Mechanistic insights into the three steps of poly(ADP-ribosylation) reversal

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Poly(ADP-ribosyl)ation (PAR) is a versatile and complex posttranslational modification composed of repeating units of ADP-ribose arranged into linear or branched polymers. This scaffold is linked to the regulation of many of cellular processes including the DNA damage response, alteration of chromatin structure and Wnt signalling. Despite decades of research, the principles and mechanisms underlying all steps of PAR removal remain actively studied. In this work, we synthesise well-defined PAR branch point molecules and demonstrate that PARG, but not ARH3, can resolve this distinct PAR architecture. Structural analysis of ARH3 in complex with dimeric ADP-ribose as well as an ADP-ribosylated peptide reveal the molecular basis for the hydrolysis of linear and terminal ADP-ribose linkages. We find that ARH3-dependent hydrolysis requires both rearrangement of a catalytic glutamate and induction of an unusual, square-pyramidal magnesium coordination geometry.
Poly(ADP-ribose) (PAR) is a polymer of ADP-ribose (ADPr) moieties, established through initial transfer of a single ADPr from β-NAD⁺ onto a protein acceptor site (termed mono(ADP-ribosylation) [MARylation]) followed by polymer extension through repeated conjugation (termed poly(ADP-ribosylation) [PARylation]). The different modification variants regulate a wide array of cellular processes including development, transcription, and the DNA damage response (DDR). Three main criteria have been established to influence functional outcomes: the conjugation site, the chain length, and the branch frequency. First, modification of the so far identified acceptor residues (including Asp/Glu, Ser, Tyr, Arg and Cys) results in O-, N- and S-glycosidic linkages, which allows target selection as well as specific regulation of signal turnover. Serine is the main acceptor in the DDR and its modification is synthesised by PARP1:HPF1 and PARP2:HPF1 complexes. Second, the polymer length may vary between 2 and 200 units, thus influencing the number of proteins able to interact with the modification. Furthermore, the charged nature of large PAR polymers can trigger condensate formation, thereby dramatically altering the physicochemical microenvironment. Finally, in addition to linear ribose(1″→2″)-ribobase linkages, infrequent addition of ribose(1″→2″)-ribobase(1‴→2‴)-ribobase conjugates leads to polymer branching and increase in signal complexity. Together, this modification heterogeneity was proposed to form the PAR code dictating the outcome of ADP-ribosylation signalling. Consequently, the degradation of PAR can be broken down into three reactions: (i) degradation of the linear ribose(1″→2″)-ribobase linkage, (ii) hydrolysis of the branch point ribose(1‴→2‴)-ribobase linkage and (iii) cleavage of the terminal amino acyl-ADP-ribose bond.

Poly(ADP-ribose)glycohydrolase (PARG) and (ADP-ribose) hydrolase 3 (ARH3) are the main hydrolases of serine PAR- and MARylation, respectively, thus playing a pivotal role in signal silencing following DNA damage. PARG is responsible for the majority of polymer degradation, but it is unable to remove the terminal ADP-ribose moiety from the protein substrate, whereas ARH3 primarily cleaves the seryl-ADP-ribose linkage, and hence terminates the signal. ARH3 is the only known enzyme to reverse serine MARylation and is consequently responsible for the regulation of hundreds of ADP-ribosylated proteins following DNA damage. In addition, ARH3 possesses weak PAR cleavage activity and it was suggested that this compensates for slower processing of short chains by PARG, or regulates PAR-induced apoptosis (Parthanatos) through degradation of free PAR chains. ARH3 deficiency leads to persistence of MARylation marks on serine residues that influences the local histone modification pattern and was proposed to alter the transcriptional activity at the ADP-ribosylated loci. Genetic deficiency in ARH3 has been recently shown to cause stress-induced childhood-onset neurodegeneration with variable ataxia and seizures (CONDIAS), anautosomal recessive neurodegenerative disorder that in severe cases can lead to childhood death.

Evolutionarily, PARG and ARH3 belong to unrelated protein families, macrodomains (Pfam CL0223) and (ADP-ribose)hydrolases (Pfam PF03747), respectively. Extensive research shows that PARG cleaves PAR preferably from the end of the polymer (exon) via a glutamate-mediated activation of the O-glycosidic bond and formation of an oxocarbenium intermediate. PARG was also shown to be able to cleave within polymers (endo) and degrade branch points, yet the details of these activities remain elusive. In contrast to PARG, the active site of ARH3 contains a catalytic binuclear magnesium centre. Recent structural studies of ARH3 using the reaction product ADP-ribose and its analogues suggest that a crucial aspect aiding substrate turnover is the exact positioning of the substrate within the active site. However, these studies showed subtle differences in ligand positioning and provide limited information of the pre-catalytic substrate binding mode, which led to the proposal of several conflicting mechanisms. Furthermore, these studies could not explain the stark differences in turnover rates for the chemically similar linkages in serine-MAR and PAR and thus hamper our understanding of the physiological role of ARH3 in the DDR.

In order to fully understand the PAR reversal reactions, we developed a synthetic route to generate well-defined branched PAR molecules. Using synthetic linear and branched PAR we demonstrate that PARG, but not ARH3, can efficiently reverse PARylation. We provide the molecular basis for these differences by solving the three-dimensional X-ray crystal structures of ARH3 in complex with a MARylated peptide and dimeric ADP-ribose. Our structural data revealed that substrate binding induces structural changes leading to repositioning of a catalytic glutamate residue as well as substrate-assisted alteration of the magnesium coordination geometry. Yet PAR binding fails to efficiently induce these structural changes in ARH3, thus providing a structural explanation for the observed low hydrolytic activity towards this substrate. Together, our findings clarify the hydrolysis mechanism of ARH3 and help to explain observed differences in its physiological activity.

