-hydroxylation, all lysis and wash buffers were supplemented with 1 mM NOG, a pan-hydroxylase inhibitor. Post treatment,
the cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris–HCl pH 7.5, 1 mM EDTA pH 7.5). Immunoprecipitation, washing, and digest was performed on a KingFisher Duo robotic station (Thermo Fisher Scientific). 5 μl of magnetic antibody bead slurry, anti-Flag-M2 beads (Sigma Aldrich), anti-myc beads (Cell signaling-9B11) and anti-GFP (GFP-Trap Magnetic Agarose-Chromotek) respectively, was dilute in 100 μl of lysis buffer and loaded in row H of a 96 deep-well plate. 500 μl of lysate was loaded into row G, 300 μl of lysis buffer were loaded into rows E and F. 300 μl of Wash buffer (150 mM NaCl, 20 mM Tris–HCl pH 7.5, 1 mM EDTA pH 7.5) were loaded into rows B-D. Row A contained the 100 μl of digest buffer (2 M Urea, 50 mM Tris–HCl pH 7.5, 1 mM DTT, 5 μg/ml porcine trypsin (Promega, Southampton, UK) 5 μg/ml GluC (Promega)). The robot picked up beads in row H, transported them to row G and

Fig. 1. A, Schematic diagram of a modified kinetic model of the HIF-1α signalling network where the assumed reactions representing asparagine dehydroxylation are removed from the original model (32) (denoted by the red cross-ed lines). Briefly, cytosolic HIF-1α proteins can be either asparaginyl-hydroxylated, denoted with subscript N(ox), by FIH; or prolyl-hydroxylated, denoted with subscript P(ox), by prolyl-hydroxylases (PHD) and targeted for Von Hippel-Lindau (VHL)-mediated degradation. In hypoxia, PHD is inactivated, leading to HIF-1α protein stabilization (unhydroxylated or asparaginyl-hydroxylated form) and translocation to the nucleus (indicated by subscript n). HIF-1α can also be exported out. Nuclear unhydroxylated HIF-1α can be asparaginyl-hydroxylated by nuclear FIH and/or prolyl-hydroxylated by nuclear PHD and targeted for nuclear VHL-mediated degradation. If no hydroxylation occurs, nuclear HIF-1α can dimerize with HIF-1β, creating a dimerized transcriptional complex (HIF1d) that can bind to HIF-response elements (HRE) of the target genes, inducing their mRNA transcription and protein translation. HIF-1 dimer can also bind to the HRE within the PHD gene, leading to up-regulation of PHD protein, leading to a negative feedback. (B–C) Comparison of the in silico predicted effects of DMOG or JNJ1935 on HIF-1α stabilization in the presence (B) or absence (C) of asparagine dehydroxylation, the former match the transient dynamics observed in experimental data (32) whereas the latter display sustained patterns instead.
released and mixed them for 2 h. Beads where picked up and released subsequently into rows F to B with 1 min mixing in between. The washed beads were then transported into row A and digested a 27 °C for 30 min under mixing. Beads where then removed and digest continued for 8 h at 37 °C. After iodoacetamide modification and acidification of the samples, the peptide mixtures were desalted using homemade C18 tips. The desalted and lyophilized peptides were resuspended in 0.1% TFA and subjected to mass spectrometric analysis by reversed-phase nano-LC-MS/MS.

