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Sensing NAD metabolites through *macro* domains

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1. ABSTRACT

The *macro* module is a globular protein domain of about 25 kDa that is evolutionarily conserved in organisms from viruses, bacteria, yeast to humans. It is generally part of proteins that have wide-ranging (and yet to be discovered) cellular functions. There are several examples of *macro* domains associated with modules showing homology to poly-ADP-ribosyl-polymerases. Many *macro* domains, including those of the human histone macroH2A1.1, bind NAD metabolites such as ADP-ribose, suggesting that *macro* domains may function in the recognition of this and related molecules. The presence of a metabolite-binding function in a repressive chromatin component opens new potential connections between chromosome structure, gene silencing and cellular metabolism. Current evidence suggests that *macro* domains also represent a novel tool for studying NAD metabolites and may be an attractive drug target for the treatment of diseases.

2. INTRODUCTION

Cellular signaling pathways contain receptor and adaptor proteins that are capable of recognizing specific signals, such as small-molecule second messengers and post-translational modifications in target proteins. Molecular recognition in signaling pathways is usually carried out by globular protein modules, independently-folded domains that recognize their ligand substrate with high specificity and, in doing so, can change the cellular localization, local concentration and specific activity of the proteins that contain them.

Since protein modules are commonly found in proteins together with domains of a different function, the recognition of a signal by a given module gets directly coupled to the recruitment of a distinct activity, such as an enzyme, which may amplify and relay the original signal. Such amplification or other feed-forward/feed-back

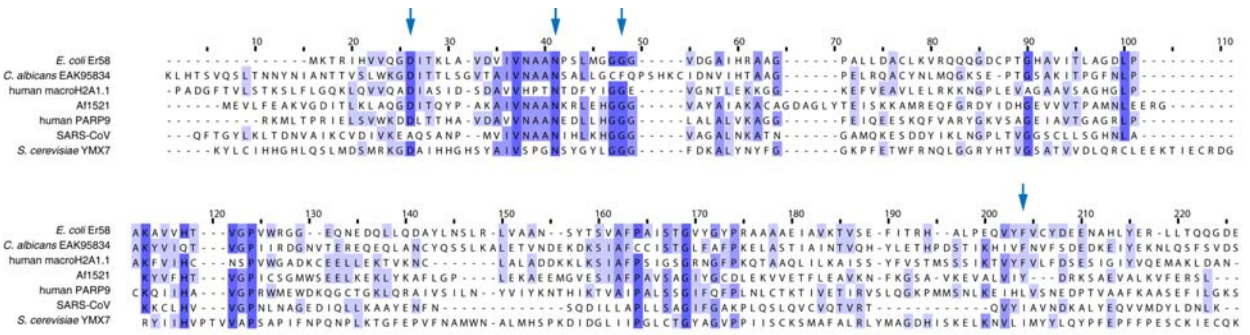

Figure 1. Sequence conservation among *macro* domain modules. Alignment of selected *macro* domains from bacteria, fungi and vertebrates. Residues are coloured according to their sequence conservation. A selection of highly conserved residues located in the ADP-ribose binding pocket are marked by an arrow. Residues D20, G42, N34 and Y176 in the *A. fulgidus* Af1521 protein and corresponding residues D203, G224, N217 and F348 of the human histone variant macroH2A1.1 are important for ligand binding (8, 11).

features are one major element of cellular signal transduction.

There are now many examples of protein modules with signaling or recruitment functions. Among them, the SH2 domains present in receptor tyrosine kinases bind phosphorylated tyrosine residues (1, 2) and are a well-known example of a protein module that couples an external signal to a downstream pathway. A second example, this time in the nucleus, are bromodomains, which directly recognize acetylated histones. In turn, this leads to the enhanced reorganization of the chromatin template through the recruitment of large bromodomain-containing cofactor complexes, such as ATP-dependent remodelers or the general transcription factor TFIID (3).

Evolution has taken advantage of such a modular way to engineer biological communication and regulatory pathways. Therefore, many modules can retain their intrinsic molecular function across species, such that the discovery of a general biological function for a module in one species generally translates well across life.

In this review we will focus on the so-called *macro* domain (4, 5). *Macro* domains are found in over 200 different proteins in all organisms ranging from thermophiles to humans. An alignment and an evolutionary tree of a set of *macro* domains are shown in Figure 1 and Figure 2. This protein domain was first described as an unusual domain present in the histone variant macroH2A (6). Evolutionally conserved *macro* domains have been identified in bacteria, viruses and mammals (4, 7). They have a size of about 23 kDa and are characterized by a compact globular fold consisting of a single seven-stranded mixed beta-sheet, five alpha-helices and a 3_{10} helix. (Figure 3). They show a limited homology to P-loop nucleotide hydrolases (4, 8). Nevertheless, the function of *macro* domains is still unclear. Recently, it was shown that *macro* domains are capable of binding NAD metabolites like ADP-ribose or *O*-acetyl-ADP-ribose *in vitro* (8). This was a first hint of a potential role of *macro* domains in NAD-dependent pathways in the cell. They may be important in DNA repair mechanism that depend on poly-ADP-ribosyl-polymerases. Or they may act in concert with sirtuin deacetylases during epigenetic regulation of gene expression, because sirtuins produce the *macro* ligand *O*-acetyl-ADP-ribose during their enzymatic reaction.

In this review, we will outline the characteristics of different *macro* domains from bacteria, viruses and humans. We will also discuss potential functions of *macro* domains and provide a perspective of how they might allow to study cellular pathways and NAD metabolism in the future.