**Results**

**Synthesis of PAR branch point.** Recent studies suggest that the PAR branching frequency is an important determinant of the cellular outcomes of ADP-ribose signalling. However, the influence of branching on polymer stability remains elusive. To gain insights, we synthesised linear, dimeric and trimeric PAR as reported earlier and describe here a strategy for the synthesis of the PAR chain branch points. The target branched ADP-ribo trimer (1) has multiple anionic pyrophosphates and was constructed via a solid-phase approach to circumvent repeated isolation of charged intermediates and increase synthesis efficiency. The synthetic route to target compound 1 involved three challenges that needed to be overcome: (i) the installation of three acid-sensitive pyrophosphate introduction in combination with known building blocks (Fig. 1) was chosen as a solid support. Through glycosylation using donor and the Glutamate residue as well as substrate-assisted alteration of the magnesium coordination geometry. Yet PAR binding fails to efficiently induce these structural changes in ARH3, thus providing a structural explanation for the observed low hydrolytic activity towards this substrate. Together, our findings clarify the hydrolysis mechanism of ARH3 and help to explain observed differences in its physiological activity.
furnished solely α-configured disaccharide 8 in high yield. Attempted deprotection of the PMBs with DDQ resulted in a low yield due to the formation of a 2,3-methoxybenzylidene side product. However, acidolysis using TFA rapidly cleaved off all PMB groups, affording triol 9 in reasonable yields. Next, the 3- and 5-OH in 9 were silylated to give TIPDS (1,1,3,3-tetraisopropyldisiloxane) protected 10, allowing selective glycosylation on 2-OH. Condensation of donor 6 and acceptor 10 using TMSOTf followed by subsequent deprotection of the PMB groups did yield the desired trisaccharide but in a low yield (24% from 10, see Supplementary Information) which can be attributed to acidic cleavage of one or both glycosidic bonds during PMB deprotection. To avoid the

**Fig. 1 Retrosynthetic analysis for branched ADPr-trimer (1).** ADPr-trimer 1 with substructures originating from the three distinct building blocks: ribofuranoside 2 immobilized on TentaGel (purple), phosphoramidite of branch point 3 (blue) and adenosine phosphoramidite 4 (orange). The dotted box shows the chemical structure of Fm (fluorenylmethyl group) and iBu (isobutyryl group). Purple sphere represents TentaGel resin. Bz benzoyl, Ac acetyl, Q-linker hydroquinone-O, O′-diacetyl.

**Fig. 2 Synthesis of protected parotriose (14).** D-Ribose is the starting material for the synthesis of donor 6 which is further coupled with acceptor 7 and donor 11 to obtain parotriose derivative 14. Yield after each step is given as percentage in brackets. PMB para-methoxylbenzyl, TMSOTf trimethylsilyl trifluoromethanesulfonate, DCM dichloromethane, TIPDS 1,1,3,3-tetraisopropyldisiloxane, TIPS triisopropylsilyl, Bn benzyl, TEA-HF Triethylamine trihydrofluoride, TBDPS tert-butylidiphenylsilyl.
Fig. 3 Synthesis of branch point phosphoramidite (3). Parotriose derivative 14 is converted into nucleotide 15 via a Vorbrüggen type glycosylation after which phosphate and phosphoramidite functionalities are installed at the primary hydroxyls of the ribose residues. Red: the arrows show the notation for the three distinct 5-OHs. Yield after each step is given as percentage in brackets. HClO₄-SiO₂ perchloric acid on silica gel, BSTFA N,O-bis(trimethylsilyl) trifluoroacetamide, DMT 4,4′-dimethoxytrityl, TBAF tetra-n-butylammonium fluoride, DCI 4,5-dicyanoimidazole, rBuOOH tert-butyl hydroperoxide, TFA trifluoroacetic acid, DIPEA N,N-Diisopropylethylamine, DMF dimethylformamide.

problematic acidolysis, we coupled acceptor 10 with a different donor 11, bearing Bn protecting groups on the 2- and 3-OH instead, using the same TMSOTf/DCM condition to give trisaccharide 12 in good yield and with excellent a stereoselectivity. Pd/C catalysed high-pressure (80 bar) hydrogenolysis of the Bn groups was followed by TFA-3HF mediated desilylation to produce 13 in 79% yield. Selective silylation of both primary hydroxyls in pentol 13 followed by acetylation of all secondary OH-groups to furnish protected parotriose 14 in 82% yield.

The synthesis continued with introduction of N²-benzoyl adenine at the anomeric position of 14 via a Vorbrüggen type glycosylation catalysed by HClO₄-SiO₂ to afford protected parotriose adenine 15 (Fig. 3)³⁹. To make 15 ready for introduction of the two phosphotriesters on the primary 5′- and 5″-OH positions, all acetyl and benzoyl esters were carefully saponified with 1 M aqueous NaOH at 0 °C to give 16 in a high yield. It is well-established that the N-benzoyl at the exocyclic amino group of N-benzoyl at the exocyclic amino group of 16 is converted into nucleotide 17 after cleavage of the DCI-catalysed phosphorylation of the 5″- and 5‴-OH groups with Fm amide 19 followed by oxidation of the resulting phosphites to phosphate triesters by rBuOOH. Next, stoichiometric amounts of TFA were used to rapidly remove the DMT leading to compound 20 with deprotected 5-OH. Finally, treatment of alcohol 20 with commercially available aminophosphorochloridate 21 and DIPEA in DMF gave key phosphoramidite 3. It is important to note that this phosphorylation needs a careful work-up procedure for the removal of DIPEA, as DIPEA is capable of cleaving one of the Fm groups. In addition, the simultaneous occurrence of an acid labile (phosphoramidite) and the base-labile (Fm) groups in compound 3 requires column chromatography with high-quality IRR silica gel (see Supplementary Information).

Eventually, starting from d-ribose, the advanced phosphoramidite 3 was prepared via a 21-steps high yielding, highly stereoselective synthetic route in sufficient amount (0.36 mmol) for the purpose of the solid-phase synthesis of branched ADPr oligomers.

After preparation of all required building blocks (see Supplementary Methods for preparation of functionalised Tentagel resin 2), we proceeded with the solid-phase synthesis of branched ADPr-trimer 1 in an iterative P(V)-P(III) procedure (Fig. 4). An α-O-methyl group was installed at the anomeric centre at the terminal ribose in immobilized 2 to mimic the native PAR stereochemistry. DBU (10%)-mediated removal of Fm protections on resin 2 was followed by the introduction of the first pyrophosphate function by a three-step procedure: (i) 5-(benzylthio)-1H-tetrazole (BTT) assisted coupling of advanced phosphoramidite building block 3 with the deprotected phosphate derivative of 2, (ii) CSO oxidation of the resulting phosphite-phosphate intermediate and (iii) DBU mediated cleavage of both CE and terminal Fm groups. The obtained immobilized intermediate 22, with two phosphonooester functions allows the simultaneous introduction of the next two pyrophosphates by the same three-step P(V)-P(III) coupling cycle using phosphoramidite 4. Finally, removing all the protecting groups and the cleavage of the product from the resin using aqueous NH₄OH gave the desired branched ADPr-trimer 1. Purification with anion-exchange chromatography led to the isolation of 0.68 mg of target branched ADPr 1. The side product of the reaction, α-1″-O-methyl-ADP-ribose 23 (meADPr), was recovered separately (Fig. 4 and Supplementary Table 1).