**Mass Spectrometry**—5 μL of the resuspended peptides were analysed by reversed-phase nano-LC-MS/MS using a nano-Ulti-3000 liquid chromatography system and a QExactive plus or Lumos Fusion mass spectrometer (both Thermo Fisher Scientific). Flow-rates were 400 nl/min. Peptides were loaded onto an self-packed analytical column (uChrom, nanoLCMS Solutions, Oroville, CA; 1.6, 0.075 mm × 25 cm) using a 67-min gradient Buffer A, 2% acetonitrile 0.5% Acetic Acid, Buffer B, 80% acetonitrile, 0.5% Acetic Acid; (0–16 min: 2% buffer B, 16–56 min: 3–35% buffer B, 56–62 min: 99% buffer B, 62–67 min 2% buffer B. The QExactive was operated in top-12, data-dependent mode with a isolation window of 1.4, AGC 5e4, HCD collision energy of 26, Scan range 70,000; AGC: 3e6 ions). MS2 was performed with an isolation window of 1.4, AGC 5e4, HCD collision energy of 26, rapid scan rate, Scan range ions). MS2 was performed in the ion trap, isolation window 0.7, Scan range ions). MS2 was performed with an isolation window of 1.4, AGC 5e4, HCD collision energy of 26, Scan range 140 to 200 ms maximum injection time. The Lumos was operated in data-dependent mode with a 30-s dynamic exclusion range. Full-scan spectra recording in the Orbitrap was in the range of m/z 350 to m/z 1400 (resolution: 70,000; AGC: 3e6 ions). MS2 was performed with an isolation window of 1.4, AGC 5e4, HCD collision energy of 26, Scan range 145–1,450 m/z, 50 ms maximum injection time and an overall cycle time of 1 s.

**Calculation of Hydroxylation Occupancy**—To calculate the relative molar ionization efficiency we devised a method that assumes a peptide exists predominantly in two molecular states, hydroxylated and unmodified. By determining the relative abundance of either species with respect to a reference intensity we estimated the relative ionization efficiency for either unmodified or hydroxylated peptides. To determine the relative ionization efficiencies of unmodified peptides, we generated a list of TNKS2/HIF1α/TRPV3 peptides, respectively, detectable in light and heavy labeled samples, excluded M-containing peptides (and N-containing peptides in the case of TNKS2) and summed up the intensities in the heavy channel of latter time point. We then divided the intensity of the unmodified containing heavy peptide by this sum to generate the relative intensity of the nonhydroxylated peptide over a reference intensity.

\[
\text{Rel. IonizationEfficiency}_{\alpha/\beta} = \frac{\text{Intensity_H(N/\beta)}}{\sum_{\text{Hif peptide}} \text{Hif peptide Intensity_H}}
\]

in the case where FIH expression induced stochiometric hydroxylation (such as N803 of HIF1α), we summed up the intensities of the same peptides in the light channel of the untreated sample (at time point 0) and divided the intensity of the hydroxylated peptide by this sum,

\[
\text{Rel. IonizationEfficiency}_{\alpha/\alpha} = \frac{\text{Intensity_L(N/\alpha)}}{\sum_{\text{Hif peptide}} \text{Hif peptide Intensity_L}}
\]

The ratio of the respective relative intensities equals the ratio of the molar ionization efficiencies.

\[
\text{Rel. IonizationEfficiency}_{\alpha/\beta} = \frac{MolarIonizationEfficiency_{\alpha/\beta}}{MolarIonizationEfficiency_{\alpha/\alpha}}
\]

If the hydroxylation appeared not to be stochiometric, such as in TRPV3, we included an additional step. We calculated the ratios of the nonhydroxylated peptide in heavy conditions. This gave us the ratio of the relative ionization efficiency of the unmodified peptide. We repeated this step for the light labeled, unmodified peptide at time point 0. The difference in the relative ionization efficiency of the unmodified peptides allows calculating the difference of occupancy between the DMOG treated sample and the control allowing us to calculate the % of occupancy of the unmodified peptide. Knowing this occupancy allows to calculate the occupancy of the hydroxylated peptide and the relative ionization intensity by multiplying the occupancy with the ratio of the light labeled hydroxylated peptide over the sum of peptides. These calculations generated a ratio of molecular ionization efficiencies of unmodified/hydroxylated peptide. We used this to transform the ratio modified/unmodified as calculate by MaxQuant into molar ratios. The molar ratios were converted into estimated occupancies by dividing the ratio by itself + 1;

\[
\text{Occupancy} = \frac{\text{Ratio}}{\text{Ratio} + 1}
\]

using the Perseus software (36). In cases where we were unable to calculate the ratio of molar ionization efficiency, we estimated it to be 1.