3. *MACRO* DOMAINS IN BACTERIA AND VIRUSES

There are over 200 proteins containing *macro* domains in bacteria, as annotated by the SMART database (9), including for example the YMBD protein in *E. coli*. The best studied bacterial *macro* domain is Af1521, a protein from the thermophile *Archaeoglobus fulgidus*. Mark Allen and colleagues solved the crystal structure of this stand-alone *macro* domain in 2003 (4). The fold of the Af1521 *macro* domain reveals homology to the P-loop family of nucleotide hydrolases, suggesting a potential connection to ADP-ribose derivatives (4, 10). Our laboratory later identified that Af1521 is indeed capable of binding both monomeric ADP-ribose and poly-ADP-ribose polymers. Binding is mediated by a well-defined surface pocket with displays a high affinity for the ligands in the nanomolar range (11). Moreover, thin-layer chromatography assays reveal that Af1521 has some enzymatic activity capable of hydrolyzing ADP-ribose-1-phosphate (Appr-1-P) a metabolite produced by cyclic phosphodiesterases during tRNA splicing. In fact, the first hint that *macro* domains may have enzymatic activity toward such substrates came from an early chemical genomics screen for proteins in the yeast proteome capable of hydrolyzing Appr-1-P (12). Thus, *macro* domains can bind to ADP-ribose-like NAD metabolites with high affinity and *in vitro* display a modest enzymatic activity. However, the functional role of the bacterial *macro* domains remains elusive. It is possible that some of these or other *macro* domains may interact with ADP-ribosylated proteins, since many bacterial mono-ADP-ribosyl-

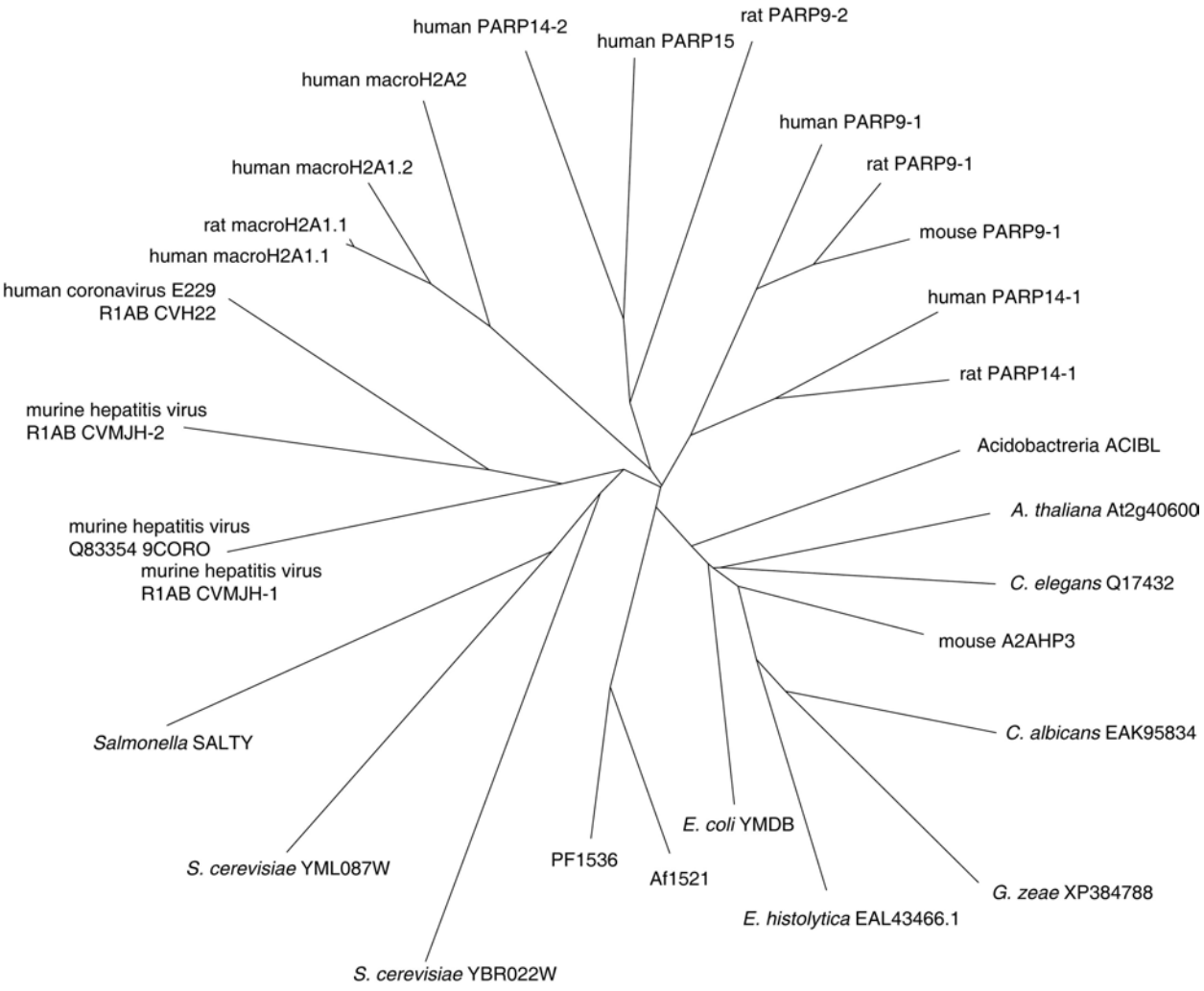

Figure 2. Representation of the evolution and conservation of annotated *macro* domain modules. *Macro* domain sequences are derived from the SMART database. The tree includes a selection of archeal, bacterial, viral, fungal and mammalian *macro* domains, including members of protein families such as predicted poly-ADP-ribosyl polymerase enzymes, histone proteins and proteins bearing both *macro* domain modules and Sir2 homology domains.

transferases have been identified [for a review on this distinct topic, see references (13-15)].

In addition to bacteria, there are positive-sense single-stranded RNA viruses that harbor *macro* domains in their genome. *Macro* or so-called X domains are present in hepatitis E virus (HEV), semliki forest virus (SFV), coronaviruses such as SARS-CoV or torovirus, alphaviruses and rubella virus (16-20). As shown for the thermophilic Af1521 *macro* domain, proteins of human coronavirus 229E, transmissible gastroenteritis virus and SARS-CoV are able to cleave the substrate ADP-ribose-1-phosphate (16, 18-20). Similar to what is observed for *S. cerevisiae macro* domains (G. Karras and A.G. Ladurner: *unpublished data*) and Af1521 (11), these *macro* domains also seem to process Appr-1-P rather slowly. These data and crystal structures of several viral *macro* domains led to the hypothesis that they rather play a role in ADP-ribose metabolism (16-18). The structure of the SARS-CoV subunit Nsp3 in complex with ADP-ribose reveals a conformational change upon metabolite binding. The movement of loops adjacent to the ADP-ribose results in a closing of the pocket around the ligand. This points to a possible induced fit mechanism upon ADP-ribose binding through *macro* domains (16). In addition, *macro* domains of HEV, SFV and SARS-CoV interact with both poly-ADP-ribose and ADP-ribose *in vitro*, which suggests a potential function in sensing ADP-ribose derivatives during viral infection (16).