PARG and ARH3 cooperate in PAR reversal. Following synthesis, we first tested the efficiency of PARG and human ARH3 (hARH3) to initiate branch point reversal by measuring their ability to degrade the synthetic PAR trimers. We used thin-layer chromatography (TLC) and liquid chromatography high-resolution mass spectrometry (LC-HRMS) (see Methods) and found that PARG was able to efficiently degrade the branch point, whereas hARH3 showed no activity under these assay conditions (Fig. 5a, b and Supplementary Figs. 1a, 2). Long time-course experiments revealed that hARH3 is able to degrade branched trimers, however, this activity is dramatically lower than the cleavage of the linear polymer and, hence, may not be physiologically significant (Fig. 5c and Supplementary Fig. 1b). This is further supported by our observations in cells: we cultured U2OS wild-type (wt) and ARH3⁻/⁻ cells and analysed cellular content...
of linear and branched PAR through isolation of di- to mono-ribosyl-adenosine (R₂-Ado/R-Ado; nucleoside utilised as indicators of branching and linear PAR, respectively) as described earlier. In the absence of PARG inhibitor (PARGi), the levels of R₂-Ado were below the technical limit of detection. However, they could be readily detected in PARG-inhibited cells, in line with PARG’s involvement in branching removal (Fig. 5d). Interestingly, we observed a ~1.5-fold lower branch frequency (R₂-Ado/R-Ado) in PARGi-treated ARH3−/− cells compared to PARGi-treated wt cells. These data suggest that ARH3 preferentially degrades linear PAR chains and argue against the importance of ARH3 for branch point removal in vivo.

Interestingly, LC-HRMS analysis of the PARG catalytically impaired mutant E756N showed marginal activity against the branch point, which allowed us to detect a meADPr-ADPr dimer as a reaction intermediate, thus identifying the cleavage of the ribose(1″–2″)ribose bond as the first step in branch point resolution (Fig. 5b and Supplementary Fig. 2). This finding is in line with earlier observations suggesting preferential branch point cleavage by PARG. Previous structural and functional studies showed that efficient hydrolysis requires the formation of a substrate:PARG complex involving coordination of the 2″-OH group by Glu755 and Glu756 and the 3″-OH moiety by N740. To gain insight into the PARG:branch point interaction, we produced energy minimised models of PARG with branched ADPr as ligand (Supplementary Fig. 3). Analysis of the ligand coordination showed that placement of ADPrₐ₋₁ (linear polymer extension; see Supplementary Note 1 for proposed PAR nomenclature) within the active site prevents E756 from coordinating 2″O (scissile bond oxygen) due to steric hindrance imposed by the [ADPrₐ₋₁] distal ribose (Supplementary Fig. 3), thus preventing hydrolysis. Furthermore the 2″ and 3″OH moieties of [ADPrₐ₋₁] may have a slight shielding effect towards the 3″OH of ADPrₐ₋₁ influencing its interaction with N740. Contrarily, placement of [ADPrₐ₋₁] within the active site does not impose these constraints on the pre-catalytic complex and a coordination comparable to that observed in PAR:dimer structure (PDB 5A7R) appears feasible (Supplementary Fig. 3). While further work is needed, our data argue for a PAR degradation mechanism in which branch pruning is required prior to polymer degradation beyond the branch point.

To assess whether the 1″-O-methyl moiety of ADPrₐ₋₁₃₅ had an influence on the reaction, we performed inhibitor studies utilising free meADPr. Our results indicate that the latter acts as an inhibitor of the ARH3 reaction with comparable potency to ADPr (Supplementary Fig. 4a). To assess whether these findings are transferable to enzyme-derived PAR, we generated different serine-linked PAR polymers formed by PARP1 variants with defined influence on polymer formation: G972R (hypo-branching), Y986H (hyper-branching) and Y986S (short chain, wt branch frequency) in the presence of HPFI. Indeed, the stability of PARP1 Y986H-derived polymers was increased in comparison to G972R- or Y986S-derived PAR (Fig. 5e), thus further supporting our finding that ARH3 hydrolysis of PAR is slowed down by the presence of branch points.

**Structures of ARH3 bound to its substrates.** Amongst the known (ADP-ribose)hydrolases—both of the macromdomain and ARH superfamilies—ARH3 stands out due to its ability to cleave diverse chemical linkages including the acetal O-glycosidic bond found in PAR and serine MARylation (Supplementary Figs. 4b, 5a and Table 1), the ester O-glycosidic bond of the metabolic intermediates 1‴-O-acetyl-ADP-ribose (OAADPr) as well as the N-glycosidic bond found in α-NAD⁺ (Supplementary Fig. 5b and Supplementary Table 1). Yet, the available ARH3:product and product analogue structures did not provide insights as to why chemically similar bonds are cleaved with different efficiency: ARH3 rapidly cleaves the seryl-ADP-ribose bond, but PAR degradation is slow. OAADPr is readily converted, but aspartate/glutamate MARylation hydrolysis is negligible. Lastly, α-NAD⁺...
is rapidly cleaved, but Arg-ADPr is an ARH3 inhibitor with nanomolar affinity. To understand the impact of the ARH3:substrate interaction on reaction efficiency, we crystallised ARH3 in complex with the chemically synthesised substrates: histone H2B peptide (aa 1–11) MARylated on Ser7 (H2BS7mar), dimeric ADP-ribose (dimer) and α-NAD⁺ (Supplementary Tables 1, 2). Furthermore, we solved the structure of wt *Latimeria chalumnae* ARH3 (LchARH3) in complex with meADPr (Supplementary Tables 1, 2). Earlier described mutations inactivating ARH3 can be broadly classified into three categories affecting (i) protein stability, (ii) magnesium coordination or (iii) ADPr binding. Some of these variants have been associated with stress-induced childhood-onset neurodegeneration with variable ataxia and seizures (CONSDIAS), an autosomal recessive disorder and are annotated in the COSMIC database for cancer-associated mutations, which may indicate an influence on disease progression in some cancer types. We tested, three involved in adenosine coordination (F143L, S148A and G150E) and five within the active site (D34G, E41A, T76R, D77N and D78N) showed severely reduced catalytic activity (Fig. 5f and Supplementary Fig. 4b). We and others have previously shown that mutation of Glu41 is compatible with the coordination of the Mg₂⁺ ion as well as ligand binding. Therefore, we chose to crystallise the hARH3 E41A substrate complexes as this prevents substrate cleavage during crystal formation, while retaining a functionally relevant magnesium coordination. The resulting structures show little divergence from the previous solved hARH3:ADPr complex (PDB 6D36) with r.m.s.d. of 0.284 Å (H2BS7mar), 0.316 Å (dimer), 0.176 Å (meADPr) and 0.362 Å (meADPr) over 278, 270, 255 and 257 Cα atoms, respectively (Supplementary Fig. 6). All ligands are well defined in the electron density with the exception of the C-terminal peptide moiety of the dimeric PAR (Supplementary Fig. 7).