**Experimental Design and Statistical Rationale**—Overall, 150 biological replicate samples were analyzed. For the determination of DMOG efficiency (two controls), 48 and 48 for the TNKS2 DMOG and TNKS2 DMOG/CHX experiments (24 controls in each sample set), 20 for the TNKS2 DFO experiment (10 controls), 6 for the TRPV3 DMOG experiment (2 controls), 16 for the HIF1α DMOG experiment (4 controls) and 4 samples for the HIF2a in vitro dihydroxylation experiment. Samples sequences for MS analysis were randomized using the excel =RAND() command. Statistical tests were performed using Graphpad Prism 8 (multiple t tests), distribution was assumed to be normal. Protein intensities and hydroxylation occupancies are shown with error bars representing standard error of mean (S.E.).

**Database Search Parameters and Acceptance Criteria for Identifications**—The MS raw data were analyzed by the MaxQuant and Andromeda (1.6.10.43) software package (37) using the pre-selected conditions for analysis (specific proteases, 2 missed cleavages, 7 amino acids minimum length). Protease was set to trypsin or trypsin+GluC, for the TNKS2/TRPV3 or HIF1α pulldowns, respectively. Carbamylation (C) was selected as fixed modification. Variable modifications were N-terminal acetylation (protein), oxidation (MPN) and heavy labeled amino acids (K8R10). FDR was set to 0.01. MS/MS spectra were searched against the human Uniprot database (09/2019 UP000005640_9606.fasta; 20,656 entries) and a the MaxQuant contaminant database (246 entries) with a mass accuracy of 4.5 ppm (for MS) and 20 ppm or 0.5 Da (MS/MS OT or IT). Peak matching was selected and was limited to within a 0.7 min elution window with a mass accuracy of 4.5 ppm.

**In Silico Modeling**—To in silico predict the effects that putative dehydroxylation may have on dynamic behavior of the HIF-1α...
signaling pathway, we modified a well-calibrated mathematical model of the HIF pathway that we developed previously (32), by removing the steps representing asparagine dehydroxylation in this model (Fig. 1A). This was done by setting the kinetic parameters describing the rate of these reactions to null in the model's ordinary differential equations. Simulations of the intact and the adjusted models under various conditions (i.e. hydroxylase inhibition by DMOG and JNK, and 1 and 3% hypoxia, Fig. 1B–1E) show that removal of the asparagine dehydroxylation steps failed to reproduce the experimental patterns of HIF-1α expression.

RESULTS

There are several analytical techniques that can be applied to the quantitation of changes in PTMs, such as the use of modification-specific antibodies or the monitoring of shifts in the apparent molecular weight in PAGE (38, 39). Unfortunately, neither of the above-mentioned methods is universal and for this reason, we decided to use quantitative MS (qMS) to investigate whether hydroxylation is a reversible process. qMS has been shown to be superior to western blotting in terms of relative quantification and is a method widely used for monitoring changes in the hydroxylation status of proteins (14, 22, 23, 30, 39). Initially, we planned to monitor the dynamics of asparagine hydroxylation on the well-described Tankyrases are members of the poly(ADP-ribose)polymerase (PARP) protein super family, which participate in the regulation of the degradation complex in the Wnt/β-catenin signaling pathway (40–42). TNKS2 has been characterized as a FIH substrate that contains the FIH consensus motif [LXXXXXV/IN] in several ankyrin repeat domains (ARD) (21, 43). As such, TNKS2 contains multiple FIH-hydroxylation sites, and furthermore, hydroxylation on TNKS2 by FIH has not been reported to have any effect on its stability. Multiple hydroxylations would allow us to monitor several sites in the same experiment, thus improving our chances of detecting if one or more hydroxylation(s) are reduced over time with statistical significance.