4. *MACRO* DOMAINS AND POLY-ADP-RIBOSYL-POLYMERASES (PARPS)

Several lines of evidence point to a connection between *macro* domains and poly-ADP-ribosyl-polymerases, a family of proteins that use NAD^+ as a co-factor and catalyze the transfer of ADP-ribose moieties onto substrates (21). As described earlier, several *macro*

domains can interact with ADP-ribose monomers, as well as with ADP-ribose polymers *in vitro*. It is presently unclear whether and how these specific, high-affinity metabolite interactions mediate important biological functions *in vivo*.

4.1. *Macro*-containing PARPs

Three members of the mammalian PARP family contain *macro* domains, in addition to their PARP domain: PARP9 (BAL1), PARP14 (BAL2) and PARP15 (BAL3). PARP9 was first identified in a genome-wide screen for genes related to diffuse large B-cell lymphoma (22). It contains a C-terminal domain with homology to the PARP catalytic region and two consecutive *macro* domains in the N-terminal half of the protein. The genes for PARP9, PARP14 and PARP15 all localize to the same region on human chromosomes, 3q21. Thus, they may be evolutionarily and/or functionally related (23). In contrast to PARP9, which lacks critical catalytic residues, PARP14 and PARP15 are catalytically active in autoribosylation assays (22, 23). All three proteins can repress transcription *in vivo* when they are tethered to different promoters (23). The fact that these *macro* domain-containing proteins repress transcription in a cellular context, suggests that *macro* domains could play a common role in gene silencing. The *macro*-domain containing macroH2A histone, for example, is a component of repressed, facultative heterochromatin. Recent studies, however, also describe PARP9 and PARP14 as transcriptional co-activators. The latter protein, which is known as *collaborator of Stat6* (CoaSt6), increases IL-4-induced gene expression (24). In addition, the over-expression of PARP9 in lymphocytes elevates transcription of interferon-controlled genes (25). Together, these early reports on *macro* domain-containing PARPs suggest a complex role of these PARPs in transcriptional regulation, which needs to be analyzed in more molecular and genetic detail. Further, while PARP14 and PARP15 can ADP-ribosylate themselves (23), it has been hypothesized that they may actually belong to a family of mono-ADP-ribosyltransferases (MARTs) (26), since they lack residues that are required for polymerization in PARP1 and PARP2 (21, 26). Significantly, the role of the *macro* domains within these so-called PARP members is unclear at present. The current assumptions are that they might help to recognize the ADP-ribosylation mark during gene regulation, may serve to mediate interaction with other proteins (including PARPs themselves) or, interestingly, may represent a way to influence the intrinsic PARP (or MART?) activity through a regulatory feedback mechanism.

4.2. Interaction of *macro* domains with PARP1

An additional link between *macro* domains and PARP enzymes is the reported interaction of the mammalian histone macroH2A1 with PARP1 (27, 28). PARP1 has been identified in macroH2A histone affinity purifications from cell extracts. Further, macroH2A leads to an inhibition of PARP1 auto-modification activity (27, 28). Thus, macroH2A and PARP1 are thought to act in concert to aid the repression of genes. For instance, the depletion of PARP1 in mouse embryo fibroblasts leads to the reactivation of a GFP transgene inserted on the inactive X-chromosome (Xi) (28). Therefore, the authors suggested that PARP1 and macroH2A together might contribute to the silencing of the Xi. Another case is the hsp70 heat-shock promoter, which is enriched for ADP-ribosylated PARP1 and macroH2A when silenced (27). Upon heat shock, both proteins appear to be released and promoter-associated proteins become ADP-ribosylated. These data suggest that macroH2A might regulate PARP1 enzymatic activity and/or recruit PARP1 to specific loci. There are several open questions about the molecular nature of an interaction between macroH2A histones and PARP1. It is not clear whether some of the reported interactions are direct, since histone-associated protein complexes can be bridged by histone-associated nucleic acids, such as broken DNA double strands. Further, only the splice variant macroH2A1.1 can bind ADP-ribose, yet its splice variant macroH2A1.2 (which cannot bind ADP-ribose; (8)) appears capable of binding PARP1 (28). Since neither macroH2A1.1 nor macroH2A1.2 can bind poly-ADP-ribose *in vitro* (8) and only macroH2A1.1 can bind to monomeric ADP-ribose, PARP1 may interact with macroH2A1.2 through indirect (e.g. DNA/RNA- but not ADP-ribose-mediated) or specific protein-protein interactions. Since there are almost twenty PARP family members in humans, it will also be interesting to determine how specific the interactions with PARP1 are. Future studies will need to address the precise mechanism of recruitment and binding of PARP family members, promising to shed light on the mode of interaction and the biological role of the interaction between a histone and PARP-family members.

5. *MACRO* DOMAINS IN HISTONES

5.1. The histone variant macroH2A

The vertebrate-specific H2A histone variant macroH2A (mH2A) was discovered by Pehrson and Fried in 1992 (6). They isolated this novel protein from histone fractions of rat liver. The human macroH2A consists of a histone fold, which is about 60% identical to the canonical H2A, and a large non-histone domain (NHR). The NHR can be divided into a basic region that is less conserved among species and the *macro* domain. MacroH2A accounts for about 2% of the H2A pool in rat liver extracts (one molecule in 30 nucleosomes) and is highly conserved in all vertebrate organisms. For example, rat and chicken macroH2A share 95% sequence homology at the protein level (7).