**ARH3 activation requires substrate-induced active site rearrangements.** Comparison of the active sites of our ARH3-substrate complexes revealed two striking features. First, substrate binding induces conformational flexibility within the Glu41-flap (composed of residues Glu41 to Tyr75), which was suggested to play a role in substrate entry and catalysis (Fig. 6a, Supplementary Fig. 8). In the closed conformation, Glu41 interacts with Mg₂⁺, which induces tighter packing of the flap against the ADP-ribose (dimer) and for 4 days. Afterwards, the di-ribosyl-adenosine to ribosyl-adenosine (R₂-Ado/R-Ado) ratio was quantified by UPLC-MS/MS analysis (see ‘Methods’). Data are shown as mean ± s.d. of biological quadruplicates, **p < 0.0032 (unpaired, two-tailed t-test).**
same enzyme state (Supplementary Fig. 8). Together, this suggests that the presence of a KS motif has an influence on the (ADP-ribosyl)hydrolase activity of ARH3, we performed ADP-ribosylation consensus motif (KS) (Fig. 6b)5,7. To test whether the presence of a KS motif has an influence on the (ADP-ribosyl)hydrolase activity of ARH3, we performed ADP-ribosylation hydrolysis experiments using previously established peptide substrates: H3 (aa 1–21), which contains an internal

domain core and was suggested to represent a post-catalytic state. In the open conformation, the Mg2+/Glu41 interaction is lost, which allows higher flexibility of the Glu41-flap, and was suggested to increase the accessibility of the active site27. Interestingly, while we observe an open conformation in the H2BS7mar- and α-NAD+)–bound structures, both the hARH3:dimer and LchARH3:meADP complexes adopt a closed conformation (Fig. 6a). To exclude influences of crystal packing on Glu41-flap flexibility, we analysed the different crystal lattices (Supplementary Table 2) of our structures and found that in the majority of chains the Glu41-flap is orientated towards the intra-crystal solvent channels (Supplementary Figs. 8 and 9). Chain-to-chain comparison within the different structures showed that even in cases where Glu41-flap crystal contacts form, the overall arrangement is similar to chains without such contacts of the same enzyme state (Supplementary Fig. 8). Together, this suggests that crystal packing does not influence the observed Glu41-flap conformations and the observed differences are mainly ligand-induced.

The AMP moiety of all four ligands is isostructurally orientated, whereas the distal riboses take up ligand-dependent poses (Supplementary Fig. 6g). The H2BS7mar peptide lies loosely coordinated within a groove running perpendicularly to the ADPr binding cleft (Supplementary Fig. 10a). It adopts no secondary structure and makes mostly water-mediated contacts with the hARH3 protein. This binding mode is incompatible with the closed conformation of the Glu41-flap and indeed stabilises the open form via a Lys6 (side chain, H2B peptide):Glu41 contact, which is part of the short serine-ADP-ribosylation consensus motif (KS) (Fig. 6b)5,7. To test whether the presence of a KS motif has an influence on the (ADP-ribosyl)hydrolase activity of ARH3, we performed ADP-ribosylation hydrolysis experiments using previously established peptide substrates: H3 (aa 1–21), which contains an internal
modification site (Ser10) within the consensus KS motif, and H4 (aa 1–23), which is primarily modified on the N-terminal Ser129. Under the assay conditions both peptides show comparable modification efficiency and similar de-modification progression (Fig. 6c). While we cannot fully exclude contribution to substrate selection of the KS lysine or other nearby residues, these results suggest that the KS lysine is dispensable for efficient ADP-ribosylation hydrolysis and that ARH3 has few limitations regarding the sequential context of the modification. This is in line with the observed broad target spectrum of ARH3 following DNA damage. In addition, the broad target spectrum suggests that substrate binding depends primarily on the ADPr moiety attached to the protein substrate. Concordantly, mutations of Phe143 and Ser148 (F143L and S148A), which coordinate the adenine base, show almost complete loss of catalytic activity. In contrast, the structurally adjacent COSMIC mutations S185P and L186V appear fully active (Fig. 5f). Mutation of residues along the peptide channel (Gly115, Asn269, Gly270, and Ile271) do not alter the enzymatic activity (Fig. 5f). Note, mutation of Tyr149 to leucine, the second residue π-stacking with the adenine base, does not influence enzymatic activity, most likely due to retention of tight packing of the adenosine in this mutant.

Our structural data further reveal that in the α-NAD+ complex Glu41 would be displaced from the axial MgII position by the nicotinamide moiety, thus forcing the observed open conformation (Supplementary Fig. 12d). In contrast, the ADPhe143 unit of the dimer binds in a relaxed conformation within the ligand site, which prevents engagement of the scissile bond with the Mg2+ ions (Fig. 6c, Supplementary Figs. 10b, 12c). Similar to the peptide substrate, the ADPhe143 makes only water-mediated contacts with the protein. While the adenosine base appears well defined with only slightly elevated temperature values (B-factors), the latter increase sharply towards the phosphate groups (Supplementary Fig. 11). The different conformations adopted by the phosphates together with the lack of reliable electron density for the distal ribose further indicate a high degree of flexibility within this part of the molecule. The axial position of MgII is occupied by a water molecule (w610) due to the E41A mutation that was introduced to allow crystallisation. However, the nicotinamide moiety sterically prevents occupation of the axial ligand position of MgII, thus creating a free valence at the Mg2+ ion and forcing the Glu41-flap to open. In addition, the nicotinamide positioning is distinctly asymmetric, reminiscent of ring slippage, which may polarise the π electron system and weaken the N-glycosidic bond (Supplementary Table 5). Noteworthy, neither the H2BS7mar nor the α-NAD+ complex have a water ligand at MgII, thus excluding the ion as a water activator during the reaction cycle (Fig. 6d and Supplementary Fig. 11d).