As an experimental approach we adapted the pulsed stable isotope labeling with amino acids in cell culture (SILAC), that has been successfully used to measure protein and PTM turnover (44, 45). HEK 293T cells were transfected with a V5-tagged FIH and a Flag-tagged TNKS2. 24 h after transfection the medium was replaced with SILAC medium (containing 13C- and 15N-labeled arginine and 13C- and 15N-labeled lysine amino acids (R10K8)) and cell lysates were prepared at different time points. To further reduce the potential incorporation of unlabeled amino acids into proteins, we additionally worked in serum-free conditions, therefore, the comprehensiveness of labelled amino acid incorporation was only limited by the isotopic purity (generally >99%) and by the recycling of unlabeled amino acids resulting from the degradation of unlabeled proteins. Within these restraints, pulsing with heavy SILAC amino acids allowed us to distinguish TNKS2 synthesized post-pulse (Heavy SILAC) from the TNKS2 present before SILAC addition (light SILAC). To avoid possible rehydroxylation of peptides after the SILAC pulse, cells were treated with dimethyl-oxalylglycine (DMOG) and the lysis buffers contained N- oxalylglycine (NOG), both of which are pan-hydroxylase inhibitors (46). Therefore, if the hydroxylation inhibition is complete, heavy-SILAC peptides should be devoid of hydroxylation. By tracking how the ratio between hydroxylated and nonhydroxylated peptide intensity changes in the light SILAC TNKS2, we can monitor if a proportion of the hydroxylation is reversed (Fig. 2A). This experimental setup allows us to track the asparagine hydroxylation levels of the initial population of TNKS2 (light) and to determine how effective DMOG inhibited the hydroxylation reaction by assaying the relative hydroxylation changes in heavy-SILAC TNKS2. To establish the concentration required to inhibit FIH-mediated TNKS2 hydroxylation, we pulsed cells with increasing concentrations of DMOG and heavy SILAC media for 4 h. We then quantified the heavy-labelled percentage of hydroxylated peptide versus the vehicle control to assess the relationship between DMOG concentration and FIH inhibition. As expected, increasing concentrations of DMOG inhibited FIH more effectively (Fig. 2B, supplemental Fig. S1), with 2 mM reducing the activity by 98%. 1 mM and 0.5 mM DMOG only partially inhibiting FIH, consequently we used the highest concentrations for all further experiments.

TNKS2 Protein Turnover is Not Altered by Hydroxylation—To determine whether SILAC media and DMOG affected TNKS2 protein stability, we first validated by western blotting that the expression of TNKS2 or FIH was not affected by either treatment at any time point (Fig. 2C). Furthermore, to estimate TNKS2 protein turnover we blocked protein synthesis with cycloheximide, a rapidly acting eukaryotic protein synthesis inhibitor (47, 48) commonly used for protein turnover studies (49). We initially monitored how CHX inhibited protein synthesis by western blotting and observed a reduction in the levels of Flag-TNKS2 and V5-FIH, with an approximate half-life of 6 h for TNKS2 (Fig. 2D). We next determined how TNKS2 hydroxylation levels change dynamically upon SILAC and DMOG pulsing. We immunoprecipitated Flag TNKS2, digested the protein on-beads, analyzed the peptides by LC-MS/MS and quantified TNKS2 expression using MaxQuant. This confirmed our initial observation that DMOG did not affect the stability of TNKs when compared with the DMSO control (Fig. 2E) and also allowed us to monitor the incorporation of heavy SILAC, by detection of the increase in heavy-labelled TNKS2 (Fig. 2F). Based on the labeling data, we calculated the TNKS2 protein half-life to be around 7 h. This matched the Western-blot-based estimation, suggesting that newly synthesized TNKS2 protein is predominantly heavy-labelled upon pulsing with heavy amino acids and that DMOG treatment does not affect the expression and stability of exogenously expressed TNKS2.

TNKS2 N-Hydroxylations Are Reversible—Having confirmed this, we began using this set-up to determine if hydroxylated proteins could be dehydroxylated in cells. Initially, we analyzed the ion chromatogram of the light-labelled TNKS2
peptides containing hydroxylated and nonhydroxylated asparagine 586, and detected a reduction in the intensity of the hydroxylated peptide associated with DMOG treatment, while at the same time we detected an increase of the absolute levels of the unmodified peptide (Fig. 2G), suggesting that hydroxylated residue can be reverted. To increase certainty, we increased the number of experimental repeats to six and analyzed several hydroxylation sites in an automated manner. We selected the peptides that we identified with a localization-specificity for hydroxylated asparagine of greater than 0.8 and where both the hydroxylated and nonhydroxylated isoform were detected in at least four of the six replicates. The hydroxylation occupancy of the peptide was estimated by first correcting the intensity ratio of the modified over unmodified peptide by the ratio of molar ionization efficiencies (Table I, Experimental Procedures). Subsequently we transformed the ratio of the hydroxylated over nonhydroxylated peptide into occupancies.