The histone variant exists in three isoforms, namely macroH2A1.1, macroH2A1.2 and macroH2A2 (29, 30). They are encoded by two distinct genes located on different chromosomes. MacroH2A1.1 and macroH2A1.2 result from alternative splicing of the macroH2A1 gene, while macroH2A2 is derived from a second gene on a different chromosome (31). The macroH2A *macro* domain structurally consists of 7 beta-stands, 5 alpha-helices and a 3_{10} helix. The overall *macro* domain fold is conserved in the genomes of thermophilic archaebacteria and of viruses (8, 32). Further, structural studies of nucleosomes reveal great similarities in structure between all nucleosomes

formed by canonical and variant H2A histones, including the H2A domain of the histone macroH2A (32, 33). There are no major changes in the interaction of macroH2A's histone fold with its heterodimeric partner histone H2B (compared to H2A), nor is the docking domain mediating H3/H4 binding affected. The main difference occurs in the L1-L1 loop interface between the two macroH2A moieties compared to two canonical H2A molecules (32). Further analysis shows that the histone domain of full-length macroH2A preferentially forms hybrid nucleosomes with canonical H2A (34), suggesting that *in vivo* the macroH2A protein may preferentially form heterotypic protein complexes. Those hybrid nucleosomes display a more rigid structure and seem to be inert to exchange by some chromatin remodeling factors (34). There is at present limited information on the structure of macroH2A-containing nucleosome core particles. Comparisons of reconstituted nucleosomes containing canonical H2A versus macroH2A by micrococcal nuclease digestion or hydroxyl radical treatment did not show significant differences (35, 36). However, the addition of a globular domain with 25 kDa size to a chromosomal histone is likely to alter the structure and packaging of the nucleosomal fiber, particularly in cells where these histones are highly expressed. In accordance with this hypothesis, DNaseI footprinting experiments reveal slight alterations in the macroH2A nucleosome (35). Future crystallographic studies with full-length macroH2A histones will hopefully shed light on the effect of the *macro* histone on the overall chromatin structure.

5.2. Differential expression patterns of macroH2A isoforms

MacroH2A subtypes are differentially expressed in tissues and during development (31, 37). Adult liver and kidney contain equal amounts of macroH2A1.1 and 1.2, while brain, thymus and testis are reported to predominantly express macroH2A1.2, but only low levels of macroH2A1.1 as analyzed by Western Blot (37). Undifferentiated mouse embryonic stem (ES) cells appear to express both types at low levels (37). During differentiation macroH2A1.2 increases in embryonic stem cells. Studies of macroH2A messenger RNA levels give similar results (31). In general, macroH2A1.2 spliced RNA is much more abundant in all tissues. Rasmusssen and colleagues also investigated gene expression in male compared to female mouse ES cells. They find a constantly high level of macroH2A1.2. On the other hand, macroH2A1.1 is hardly detectable in male cells, but is present at lower and constant levels in female ES cells. Here, mH2A expression does not change during cell differentiation (31). In summary, mH2A1.1 is mainly present in adult tissues containing non-dividing cells. Unlike mH2A1.1, the second splice variant mH2A1.2 is highly expressed in several tissues with ongoing cell proliferation. The third isoform macroH2A2 is also present in liver and kidney tissues but does not display an identical distribution to macroH2A1.2 (30). It remains to be seen whether there is any correlation between the cellular content of mH2A, chromatin structure and cell proliferation. Further insights might arise from the investigation of cancer cells, since mH2A histones have been reported to be a feature of senescent cells.

Interestingly, macroH2A expression not only differs according to the differentiation state or the cell-type, but can also change upon changing environments (38). The common carp *Cyprinus carpio* adapts to the cold during wintertime by reprogramming its cellular functions. Winter adaptation is accompanied by the condensation of chromatin, reorganization of the nucleolus and silencing of ribosomal RNA genes. Despite some changes in common histone modifications, RNA and protein levels for the histone variant mH2A2 increase drastically (38). Using immunofluorescence assays, a strong signal of the *macro* histone is visible in nuclei of winter cells compared to summer cells. This study shows that mH2A expression is regulated through environmental conditions, which is highly interesting considering the ability of at least some macroH2A histones to recognize cellular NAD metabolites. It is well known that metabolic activity in an organism changes depending on environmental temperature. The carp data from Bouvet's laboratory provide an interesting early glimpse into how our environment can modulate the chromatin state through altering the expression and incorporation of histone variants.

5.3. The histone macroH2A is enriched in the inactive X chromosome

Initial localization studies of the histone mH2A showed a strong enrichment in the inactive X chromosome (Xi) of female mammals (39). The observed staining suggested a role for mH2A in transcriptional gene silencing. Consistently, macroH2A can be preferentially associated with centromeric heterochromatin depending on the cell type and stage of development (40, 41). Targeting of the histone variant to the X chromosome does not depend on the *macro* domain alone, but is also directed by the histone fold, as studies with chimeric histone proteins showed (42). Moreover, regions in the mH2A histone fold responsible for directing it to the Xi were mapped to the helix alpha1, the L1 loop, the docking domain and the C-terminal part of the histone fold. Thus, several short and dispersed sequence elements within the histone-fold of macroH2A contribute to the specific localization of mH2A (43). Those sequences cluster in a surface-exposed domain when mapped onto the crystal structure of mH2A nucleosomes, suggesting that they may form a surface patch capable of interacting with chromatin remodeling factors, for example (43).

MacroH2A is not only associated with the inactive X chromosome in somatic cells, but also forms nuclear sub-structures that do not colocalize with the Xi (39, 44), including senescence-associated heterochromatin foci (see below) and centrosome-associated structures outside the nucleus (41, 45). At the time of their identification, they were called macrochromatin bodies (MCBs). Additionally, a diffuse signal of mH2A is observed in the entire nucleus in female (XX) and male (XY) cells. In undifferentiated ES cells, a MCB is present in XY and XX cells before X inactivation takes place. The MCB disappears during differentiation in male cells, while the MCB colocalizes with the Xi in female cells. This colocalization only occurs after initiation of X inactivation and coating of the X chromosome with Xist RNA.