Glu41 positioning leads to substrate activation. Given that the E41A mutation allows transition between the open and closed conformation while remaining catalytically inactive, the maintenance of a closed conformation in the absence of substrate cannot be the only function of this residue. Our structural analysis suggests that this residue is required for further substrate activation following binding. In the hARH3:H2BS7mar complex, Glu41 is shielded from the aqueous environment by the peptide and is no longer able to form a first-order coordination bond with MgII. In the structure, we observed a water molecule (w592; chain A) residing within the second coordination sphere of MgII, which coincided with the carbohydrate group of a naïvely placed favourable glutamate rotamer. Energy minimisation modelling of this conformation into the water density leads to the formation of second coordination sphere interactions with both Mg2+ ions (Fig. 7a and Supplementary Fig. 13). This conformation would allow interaction with the axial water ligand of MgII, which in turn could activate and position the molecule for a nucleophilic attack from the scissile bond oxygen. While a similar Glu41 conformation in the α-NAD+ complex is possible, the differences between the O- and N-glycosidic bonds render an initial nucleophilic attack on a α-NAD+ superfuous for hydrolysis. Furthermore, the absence of the peptide backbone imposes less restrictions on potential Glu41 side-chain conformations and an interaction between Glu41 and the pyridine ring amide appears feasible. This interaction could fix the relative positioning of the ring towards the magnesium ion and further withdraw electrons from the ring system, thus contributing to the weakening of the nicotinamide-ribose bond.

Taken together our data support a mechanism in which both the achievement of a high energy MgII state as well as a repositioning of Glu41 are prerequisites to catalysis. The former is demonstrated by the inability of hARH3 to cleave meADPr despite the presence of an only five-coordinated magnesium ion (Supplementary Figs. 4a and 12d). This lack of activity against meADPr, however, raises the question why OAADPr can be efficiently cleaved by ARH323,54? While we were unable to co-crystallise ARH3 with 1’-O-AADPr, which is the confirmed substrate isomer, due to transesterification, which disfavours this linkage under our crystallisation conditions, our data strongly suggest that this ligand would need to displace Glu41. Most likely displacement is achieved by a Mg2+-substrate interaction that does not involve the scissile bond oxygen, but rather the adjacent carbonyl group. We modelled this interaction and found that the scissile bond would be orientated towards the water ligand of MgII, thus favouring a nucleophilic attack (Fig. 7b and Supplementary Figs. 12g and 14). In addition, this interaction would place the terminal carbon of the acetate facing away from the peptide binding site, disfavouring the binding of peptides containing Asp- or Glu-linkages within the active site and thus providing an explanation for the inability of ARH3 to cleave these modifications29.

ARH3 reaction progresses through an oxocarbenium ion transition state. To clarify reaction progression post initiation, we aim to distinguish between the proposed formation of an
panels) meADPr (M increases experimental background. Source data are provided as a Source chromatograms were extracted setting tolerance at 10 ppm. Note, that this intermediate55. Indeed, we observed the ARH3-dependent observable as reaction product if the reaction progress through alternative nucleophile. Methanol has a higher propensity to react (w1, axial water) or to the template PAR and resolution mass spectrometry analysis of solvolysis of H2BS7mar, trimeric model Glu41, w1 and scissile bond oxygen are shown in black. magnesium coordination bonds are shown in yellow and distances between model Glu41, w1 and scissile bond oxygen are shown in black. e High-resolution mass spectrometry analysis of solvolysis of H2BS7mar, trimeric PAR and α-NAD⁺ in the presence and absence of methanol. Shown are the total negative ion counts for ADP‘ [M–H]⁻ with a m/z of 558.06 (left panels) meADP‘ [M–H]⁻ with a m/z of 558.06 (right panels). Ion chromatograms were extracted setting tolerance at 10 ppm. Note, that trimeric PAR contains meADP‘; which is released upon cleavage and increases experimental background. Source data are provided as a Source data file.

oxocarbenium ion or seryl alkoide10,27–29 intermediate by performing solvolytic experiments in presence of methanol as alternative nucleophile. Methanol has a higher propensity to react with oxocarbenium ions than water and meADP‘ should be observable as reaction product if the reaction progress through this intermediate55. Indeed, we observed the ARH3-dependent formation of meADP‘ regardless of the used substrate (H2BS7mar, PAR trimer and α-NAD⁺; Supplementary Fig. 7c). This commonality in the intermediate formation suggests that, while difference in reaction initiation may exist, the later steps of the ARH3 catalytic cycle are substrate independent. Future work is needed to determine whether the reaction progresses step-wise with a solvent-equilibrated intermediate or via a preassociated/concerted mechanism with an oxocarbenium ion-like transition state56.

Discussion

The dynamics of PAR signal turnover are a crucial determinant of its physiological outcomes. However, the complexity of the PAR molecule with the apparent stochastic nature of branch point introduction has long hampered efforts to study its degradation directly. Thus far, only linear PAR fragments were available either by liquid chromatographic purification of enzyme-derived polymers or chemical synthesis32,57. Due to the unavailability of defined branch points, observations of their cleavage have relied on the analysis of mixed, enzyme-derived polymers to infer hydrolyase properties36,38. Here we describe the de novo synthesis of branched point polymers, thus making this elusive PAR building block available for targeted studies. We utilised this approach to confirm that the PAR degradation step is primarily driven by PARC, which can efficiently cleave linear and branched polymers. Long incubations with a high ARH3 concentration showed detectable activity against branch points, but this very low activity may be physiologically insignificant. Our findings suggest that introduction of PAR branch points imposes a further level of specific regulation: not only providing these structural binding platforms for specialised binders, such as APLF31, but also regulating signal duration, thus altering physiological outcomes. This is in line with recent studies that show a correlation between branch frequency and physiological response50,51.