Using this automated analysis method, we monitored how the occupancy of several light-labelled hydroxylated peptides changed over time (Fig. 2H, supplemental Fig. S2, supplemental Table S1). Six sites showed a statistically significant decrease in hydroxylation occupancy relative to a negative control. The occupancy of some sites was only altered by a few percentage points, whereas others by up to 50%, suggesting a gradient of reversibility.

To rule out the possibility that the pulsing with heavy amino acid did not comprehensively abrogate protein synthesis of light-labelled protein, we repeated the experiment using CHX as an additional control. We combined CHX with a heavy SILAC and DMOG/DMSO pulse in order to abrogate TNKS2 synthesis during the time course of DMOG treatment. Heavy-SILAC pulsing further allowed us to determine how efficiently CHX abrogated protein synthesis. We initially monitored how CHX inhibited protein synthesis by LC-MS and observed a near complete abrogation of heavy-labelled TNKS2 production when compared with a control (Fig. 3A). Overall, TNKS2 synthesis was inhibited by over 98% in the presence of CHX and can therefore be considered residual. Once this was confirmed, we immunoprecipitated Flag TNKS2 from HEK293T cells post pulse and analyzed the hydroxylated by LC–MS/MS as above. When monitoring how the relative occupancy of the light-labelled hydroxylation changed upon pulsing with DMOG, we were able to observe a significant reduction of several asparagine residues (Fig. 3B–3D, supplemental Table S2). Surprisingly, the reduction of the hydroxylation occupancy observed was smaller when compared with the previous experiments. We further failed to observe the proportionality between incubations times and the reduction in hydroxylation occupancies. This initially puzzling observation could suggest that what we underestimated dehydroxylation in the previous experiment because of a significant de novo synthesis of light-labelled TNKS2. If this would be the case, roughly three quarters of the newly synthesized TNKS2 would have to be light labelled. This is inconsistent with our previous observations where half-life estimations based on heavy-label incorporation match estimations based on CHX-pulse experiments. An alternative explanation is that the dehydroxylation or an essential co-factor is rapidly degraded upon CHX treatment. Should the enzyme/s catalyzing the dehydroxylation reaction have a short half-life, this would result in partial dehydroxylation during the initial time points of the experiment, followed by a plateau. This is a profile not dissimilar to what we have observed (Fig. 3B–3D).

To rule out a nonspecific side-effect of DMOG treatment, we utilized a second, structurally and functionally unrelated

| Gene  | Peptide                  | Ionization Efficiency | Unmodified/Modified |
|-------|--------------------------|-----------------------|---------------------|
| TNKS2 | VN<sub>427</sub>ALDNLGQTSLHR | 0.500652              |                     |
| TNKS2 | KGAIN<sub>394</sub>EK    | 1.82884               |                     |
| TNKS2 | HGAVN<sub>586</sub>VADLWK | 0.745932              |                     |
| HIF1A | VN<sub>103</sub>APIQGSR   | 0.93733               |                     |
| TRPV3 | RQGDIAALLIAAGADVN<sub>242</sub>AHAK | 0.848233 |                     |