MacroH2A does not seem to be implicated in this initiation process (44). Hence, it is thought that mH2A may rather contribute to the maintenance of the inactive state. However, the conditional deletion of Xist following the establishment of X-inactivation results in the loss of mH2A and the MCB, but does not lead to a total defect in X inactivation: the Xi remains hypoacetylated at histone H4 and replicates late (46). This might argue against a role of macroH2A in maintaining X inactivation. Nevertheless, macroH2A localization seems to depend on the Xist RNA and on the SPOP/cullin3 E3 ubiquitin ligase (47). Conditional deletions of Xist or RNAi-mediated knock-down of SPOP/cullin result in the reactivation of a GFP transgene inserted at the Xi. The Xi as a whole remains inactive and replicates late (47, 48). Thus, in both cases macroH2A might account for the observed reactivation of genes and therefore play a role in maintaining the Xi inactive state. A different scenario has been observed in pre-implanted mouse embryos (49). In the blastocyst, mH2A recruitment still takes place after Xist expression but occurs before histone and DNA modifications. Taken together, this rather points to a role mH2A in the initiation of X inactivation. It is possible that these ambiguous data arise as a result of the different models (ES cells *versus* blastocysts) or that it mirrors general differences between the random X inactivation observed in ES cells and imprinted X inactivation in embryos. Moreover, other modifications like H3K27 methylation can also occur early during development like Xist expression (50) and the process of imprinted X inactivation may vary from one organism to the other (51). Therefore, additional experiments are necessary to investigate the sequential process of X inactivation in different species.

It has also been reported that the localization of mH2A changes in a cell cycle dependent fashion (40, 41). In undifferentiated ES cells and during G2-M phase, mH2A staining increases at centrosomes. The staining of the inactive X is predominantly present during S-phase and then decreases until mitosis starts. The localization of Xist RNA changes similarly, but is released only shortly before mitosis and its expression reoccurs much earlier after mitosis than the deposition of mH2A. Targeting of mH2A to the centrosome might lead to its degradation through the proteasome, because inhibition of the latter leads to accumulation of mH2A1 at centrosomes (40). However, this is not the case for mH2A2. It therefore remains unclear what function the apparent increase in macroH2A accumulation at centrosomes has.

A recent publication describes the increase of a phosphorylated form of mH2A during mitosis, which is not enriched on the inactive X chromosome (52). The authors suggest that phosphorylation of the histone might disrupt binding of mH2A to Xist RNA. Although, mH2A deposition depends on Xist, it is not clear whether mH2A can bind RNA. Preliminary analysis in our laboratory has failed to detect specific interactions between the *macro* domains of macroH2A isoforms and RNA-based oligonucleotides (G. Kustatscher and A.G. Ladurner; *unpublished observations*). Recruitment of mH2A to the Xi also is reported to require a E3 ubiquitin ligase SPOP/cullin3 (47). Knock-down of the ligase affects deposition of mH2A at the inactive X, but does not alter its protein stability. One could imagine that the phosphorylation of mH2A marks this histone variant for transfer to the centrosome during mitosis and degradation by the proteosome. This posttranslational modification may also modulate the interaction with yet to be identified effector complexes and thus lead to the removal of mH2A from the Xi.

5.4. MacroH2A- a marker for heterochromatic regions?

5.4.1. How does macroH2A influence heterochromatin formation?

Structural studies of mH2A-containing nucleosomes reveal a slightly different conformation of chromatin (32, 35), which might affect accessibility of the chromatin template by transcription factors. Indeed, incorporation of mH2A into nucleosomes can affect binding of the transcription factor NFkappaB and chromatin remodeling through the SWI/SNF complex *in vitro* (35). NFkappaB binding, however, is only impaired when its binding sites is close to the dyad axis of the nucleosome core particle. Although the remodeling complex SWI/SNF binds equally well to H2A and mH2A nucleosomes, the sliding in the latter case is negatively affected. Recent data further show that macroH2A not only blocks chromatin remodeling but also polymerase II transcription (53). Specifically, transcription mediated by the histone acetyltransferase p300 is inhibited, resulting in a loss of the activating acetylation mark of histones. Supporting a functional connection between mH2A and hypoacetylated histones, *macro* domains also interact with histone deacetylases HDAC1 and HDAC2 (32). It is presently unclear whether macroH2A mediates histone deacetylation-dependent functions *in vivo*. Furthermore, we do not know whether and how mH2A recruits HDACs to the chromatin. Is the impairment of chromatin remodeling a direct effect of the interaction between *macro* domains and remodeling factors or is it due to sterical hindrance by the increased size of mH2A-containing nucleosome structure? Binding of SWI/SNF or Gal4-VP16, which recruits p300, to macroH2A nucleosomes appears to be similar to the binding of canonical H2A-containing nucleosomes. These data argue against an impairment of chromatin remodeling as a result of sterical changes in the chromatin fiber. Future experiments will need to elucidate the mechanism of how macroH2A is incorporated into chromatin and the effect of its deposition on the overall structure, function and activity of chromatin.

5.4.2. MacroH2A and senescence-associated heterochromatin foci (SAHF)

The histone variant macroH2A has been identified as a marker of facultative heterochromatin of the inactive X chromosome in mammals. In addition, it is shown to be enriched in a form of facultative heterochromatin that occurs in senescent cells (54, 55). Senescent cells are marked by a different cell shape, cell cycle exit and formation of heterochromatic structures in order to repress proliferation- promoting genes. So-called senescence-associated heterochromatin foci in the nuclei of senescent cells show enrichment of HP1 protein, H3K9

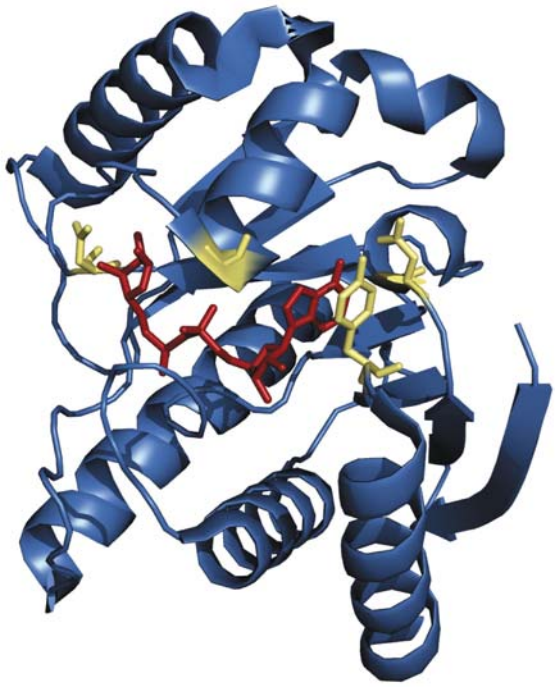

Figure 3. Structure of the *Archaeoglobus fulgidus* Af1521 *macro* domain (blue) in complex with the ligand ADP-ribose (red) (11). Residues D20, N34, G42 and Y176, which play an important role for ligand binding, have been highlighted (yellow).

methylation and all three isoforms of mH2A (55). Deposition of mH2A is a late event during the onset of senescence, since the mH2A signal appears after formation of SAHFs. SAHF formation depends on a protein complex consisting of HIRA and ASF1a, a histone chaperone. It is speculated that both proteins are also responsible for mH2A recruitment to SAHF. However, no direct interaction between the proteins could be detected (55). It is possible that ASF1a may alter chromatin structure and allow incorporation of mH2A. Future biochemical and cellular assays will identify which factors mediate mH2A recruitment, deposition and exchange.