While PARG is only able to cleave the O-glycosidic PAR bond, ARH3 is known to process chemically distinct substrates12,16,31,54,59. Amongst the more surprising findings in substrate selection and turnover is that ARH3, despite the similarities in bond nature, is able to process Ser-ADP and OADP‘ efficiently, whereas PAR degradation by ARH3 is a much slower process12,16. Our structural work gives rise to mechanistic insights into these differences and the molecular factors governing substrate activation and hydrolysis initiation.

First, the substrates directly interact with the magnesium centre and induce a square pyramidal coordination geometry at MgβII. This unusual coordination can be found in several chemical compounds but has so far only been observed twice in protein structures60,61. The NG GTPase domain of the Ffh subunit of the prokaryotic signal recognition particle (SRP) contains a mononuclear, catalytic magnesium centre. In the GDP-bound form, the ion is usually octahedrally coordinated by four water molecules, the GDP β-phosphate and a threonine side-chain oxygen. However, a second, square planar, state was observed, which was stabilised through rotation of a glutamate carboxyl into the second coordination sphere (PDB 1087). These changes in the metal coordination share similarities with our observed H2BS7mar structure; however, in the Ffh GTPase a post-catalytic state was represented, as the GTPase substrate activation involves octahedral coordination of β- and γ-phosphate at the magnesium centre. Our structural models predict that Glu41 enters the second coordination sphere of both Mg2⁺ ions after displacement from its axial MgβII coordination position, which in wt ARH3 may help stabilise the observed five-coordinated intermediate. Furthermore, Glu41 positioned in this way may activate the axial water at MgβII for a nucleophilic attack onto the 1°O (Figs. 4a, 5). The second example of a square pyramidal coordination was observed in a Lon AαA + protease (LonA) crystallised in presence of magnesium and the covalent inhibitor bortezomib (PDB 4YPM)61. Attachment of the inhibitor occurs in close proximity to the metal-binding site and sterically blocks access of water molecules to one of the ligand sites. This mirrors the binding configuration we observe in our α-NAD⁺ structure in which the nicotinamide ring sterically prevents access of water or Glu41 to the MgβII centre. The relative distance between magnesium ion and nicotinamide in our structure suggests that rather than engaging in metal coordination, the ligand displacement leads to a free valence and a high energy magnesium state. Together with slippage-like positioning of the nicotinamide ring, which introduced polarisation into the π electron system, it likely provides the energy for bond cleavage.

Second, the substrate needs to induce the open conformation of ARH3 through displacement of Glu41 from the MgβII coordination. Such a displacement not only allows the engagement of
the substrate with the magnesium centre but also leads to the repositioning of Glu41 into the catalytic position. The importance of this step can be observed in our LchARH:meADPr structure in which the ligand engages with the magnesium centre, but fails to displace Glu33 (Glu41 in hARH3) from MgII. Therefore, meADPr can act as an inhibitor of ARH3 instead of being hydrolysed. Our structures furthermore suggest that this engagement is the rate-limiting step in the hydrolysis of PAR. Within our hARH3-dimeric structure, crystal packing does not impact the positioning of the ADPr$_{a1-m-1}$ moiety. Both ADPr groups of the dimeric PAR adopt a relaxed conformation and do not engage with the MgII centre to displace Glu41. The most likely reason for this unproductive binding mode is the strained position the ADPr$_{a1-m-1}$ would need to adopt as well as the lack of a further coordination groups (like the serine backbone carbonyl) to stabilise the binding.

It is worth noting that earlier apo and ADPr-bound structures of hARH3 showed a μ-aqua ligand at the metal centre (Supplementary Fig. 12a,c,29,31) which was suggested to play a catalytic role. We were, however, unable to observe this ligand in any of the here reported structures (Supplementary Fig. 12) or in our earlier reported structures of LchARH3 product (analogues) complexes. This absence, together with the unusually short coordination bonds observed in the earlier structures, suggests that the μ-aqua ligand is dispensable for catalysis and may have rather a function in the stabilisation of the binuclear magnesium centre in absence of a substrate or ligand.

Together these observations clarify the initial steps of the reaction (Fig. 8), but the reaction progression post glycosidic bond cleavage is not immediately obvious and depends on which of a variety of possible intermediates, including the earlier proposed oxocarbenium ion and seryl alkoxide,27–29, is formed. Introduction of methanol to the reaction leads to the formation of meADPr, thus strongly suggesting that an oxocarbenium intermediate or a transition state with strong oxocarbenium ion-like characteristic is formed. It is a generally held view that oxocarbenium ions are too unstable to exist as a free intermediate and hence stabilisation in the reaction cycle would be expected. However, we could not observe any suitably coordinated nucleophilic water, needed for the resolution of a free oxocarbenium ion via an attack on the C1" position, or stabilising residues in our crystal structure. The steric limitation within the active site further suggests that the nucleophilic attack can only be realised from the α-face of the ribose, which, unlike in macromolecular-type hydrolysis, preserves the configuration at the stereocentre. A likely explanation could be that the required water molecule is part of the outer magnesium coordination spheres and thus prone to enter the active site following coordination changes during substrate turnover (Fig. 8).

In conclusion, our synthesis strategy for branch points within PAR polymers made it possible to gain insights into the substrate selection and hydrolysis of PARG and ARH3. The synthesis strategy is scalable and can be used in future studies to overcome limitations in the availability of well-defined substrates that has long hampered investigations in this important area. Here we provide a more complete understanding of PAR removal from branch point cleavage to serine MARylation hydrolysis. The reduced activity of PARG and ARH3 against branch points observed in this study provides a plausible explanation for the phenotypically observed impact of branch frequency on cellular outcomes of ADP-ribosylation signalling.30,31 Furthermore, our findings give further insights into the molecular mechanism of abnormal ADP-ribosylation signalling identified in several...
diseases. For instance, (ADP-ribosyl)hydrolase deficiencies readily lead to neurodegeneration22,23,64 and in particular ARH3 was associated with alteration of the histone code25 and failure to revert back to a pre-damage chromatin state after DNA repair23.