Fig. 2. A, Schematic illustration of the MS-based hydroxylase screen. Light-labelled cells/protein are in blue and protein synthesized post-pulse are in red. Cells are pulsed with heavy SILAC amino acids in the presence of hydroxylase inhibitors. Cell are lysed and proteins are digested. Changes to the intensity ratios of unmodified and hydroxylated peptide pairs over time (bottom panel) indicates the reversal of the post-translational modification. B, HEK293T cells were transfected with FLAG-tagged TNKS2. 24 h post-transfection the media was replaced by heavy SILAC. Cells were treated with different concentrations of DMOG or vehicle for four hours. FLAG-tagged proteins were immunoprecipitated, digested and analysed by MS. Graph represents the average percentage of heavy-labelled hydroxylated TNKS2 peptides in the presence of DMSO/DMOG, with 100% set to the DMSO level. C, HEK293T cells were transfected with FLAG-tagged TNKS2. 24 h post-transfection the media was replaced by heavy SILAC and cells were treated with different times of DMSO or DMOG. The cells were lysed and proteins separated on PAGE, electroblotted and detected with the indicated antibodies. D, HEK293T cells were transfected with FLAG tagged TNKS2 and V5 F1H. 24 h after transfection cells were treated with CHX and DMOG. Cells were harvested and lysed at different times of CHX/DMOG/SILAC treatment, followed by Western blot analysis with the indicated antibodies. E, FLAG-tagged proteins were immunoprecipitated, digested and analysed by MS. Diagram represent the intensities of immunoprecipitated light FLAG-TNKS2 across the DMSO/DMOG treatment. Error bars are standard deviation. F, As in E but representing heavy FLAG-TNKS2. G, XIC of hydroxylated/unmodified light TNKS2 peptide (HGAVN-VADLWK) along DMOG treatment from 0, 2, 6 and 9 h. H, Graphs represent the hydroxylation occupancy of light SILAC TNKS2 peptides. The error bars represent S.E. and n = 6.
FIG. 3. A, HEK293T cells were transfected with FLAG tagged TNKS2 and V5 FIH. 24 h after transfection cells were treated with CHX and DMOG and the media was replaced by heavy SILAC. Bar graphs represent the ratio of intensity heavy/light SILAC of FLAG TNKS2 during the control/CHX treatment. The error bars represent S.E. and \( n = 6 \). B–D, Graphs represent the hydroxylation occupancy of light SILAC TNKS2 peptides. The error bars represent standard deviation and \( n = 6 \). E, HEK293T cells were transfected with FLAG tagged TNKS2 and V5 FIH, the media was replaced by heavy SILAC and cells were treated with DFO. Cells were harvested and lysed at different times of DFO/SILAC treatment. Proteins were separated by PAGE, electrobotted and detected with the indicated antibodies. F, FLAG-tagged TNKS2 proteins were immunoprecipitated, digested and analysed by MS. Bar graphs represent the intensities of immunoprecipitated light FLAG-TNKS2 across the DFO treatment. Error bars are standard deviation \( n = 2 \). G, Bar graphs represent the intensity of heavy FLAG TNKS2 during the DFO treatment. Error bars are standard deviation \( n = 2 \). H–I, Graphs represent the hydroxylation occupancy of light SILAC TNKS2 peptides. The error bars represent S.E. and \( n = 2 \).
inhibitor, deferoxamine (DFO), which inhibits FIH by chelating the iron in the active center (50, 51). Firstly, as we did previously for DMOG, we checked by Western blot and MS that DFO did not affect the stability of the protein (Fig. 3E) and the incorporation of heavy SILAC (Fig. 3F, 3G). Secondly, we quantified the occupancy for two previously characterized sites. As previously, we observed a reduction in the hydroxylated/nonhydroxylated ratio upon DFO treatment in the Light SILAC samples (Fig. 3H, 3I, supplemental Table S3).

### Rapid Reversal of TRPV3 N-Hydroxylation

To determine whether dehydroxylation takes place on other substrates we decided to investigate the reversibility of a hydroxylation site on TRPV3. As mentioned in the introduction, TRPV3 is hydroxylated by FIH on asparagine 242 (25) and the hydroxylation regulates TRPV3 activity without affecting its expression. Interestingly, TRPV3 activity responds rapidly to hydroxylase inhibition, suggesting that dehydroxylation could be taking place. Replicating our experimental setup in HEK 293T cells and over-expressing myc-tagged TRPV3, we initially confirmed that inhibition of FIH by DMOG and incubation with SILAC media did not affect TRPV3 protein expression. By western blotting we confirmed previous observations that TRPV3 protein stability was not affected by hydroxylase inhibition (Fig. 4A). We then immunoprecipitated myc-TRPV3, digested with trypsin and analysed resulting peptides by MS. Overall intensity of the light-labelled TRPV3 was altered over 2 h and we detected less than 20% incorporation of the heavy label (Fig. 4B), suggesting that TRPV3 is a protein with a long half-life. In addition, we readily detected the reported hydroxylation of N242. We could further determine that for light-labelled N242 the hydroxylation occupancy was rapidly reduced over time upon DMOG treatment (Fig. 4C, supplemental Fig. S3A, supplemental Table S4). Together, these data suggest that the hydroxylation of N242 in TRPV3 is reversible.