5.4.3 Patterns of macroH2A chromatin deposition

MacroH2A has mainly been implicated in X inactivation, although it seems to be widely distributed throughout the chromatin. Moreover, one study suggests that the enrichment of mH2A on the Xi may be due to a higher density of nucleosomes, rather than reflecting a genuine enrichment of macroH2A compared to canonical H2A (56). Since macroH2A levels differ between distinct cell-types, it is also quite plausible that macroH2A enrichment on the Xi is sometimes seen as a prominent feature and might be absent in a different cellular context. Several studies address the question of mH2A distribution in more detail. Purification of mH2A-containing nucleosomes by binding to thiopropyl sepharose shows enrichment of the histone on the Xi in females, but also at repeat sequences and retroviral elements in both males and females (57). As expected, actively transcribed genes and genes that escape X inactivation are devoid of mH2A. In contrast, it is present in pseudoautosomal regions with an equivalent distribution in males and females (57, 58). Thus, mH2A is preferentially deposited in silenced regions of the X chromosome or in repetitive regions of our genome, but it is also found at other gene loci such as the protamine cluster (57). MacroH2A can also be found in autosomal imprinted regions. Chromatin immunoprecipitation assays reveal localization of mH2A to methylated CpG islands in promoter regions of paternally or maternally imprinted genes (59). The enrichment at those gene loci is even higher than on X-linked genes. Therefore, mH2A might be a key player during gene silencing. At present, this hypothesis is challenged by results obtained from mH2A1 knockout mice. These mice do not display obvious defects in X inactivation (60), since females are born at normal, wild-type ratios. Interestingly, knockout macroH2A1 cells display a de-repression of endogenous murine leukemia viruses. Thus, macroH2A might be specifically enriched at retroviral loci to mediate silencing (61). This first knockout paper also reports a metabolic phenotype in macroH2A1 knockout male mice. Specifically, they show a statistically-significant reduced ability to clear a *bolus* injection of glucose, implying potential changes in metabolism due to altered insulin signaling or glucose uptake. The expression of genes involved in fatty acid metabolism is affected in the liver of these knockout mice, which may alter glucose metabolism. There are additional reports, that macroH2A may be able to repress highly specific genes in certain cell types. The Thanos laboratory, for example, has reported that in Namalwa cells, the IL-8 promotor is marked by mH2A nucleosomes and is resistant to binding of activating factors. This process seems to be specific to this cell line and depends on binding of ATF-2 to the IL-8 promotor (62). ATF-2, in turn, can directly bind to macroH2A. In summary, the knockout data for macroH2A1 argue against a vital role for this histone in mammals. However, there may be genetic redundancy with macroH2A2, whose knockout phenotypes in isolation or in conjunction with macroH2A1 have not yet been reported. Overall, it is likely that macroH2A might rather help to fine-tune gene expression of single genes instead of acting as a general, vital silencer of gene expression at the level of heterochromatin structure. Future studies will need to address whether and how additional transcription factors are involved in the recruitment or function of macroH2A at specific genomic locations, such as the promoters of RNA polymerase II-dependent genes.

5.5. The histone variant macroH2A1.1 and its ligand ADP-ribose

Several *macro* domains are capable of interacting with ADP-ribose and with other NAD derivatives. The thermophilic Af1521 and the PARP9/BAL *macro* domains bind mono- and poly-ADP-ribose *in vitro* (11). The structure of Af1521 in complex with ADP-ribose and the residues important for binding are shown in Figure 3. In contrast, the *macro* domain of the human histone macroH2A1.1 is able to bind the ADP-ribose monomer with micromolar affinities, but shows no affinity (or enzymatic activity) towards the polymer (8). Nevertheless, macroH2A1.1 does interact with an additional ADP-ribose

derivative, *O*-acetyl-ADP-ribose (AAR) (8). This metabolite is produced by sirtuins, Sir2-related enzymes which catalyze deacetylation reactions using NAD as a co-factor. AAR is a direct product of the reaction. Sirtuins and macroH2A are both implicated in gene silencing and heterochromatin formation. While direct interactions between the two proteins remain to be determined, the fact that the *macro* domain of macroH2A1.1 can bind the AAR metabolite opens up a potential new level of regulation of chromatin structure and function through NAD metabolites. Surprisingly, the two other isoforms of macroH2A, macroH2A1.2 and macroH2A2, do not bind any of the related nucleotides ADP-ribose, NAD, ADP or AAR (8). Structural studies of the two splice variants clearly show how the alternatively-spliced exon leads to an insertion of three residues in the ligand binding pocket of macroH2A. These observations provide convincing structural insight into the distinct binding behavior of these two histone variant splice isoforms. This would allow chromatin to incorporate two different functions into chromatin, perhaps allowing for an individual response of the chromatin fiber to changes in cellular NAD metabolites.