**Methods**

### Plasmid construction

Expression vectors for hARH3, LcARH3, HPF1, PARP1 and PARG were described earlier1–8. All indicated mutations were introduced via PCR based site-directed mutagenesis (Supplementary Table 6). The hARH3 E41A crystallisation construct was generated by PCR based site-directed mutagenesis altering the encoded N-terminal sequence: parts of the linker region as well as aa 1–18 of ARH3 were replaced by a HRV3C cleavage site (N-terminal protein sequence of construct utilised in the biochemical analyses MGSHHHHHHDDTSLTDLFNLNPRWBAAGGAGASLR) and was altered to MGSGHHHHHDDTSLFNLFPGPGSSLR [for crystallisation] (Supplementary Table 6).

### Protein expression and purification

**For biochemistry.** Expression of recombinant proteins in Rosetta (DE3) cells grown in LB medium was induced at OD$_{600}$ 0.6 with 0.4 mM IPTG, cells were grown overnight at 290 K and harvested by centrifugation. Recombinant His-tagged proteins were purified using a HiTrap HP column (GE Healthcare) according to the manufacturer’s protocol using the following buffers: all buffers contained 50 mM Tris-HCl (pH 8) and 500 mM NaCl; additionally, the lysis buffer contained 25 mM, the washing buffer 40 mM, and the elution buffer 500 mM imidazole. For purification of the ARH3 proteins all buffers also contained 10 mM MgCl$_2$, 1 mM DTT, and protease inhibitors (1 mM PMSF, 500 μM Tris-HCl (pH 8), 200 mM NaCl, 1 mM DTT and 5% (v/v) glycerol). Proteins were removed from dialysis using Amicon Ultra centrifuge filters (3000 MWCO; Sigma-Aldrich). Flow-through was stored at −80 °C until analysis.

**For crystallisation.** LcARH3 wt and hARH3 E41A underwent affinity purification over a HiTrap HP column (GE Healthcare), followed by dialysis against 50 mM Tris-HCl (pH 8), 500 mM NaCl, 20 mM imidazole and 1 mM DTT in presence of 0.75, 1.5 or 2.0 M NaCl to a total concentration of 1000 mM, followed by elution of bound protein with the addition of 250 mM imidazole. The eluate was diluted fivefold with 25 mM Tris, pH 7, 100 mM NaCl, 0.5 mM TCEP, and lysed by high-pressure homogenisation. The proteins were purified using an AKTA FPLC system (GE Healthcare Life Sciences) at 277 K via affinity chromatography using a HiTrap HP column (GE Healthcare Life Sciences). After lyse application, the column was washed with lysis buffer supplemented with, first, 50 mM imidazole and, second, NaCl to a concentration of 1000 mM, followed by elution of bound protein with the addition of 250 mM imidazole. The eluate was diluted fivefold with 25 mM Tris, pH 7, 100 mM NaCl, 0.5 mM TCEP, and applied to a HiTrap Heparin column (GE Healthcare Life Sciences) equilibrated in the same buffer, eluted with a linear NaCl gradient to 1000 mM. Fractions containing PARP1 were pooled and loaded on a Superdex 200 Increase 10/300 GL, and eluted with 200 mM NaCl, 0.5 mM TCEP.

### Chemical synthesis

The synthesis of linear ADPr polymers and the H2BS7mar peptide were described earlier32,36. The synthesis of poly(ADP-ribosyl) branch peptide were described earlier32,36. The synthesis of poly(ADP-ribosyl) branch point or linear trimer, was incubated with 10 μM PARP1, 0.6 μM HPF1 and activated DNA (Trevigen) in assay buffer (50 mM Tris-HCl [pH 8], 200 mM NaCl, 1 mM MgCl$_2$, 1 mM DTT, 25 μM NAD$^+$ and 1 μM PARGi). Reactions were incubated for 30 min at 30 °C and stopped by addition of 0.1% SDS to a total concentration of 0.05%. To generate modified PARP1 mutants, the same reactions were performed excluding the histone peptides and utilising a higher NAD$^+$ concentration (50 μM). Reactions were further incubated in presence of 1 μM ARH3 for 45 min at 30 °C. Reactions were stopped by addition of LDS sample buffer (Life Technologies) and incubation at 95 °C for 3 min. Samples were then analysed by SDS-PAGE and autoradiography. Alternatively, reactions were performed as described in absence of 32P-NAD$^+$ and analysed by SDS-PAGE and immunoblot using rabbit monoclonal anti-pan-ADP-ribose binding reagent (Millipore, MABE1016; RRID: AB_2665466) and mouse monoclonal anti-His antibody (Takara, 631212; RRID: AB_2721905) as primary antibodies and polyclonal anti-mouse immunoglobulins/HRP (Dako, P0447; RRID: AB_2617137) and polyclonal swine anti-rabbit immunoglobulins/HRP (Dako, P0399; RRID: AB_2617411) as secondary antibodies. For inhibitor study, the ARH3 was pre-incubated with the indicated amount of inhibitor for 5 min at RT.

### LC-HRMS analysis

**LC-HRMS analysis.** ADP$_r$ and other nucleotides were analysed by a method previously developed in ref. 69. Briefly, a Thermo Exactive mass spectrometer equipped with Waters Acquity liquid chromatography system was used for high-resolution mass spectrometry. Thermo Xcalibur software was used for controlling the instrument. Mass resolution was set to ultra-high (100,000 at 1 Hz) according to the manufacturer manual to achieve measurement accuracy of 0.025 ppm at m/z 200,000. Before each measurement, the system was calibrated and its accuracy with external calibration was confirmed to be better than 5 ppm. We adjusted the Electrospray source conditions to maximise sensitivity, and detection mode was set to detect both positive (+) and negative (−) ions. UV-visible chromatograms were recorded at 200 nm. Mass chromatograms were separated over a SeQuant™ ZIC® HILIC column (100 × 2.1 mm, 5 μm particle size, 200 Å pore size; Merck) using the following buffer systems: buffer A contained 20 mM ammonium acetate (pH 7.5) in MeCN (Honeywell, CHROMASOLV™ 99.9%); water 9:1 (vol/vol) and buffer B 20 mM ammonium acetate (pH 7.5) in water. The column was equilibrated to 40 °C and sample eluted at 0.2 mL/min using the method: [0–1 min]: 100% (B); [1–22 min]: 100% (B) linearly changed to 50% (A): [22–25 min]: 50% (A); [25–26 min]: 50% (A) linearly changed to 80% (A); [26–30 min]: 80% (A); [30–31 min]: 80% (A) linearly changed to 100% (A); and [31–45 min]: 100% (A). All compounds were eluted within 25 min and the gradient was kept 100% (A) from 31–45 min to assure the column return to equilibrium before the next injection. MS data were recorded from 0–25 min. Analysis of the LC-HRMS data was performed using MNova software (v13, Mestrelab Research).