### HIF N803 Hydroxylation is Reversible

Finally, we checked if our finding could be extrapolated to the best-studied sub-strate of FIH, the alpha subunit of HIF1α (19, 20). HEK293T were transfected with a GFP tagged HIF1α plasmid in order to overexpress the protein. As previously, we checked if the total levels of GFP HIF1α were affected by DMOG or heavy SILAC treatments. By western blotting we surprisingly detected that HIF1α expression appeared to be stable under normoxic conditions and that DMOG only marginally, if at all, increased HIF1α protein levels (Fig. 5A). We repeated the same experiment and using MS we observed a reduction in the levels of light-labelled HIF1α, at the same time that we detected an induction of the heavy-labelled GFP HIF1α (Fig. 5B). Finally, we studied the oxidation levels of N803 (the asparagine residue hydroxylated by FIH) and we observed that the ratio of oxidation of N803 was rapidly reduced upon DMOG treatment (Fig. 5C, supplemental Fig. S3B, supplemental Table S5). As we expressed full-length HIF1α we decided to additionally monitor the hydroxylation of both reported proline sites. Whereas we identified the unmodified peptides with ease and over 50 MS/MS, we were not able to detect a single MS/MS identifying the 402 and 564 proline hydroxylation sites with confidence. We have therefore concluded that overexpressing HIF1α overwhelms the capacity of the endogenous PHD1-3 enzymes to hydroxylate the proline residues stoichiometrically, delivering an explanation as to why we can detect HIF protein expression in normoxia. Overall, these results match the data that we obtained for TNKS2 and TRPV3, suggesting the presence of an asparagine dehydroxylation reaction. To determine whether other modifications can occur on the hydroxylated peptide we repeated the data analysis including the dependent-peptide matching option, which allows for the unbiased identification of modified derivatives of “base” peptides. The algorithm detected several modifications localized to N803, one of which was the elimination of two hydrogens (supplemental Fig. S4). We could only detect this modification on light-labelled peptides, suggesting that presence of DMOG reduces the amount of the precursor, indicating that
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didehydrogenated N803 is derived from hydroxylated N803. This would be consistent with an intermediate product in the potential dehydroxylation reaction where water is eliminated from the hydroxylated side-chain. Nevertheless, this is speculation as the abundance of didehydrogenated peptide is miniscule preventing us from reliably quantifying it across the samples.

To determine if we could observe asparagine dehydroxylation in a cell-free reaction, we generated hydroxylated protein by incubating the recombinant C-terminal transactivation domain of HIF2α (CAD) with recombinant FIH. To prevent re-hydroxylation by FIH, hydroxylated CAD was re-purified and subsequently incubated with cellular lysates derived from FIH-knockout HeLa cells (35). After 3 h the CAD was affinity purified, digested, and analyzed using MS. The ratio of nonhydroxylated peptide over hydroxylated peptide was approximately double that of the control sample that was immediately purified upon mixing with the lysate (Fig. 5D–5E). Importantly, we observed an increase in nonhydroxylated peptide. Although the overall amount is small, the ratio increased to less than 1/100 of the hydroxylated intensity, this is not unexpected given the supraphysiological levels of hydroxylated peptide that were incubated with the HeLa cellular lysates.

Fig. 5. A, HEK293T cells were transfected with GFP HIF1α and V5 FIH. 24 h after transfection cells were treated with DMOG and the media was replaced by heavy SILAC. Cells were harvested and lysed at different times of DMOG/SILAC treatment. Total lysates were probed for the indicated proteins for immunoblotting. B, GFP-tagged proteins were immunoprecipitated, digested with Trypsin and GluC and analysed by MS. Bar graphs represent the intensities of immunoprecipitated light and heavy GFP-HIF along the DMOG treatment. Error bars are standard deviation n = 2. C, Graph shows the estimated hydroxylation occupancy of light SILAC HIF peptide during DMOG treatment. Error bars represent S.E. and n = 2. D, Representative XIC of unmodified and hydroxylated HIF2α peptide before and after incubation for 3 h with FIH-/-HeLa lysate. E, Bar graph shows the hydroxylation ratio of the unmodified over hydroxylated HIF2α peptide before and after incubation with FIH-/-HeLa lysate. Error bars represent S.E. and n = 3.
Taking together, the data obtained from three different FIH substrates supports the hypothesis that asparagine hydroxylation is a post-translational modification that can be reversed within cells, with additional evidence that the hydroxylation can be reversed in vitro.