6. *MACRO* DOMAINS AND SIRTUINS- DIRECT AND INDIRECT LINKS

6.1. Regulatory role of *macro* domains in Sir2-mediated catalysis

The discovery that Sir2 metabolites can bind the histone variant macroH2A1.1 with high micromolar affinity provides the first experimental evidence potentially linking *macro* domain function to Sir2-mediated deacetylase activity (8). Further, it is also the first example of a metabolite-binding capacity in a human chromatin component. Conceptually, this is unprecedented and will lead to new research addressing the connections between metabolism, chromatin structure and gene expression. However, we need to be cautious about the physiological significance of the current *in vitro* experimentation. For now, the most important question is what the effect of *O*-acetyl-ADP-ribose binding to macroH2A1.1 might be? Since our provocative discovery of the ADP-ribose and AAR-binding function in the histone macroH2A1.1, no progress has been made in the chromatin field that would allow us to advance our findings to the next, necessary level. However, we can put forward and discuss at least three out of several plausible scenarios with regard to the ADP-ribose and AAR binding functions. The same three scenarios could apply to other binding functions involving mono-ADP-ribose-related moieties. First, macroH2A1.1 may act as an enzyme and degrade the Sir2 metabolite, thus being an integral part of a Sir2-mediated enzyme chain. Second, binding of the metabolite to the *macro* domain of macroH2A1.1 may lead to an altered conformation and/or activity of the Sir2 enzyme, thus providing feedback regulation. And third, binding of the metabolite to macroH2A could change the structure of the histone and of the chromatin fiber itself, thus acting as a metabolic sensor or as a signaling receptor.

6.2. An active role of *macro* domains in Sir2 biology

The crystal structure of a Sir2 enzyme crystallized with NAD and acetylated histone substrates surprisingly revealed the formation of a complex between the histone substrate and the product metabolite *O*-acetyl-ADP-ribose (63). Thus, the crystallization process had serendipitously captured one of the products of the reaction, providing detailed molecular insight into how this unusual metabolite is recognized by Sir2 family enzymes. The diffraction data clearly show a non-covalently bound *O*-acetyl-ADP-ribose molecule in the active site. This directly suggests that the product metabolite may act as a competitive inhibitor of the Sir2 enzyme. Any accumulation or reduction of the metabolite in the cell would therefore be expected to alter the overall level of Sir2 activity. One hypothesis is that nuclear-localized *macro* domain proteins, including the *macro* domain within the repressive histone macroH2A1.1, could act as enzymes and degrade the Sir2 metabolite into smaller products, which would fail to bind Sir2, thus releasing the AAR-mediated inhibition of Sir2. Since Sir2 and macroH2A have both been implicated in the formation and maintenance of heterochromatin, it could make sense that they cooperate at this functional level through the Sir2 metabolite. This would be akin to an enzyme chain in biosynthetic pathways. There are clearly many precedents in the field of metabolism where the activity of one enzyme in a given metabolic pathway is directly coupled to the following enzyme through direct protein contacts. For this to occur, macroH2A1.1 would need to be an enzyme and, as mentioned earlier, several *macro* domains have indeed been reported to act as enzymes toward phosphorylated forms of ADP-ribose (11, 18, 64). The structure of the ADP-ribose binding macroH2A1.1 *macro* domain pocket reveals several surface residues that in principle could contribute to catalytic events, such as surface histidine residues. In an effort to test whether macroH2A histones may aid Sir2 function through degrading its AAR product, we incubated *macro* domains with the Sir2 metabolite under different conditions. These experiments have to-date failed to reveal a catalytic or degradation role toward the Sir2 metabolite (G. Kustatscher and A.G. Ladurner; *data not shown*). However, it is possible that catalysis may necessitate as-yet unknown cofactors (metals, proteins) and appropriate assay conditions. Based on the evidence from genes in fungi and microbial parasites that connect a *macro* domain directly to a Sir2 domain (described below), our field will likely continue to pursue the identification of genuine and biologically relevant catalytic functions among *macro* domain containing proteins.

6.3. *Macro* domains may buffer Sir2 catalysis

A molecularly simpler role of macroH2A1.1 (and of other *macro* domain proteins localized to the nucleus) could be a metabolite squelching function. Any protein binding to AAR would effectively lower the concentration of free metabolite in the nucleus and thus positively contribute to Sir2 activity. Obviously, such a passive role of a metabolite-binding domain, might only serve a transient buffering function, until all binding sites are occupied under situations of acutely high Sir2 activity, unless the number of binding sites is indeed substantial. There is currently little quantitative insight into the number (and cellular sublocation) of *macro*-domain containing proteins, but macroH2A1.1 is a histone, and histones are clearly highly abundant. Specifically, biochemical assays

have provided an estimate of macroH2A histone levels in liver cell nuclei of about 2% relative to canonical histone H2A (6). This abundance suggests that there could indeed be a substantial, albeit passive, buffering capacity for Sir2 metabolites in human chromosomes. If this is the case, then *macro* domains may be able to act as a negative feedback regulator for Sir2 function once 'free' AAR-binding sites have all been occupied.

Future studies will need to reveal the extent to which the AAR-binding macroH2A1.1 histone contributes to cellular Sir2 function and NAD homeostasis. Detailed and quantitative studies in the recently reported macroH2A1 knockout mice will need to be conducted to test how macroH2A1.1 contributes to sirtuin-mediated biological functions. The Guarente lab has shown that SirT1 is involved in mediating insulin secretion upon glucose signaling in cultured insulinoma cell lines (65). Interestingly, macroH2A1 knockout studies do suggest a phenotype related to altered glucose metabolism, so it possible that (specific) sirtuins and macroH2A1 may operate in related biological pathways (60). Future studies will aim to determine how the NAD-metabolite binding function of the nuclear macroH2A1.1 histone may directly contribute to the changes in macroscopic physiological parameters.

6.4. Conformational responses to Sir2 metabolites by *macro* domains

Classic signaling cascades generally rely on changes in protein activity upon receipt of a given signal, often through ligand-induced conformational changes in critical signaling components. Intriguing data from the budding yeast, suggest that a large multi-subunit protein assembly known as the SIR complex alters its three-dimensional conformation upon binding the Sir2 metabolite AAR (66). This study establishes the principle that a eukaryotic chromatin-associated component and important gene repression regulator alters its shape and activity in response to AAR, suggesting that this Sir2 metabolite may acts a second messenger. While the precise molecular region or subdomain of the SIR complex that recognizes and responds to AAR metabolite remains to be identified, it is possible that *macro* domains may also show changes in conformation upon metabolite binding. The existing crystal structures between *macro* domains and the NAD metabolite ADP-ribose, in general, do not reveal substantial changes in structure. The thermophilic Af1521 protein only marginally alters its structure and binding sites in response to ADP-ribose or MES buffer (a pseudo-ligand) binding (4). Others, such as the *macro* domain of the SARS coronavirus display a induced-fit closure of the pocket upon ligand binding (16). However, it is possible that a related but distinct metabolite, such as AAR, may elicit conformational changes not seen with the smaller ADP-ribose ligand. This idea needs to be addressed through experimentation and the use of techniques that allow the study of protein dynamics, such as NMR spectroscopy. It is premature to speculate whether human chromatin might contain the ability to sense NAD metabolites and respond to these signals as a receptor. But it is possible that macroH2A-binding proteins may modulate the affinity and binding/dissociation kinetics of the Sir2 metabolite or that ADP-ribose like small molecules may regulate the affinity and binding kinetics for macroH2A-associated proteins. It is likely that any of these potential functions would have a significant impact on chromosome structure and gene expression.