### Analysis of branch frequency of in vivo generated PAR

UPLC-MS/MS analyses of PAR were performed as described previously44,60,65, with some modifications. Control and ARH3−/− U2OS cells were cultured in DMEM (Sigma) containing 10% FCS and penicillin/streptomycin (Thermo Fisher Scientific). The cells were treated with DMSO or 25 μM PARGi for 4 days. Afterwards, the media was removed, cells were washed with PBS and lysed by addition of ice-cold 20% TCA and detached mechanically using cell scrapers. Precipitates were pelleted by centrifugation at 3000 x g for 5 min, pellets washed with 70% ice-cold EtOH, air-dried for about 1 h and further stored at −20 °C. 1 μL of sample was incubated with 6 μL 25 mM MgCl$_2$, 2.5 μL 100 mM CaCl$_2$, 12.5 μL 2 mg/ml DNase (Roche) and 2.5 μL 1 mg/ml RNase (Thermo Fisher Scientific), and incubated for 3 h at 37 °C and recorded using a SpectraMax M5 plate reader with the SoftMax Pro software (Molecular Devices). Data were analysed using Prism (v9.1, GraphPad).

Enzymatic activity assay using PARP1-derived MAR and PAR. ARH3 activity assays were performed essentially as described in1–5. Briefly, histone H3 (α 1–20, biotinylated) or H4 (α 1–23, biotinylated) peptides were modified by incubation with 0.5 μM PARP1, 0.6 μM HPF1 and activated DNA (Trevigen) in assay buffer (50 mM Tris-HCl [pH 8], 200 mM NaCl, 1 mM MgCl$_2$, 1 mM DTT, 25 μM NAD$^+$ and 1 μM PARGi). Reactions were incubated for 30 min at 37 °C and stopped by addition of 100 μL of 0.1% SDS. To generate modified PARP1 mutants, the same reactions were performed excluding the histone peptides and utilising a higher NAD$^+$ concentration (50 μM). Reactions were further incubated in presence of 1 μM ARH3 for 45 min at 30 °C. Reactions were stopped by addition of LDS sample buffer (Life Technologies) and incubation at 95 °C for 3 min. Samples were then analysed by SDS-PAGE and autoradiography. Alternatively, reactions were performed as described in absence of 32P-NAD$^+$ and analysed by SDS-PAGE and immunoblot using rabbit monoclonal anti-pan-ADP-ribose binding reagent (Millipore, MABE1016; RRID: AB_2665466) and mouse monoclonal anti-His antibody (Takara, 631212; RRID: AB_2721905) as primary antibodies and polyclonal anti-mouse immunoglobulins/HRP (Dako, P0447; RRID: AB_2617137) and polyclonal swine anti-rabbit immunoglobulins/HRP (Dako, P0399; RRID: AB_2617411) as secondary antibodies. For inhibitor study, the ARH3 was pre-incubated with the indicated amount of inhibitor for 5 min at RT.
300 rpm in a thermostorer. Afterwards, 1.25 µl of 40 µg/ml protease K (Roche) was added and samples were incubated at 37 °C and 300 rpm overnight. Then, PAR was purified using the QIAquick PCR purification kit (Roche) according to the manufacturer’s instructions. Briefly, 300 µl of sample were mixed with 624 µl Binding Buffer and 400 µl Enhancer and loaded onto the column assembly (High Pure Filters on Collection Tubes). The column assembly was centrifuged at 15,700 × g for 30 s, washed once with 300 µl and once with 200 µl Wash Buffer and finally disassembled and air dry to dry columns completely. PAR was eluted from 100 µl Milli-Q H2O and centrifuging again for 1 min. To digest purified PAR to nucleosides, samples were incubated for 3 h at 37 °C and 400 rpm on a thermostorer in a solution containing 10 µl alkaline phosphatase (Sigma), 0.5 U phosphatase stress (Affymetrix), 1.4 mM Mg(OAc)2, and 34 mM NH4HCO3. Afterwards, samples were loaded onto Nanosep 10 K Omega columns (Pall) and centrifuged at 15,700 × g for 10 min. Samples were dried in SpeedVac vacuum concentrator UnivaP 100 ECH (Uni Equip) and resuspended in 25 µl Milli-Q H2O prior to MS measurement. After centrifugation at 15,700 × g for 5 min, 20 µl of each sample was transferred to an MS vial. Subsequently, ribosyl-adenosine (R-Ado) and di-ribosyl-adenosine (R2-Ado) were quantified by isotope dilution UPLC-MS/MS.

**Data availability**
The atomic coordinates and structure factors for the hARH3 E41A:H2BS7mar, hARH3 E41Adimer, hARH3 E41An:NAD+ and LchARH3:ADPr structures reported in this paper have been deposited in the RCSB Protein Data Bank (www.rcsb.org) under accession codes 7AKS, 7AKR, 7ARW, and 7AQM, respectively (see ‘Methods’ and Supplementary Table 2). Earlier deposited structural data used in this study are available in the RCSB Protein Data Bank (www.rcsb.org) under accession codes 2FOZ (hARH3 apo form), 5A7R (PARG:dim)er, 6D36 (hARH3:ADPr), 6HGZ (LchARH3:ADPr) and 6LH3 (LchARH3:ADP-HPD). The somatic mutation data used in this study are available in the COSMIC database (https://cancer.sanger.ac.uk/cosmic) under accession codes COSM503871 (D34G), COSM5992851 (776R), COSM3727906 (S185P), COSM38890 (L186V), and COSM6262988 (G270C) (Supplementary Table 3). Further requests for information, resources and reagents should be directed to and will be fulfilled by the corresponding authors. Source data are provided with this paper.

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Author contributions
J.G.M.R. performed biochemical experiments, protein crystallisation and data analysis, Q.L. synthesised and validated linear and branched PAR and meADPr, V.Z. and A.A. solved, refined and validated structures, V.Z. carried out structural modelling, J.V. synthesised and validated the H2B857mar peptide, K.H.E. performed HRMS analysis, J.M.R. and S.C.K. performed branching analysis in cells, J.G.M.R. and I.A. wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

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