DISCUSSION

Protein functions can be switched on and off by distinct PTMs, such as phosphorylation, glycosylation and others (1–4), which allows cells and organisms to respond dynamically to changes in the environment. The postulated reversibility of hydroxylation could explain how acute hypoxia and reoxygenation elicits rapid responses irrespective of protein degradation. This may be especially important in tumors as cyclical/intermittent hypoxia and protein hydroxylation are cancer hallmarks (52, 53).

We have not yet identified the enzyme/s responsible for the postulated reaction. We initially took a candidate approach and knocked-down proteins that were commonly binding to several N-hydroxylated proteins. However, none of these perturbations had an effect on the base-line hydroxylation levels, which suggests that the best option would be to devise a genome-wide screen and using N-hydroxylated-dependent protein-protein interactions or antibodies as a readout. Identification of the enzyme responsible would also allow design of a tailored in vitro assay that could reveal parameters and molecular mechanisms of the reaction, the precise nature of which we can currently only speculate on. Unexpectedly, the experiments we conducted in presence of CHX may have narrowed the search area. It is attractive to speculate that the dehydroxylase is a short-lived protein, as this would enable rapid and dynamic regulation of its activity. Rapid degradation would also bestow the system with the ability to adapt to the cellular needs dynamically. It is also plausible that such a system could include additional regulation downstream of hydroxylases, either as transcriptional target of the HIF-pathway or as direct PHD/FIH substrate. Indeed, most reported PHD-mediated hydroxylations result in protein stabilization, although some discussion has emerged whether PHDs have any substrates apart from HIFs (54). Either way, such feed-forward loops are extensively used in signaling networks as they increase the dynamic response of a system when facing perturbations. Furthermore, rapid turnover of the postulated dehydroxylase delivers a possible explanation as to why dehydroxylation was not observed using CHX-chase experiments (Singleton et al., 2011). A different difference between our work and attempts to detect dehydroxylation, is the addition of NOG to our buffers. This prevents re-hydroxylation post-lysis when the concentration of the intercellular inhibitor is diluted, possibly a crucial step if longer processing of non-denatured samples is required, such as during immunoprecipitations.

When searching for the potential dehydroxylase we can also look at how similar reactions are catalyzed in cells. Typi-cally, these involve the formation of a short-lived intermediate by elimination of water, followed by the hydrogenation of the resulting unsaturated bond. Unbiased screening of aspara-gine modifications indeed detected this potential intermediate, but future work will have determine whether this is the dehydroxylation mechanism. The discovery of the dehydroxylation enzyme/s would also provide a set of likely new therapeutic targets to treat cancer or other human pathologies. For this reason, identification of the enzyme should be a priority and is part of our plans.

DATA AVAILABILITY

MS-data are deposited at Proteomexchange PXD013116.

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Conflict of interest—Authors declare no competing interests.

Abbreviations—The abbreviations used are: 2OG-ox, 2-Oxoglutarate dependent dioxygenases; ARD, ankyrin repeat domains; CAD, C-terminal transactivation domain; CHX, cycloheximide; DFO, deferoxamine; DMOG, dimethyl-oxalylglycine; FIH, factor inhibiting HIF; HIF, hypoxia inducible factor; HRE, HIF responsive element; MBP, maltose binding protein; NOG, N- oxalylglycine; PARP, poly(ADP-ribose)polymerase; PHD, HIF prolyl-hydroxylase; PTM, post-translational modification; qMS, quantitative mass spectrometry; SILAC, stable isotope labeling with amino acids in cell culture; TNKS2, tankyrase-2; TRPV3, transient receptor potential vanilloid 3; Trx-6H, Thioredoxin-6 histidine; VHL, von Hippel-Lindau ubiquitin ligase.

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