6.5 Auto-inhibitory events in proteins combining Sir2 and *macro* folds

While many independent reports have validated the general notion that *macro* domains bind monomeric ADP-ribose-related NAD metabolites, such as the Sir2 metabolite AAR and the PARG-catalyzed PAR breakdown product ADP-ribose, there is as yet no experimental evidence suggesting direct protein-protein interactions between Sir2 (or PARG) and *macro* domains. Yet, there exist a surprisingly large number of genes in a variety of organisms (predominantly fungi and microbial parasites; see genomic databases), whose predicted polypeptide sequence combines a *macro* domain with a Sir2-related protein fold. Furthermore, these two domains are juxtaposed, with the *macro* domain sequences immediately preceding or following sequences for the canonical Sir2 fold. It is unclear how these proteins may have evolved, or what the biological role of these proteins is. However, their existence suggests that in these organisms macro domain function is linked to Sir2 pathways. To date, empirical evidence shows that Sir2 proteins in lower organisms are either NAD-dependent acetyl-lysine deacetylases and/or ADP-ribosyltransferases. It is therefore tempting to speculate that macro domains in these macro/Sir2 'combo' proteins may either be involved in the enzymatic degradation of the AAR metabolite or may serve to alter the catalytic activity of Sir2, for example, through binding of an auto-ADP-ribosylated form of the Sir2 enzyme. Structural analysis of these proteins will reveal whether *macro* domains directly interact with Sir2 domains, opening up the prospect for related intermolecular interactions in higher organisms.

7. *MACRO* DOMAINS AS A POTENTIAL THERAPEUTIC TARGET

The *macro* domain is the first globular protein module known to bind ADP-ribose and related metabolites (8, 11, 16). As such, it has the potential of becoming a useful tool for the study of NAD-derived metabolites, whether they be soluble metabolites, such as AAR, or substrate-linked modifications, such as mono- and poly-ADP-ribosylated substrates, such as proteins. Over the next years, the field will exploit *macro* domains for the discovery of novel principles of biological regulation and determine their roles in cellular signaling, NAD metabolism and energy homeostasis *in vivo*. Structural studies of *macro* domains bound to ligands, including oligomeric ADP-ribose and protein-bound ADP-ribose, will provide insight into metabolite binding modes and allow structure-based searches for small-molecule NAD metabolite analogs, which can then be tested for binding to *macro* domains *in vitro* and refined through synthetic chemistry. Their use in biological assays, in turn, will aid the characterization of *macro* domain pathways and their biological role in sirtuin, PARP and other NAD-dependent cellular pathways.

Beyond their use as a small molecule tool in pathway discovery, it is possible that small molecule analogs of ADP-ribose that bind the ligand pocket of *macro* domains may be of therapeutic value in a number of areas of medical interest. Four sets of potential target families are particularly interesting. First, *macro* domains may have a connection to the family of sirtuin enzymes due to their ability of binding ADP-ribose-related metabolites that sirtuins produce. It was recently shown that sirtuins play important roles for the aging process by mediating responses to caloric restriction and insulin secretion, as well as being implicated in diseases such as metabolic or cardiovascular disorders (67, 68). Second, *macro* domains might be important for PARP1-mediated DNA damage recognition, DNA repair or cell death, as the macroH2A´s *macro* domain appears capable of negatively affecting PARP1 activity *in vitro* (27, 28). Third, macroH2A1.1 is a marker for facultative heterochromatin (such as in SAHF) and can be found to be enriched in differentiated cells. It will be interesting to test whether the level of macroH2A can be correlated to the proliferation state of a cell and thus, may potentially play a role in tumor biology. Finally, a large number of viruses and microbial parasites have *macro*-containing proteins. Some of these proteins are necessary for host cell infection and viral replication (16, 18). Therefore, molecules targeting *macro* domains might restrict viral infections and other human diseases.

8. CONCLUSIONS

In this review we sought to summarize known facts about distinct *macro* domains and outline potential functions of this module in NAD-dependent pathways. In conclusion, *macro* domains are likely to be involved in poly-ADP-ribosyl-polymerase reactions, such as during DNA repair or cell death, as they were shown to bind NAD metabolites like ADP-ribose and to interact with PARP1 directly . Other NAD derivatives such as AAR may link *macro* domains to sirtuin deacetylases and thus to chromatin silencing functions. However, this metabolite ligand binding feature is not conserved throughout all *macro*-containing proteins. While AF1521, PARP9 and macroH2A1.1 bind ADP-ribose, the other two macroH2A isoforms do not bind any of the nucleotides we tested. A distinct case of specialization in protein function is seen for the reported phosphatase activity of *macro* domains. Although low-level activity can be detected for certain thermophilic and viral *macro* domains, as well as in the yeast protein YBR022W, the enzymatic activity is not conserved among tested human *macro* domain proteins. Thus, it is possible that a subset of *macro* domains rather than having an enzymatic role serve as protein-interaction modules. Especially, the distinct binding behaviors of the variants macroH2A1.1 and 1.2 hint at a regulation of processes through alternative splicing. Already tissue- and development-specific differences in expression level between the two variants have been observed. Further investigation of human tissues and cell-types, of the macroH2A knockout mice or cancer tissues might help to determine the function of the histone´s *macro* domain.

In a broader perspective, *macro* domains that bind ADP-ribose derivatives (and most likely also ADP-ribosylated substrates) could be exploited to analyze or even manipulate NAD-dependent pathways in the cell. In the long-term, small molecules may be synthesized that block *macro* domain function and thus could be of therapeutic relevance, such as during viral infections or in DNA repair pathways.

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