The active rather than intrinsic mechanism of nucleosome depletion by poly(dA:dT) tracts

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Abstract: Poly(dA:dT) tracts cause nucleosome depletion in many species, e.g., at promoters and replication origins. Their intrinsic biophysical sequence properties make them stiff and unfavorable for nucleosome assembly, as probed by *in vitro* nucleosome reconstitution. The mere correlation between nucleosome depletion over poly(dA:dT) tracts *in vitro* and *in vivo* inspired an intrinsic nucleosome exclusion mechanism *in vivo*. However, we compile here published and new evidence that this correlation does not reflect mechanistic causation. 1) Nucleosome depletion over poly(dA:dT) *in vivo* is not universal, e.g., very weak in *S. pombe*. 2) The energy penalty for incorporating poly(dA:dT) tracts into nucleosomes is modest (<10%) relative to ATP hydrolysis energy abundantly invested by chromatin remodelers. 3) Nucleosome depletion over poly(dA:dT) is much stronger *in vivo* than *in vitro* if monitored without MNase and 4) actively maintained *in vivo*. 5) *S. cerevisiae* promoters evolved a biased poly(dA) versus poly(dT) distribution. 6) Nucleosome depletion over poly(dA) is directional *in vivo*. 7) The ATP dependent chromatin remodeler RSC preferentially and directionally displaces nucleosomes towards 5′ of poly(dA). Especially bias and directionality would not be expected for an intrinsic mechanism. Together, this argues for a mechanism where active and species-specific read out of intrinsic sequence properties, e.g., by remodelers, shapes nucleosome occupancy.

Keywords: nucleosome; chromatin; poly(dA:dT); remodeling; RSC;

Nucleosomes are depleted *in vivo* over poly(dA:dT) tracts in many species

Nucleosome occupancy is prominently low over homopolymeric poly(dA:dT) sequence tracts in a wide variety of species including *S. cerevisiae* and other yeasts, fly, worm, mouse and human cells [1-3]. Nucleosome depletion scales directly with tract length. Clear effects usually start with five and more dA:dT base pairs (bp) in a row and are increasingly diminished with the number of interruptions in the homopolymeric sequence. As most nucleosome mapping is based on limited digests with Micrococcal Nuclease (MNase) [4, 5] and as MNase has a sequence preference for dA:dT-rich sequences [6, 7], there is the concern that the depletion of nucleosome signal in MNase-seq and related techniques reflects a technical bias. Indeed, it is recognized since long [8-11] and sometimes explicitly controlled for (e.g., [10-12]) that nucleosomes with dA:dT-rich DNA are depleted faster during MNase digestion kinetics. The depletion over poly(dA:dT) tracts *in vivo* is mostly (see below) stronger than can be explained by this MNase bias and, more to the point, confirmed, at least for some species like *S. cereivisae*, in MNase-independent methods like anti-histone ChIP with sonication [11, 13], ChIP-exo [14], chemical mapping [15, 16] and DNA methylation footprinting [17-19].

Nucleosome depletion is functionally important and caused by poly(dA:dT) tracts

Poly(dA:dT) tracts and their associated low nucleosome occupancy is linked to functional genomic regions like promoters [20-24] and replication origins [25]. As the assembly of protein complexes like the transcription or replication machinery on these DNA regions

would be hindered by nucleosomes, it is functionally important that there are mechanisms that remove nucleosomes from these regions. Poly(dA:dT) tracts constitute one of these mechanisms as their direct causality was demonstrated *in vivo* by introduction or removal of such tracts and the corresponding decrease or increase, respectively, in DNA accessibility/nucleosome occupancy [21, 22, 26, 27].

Special intrinsic properties of poly(dA:dT) tracts were suggested to cause nucleosome depletion *in vivo* by a nucleosome-intrinsic mechanism

Many biophysical studies established intrinsic properties of poly(dA:dT) tracts that deviate from those of generic DNA sequences [3]. These properties include a shorter helical repeat, a more narrow minor groove, more extensive hydration, more extensive base overlap within one strand, and lead overall to increased stiffness (higher deformation energy [28]) and higher energetic costs for the wrapping and twisting of DNA around the histone octamer during nucleosome assembly. These costs can be observed and quantified in competitive nucleosome reconstitutions where several DNA sequences compete for nucleosome assembly [29-31]. The classical reconstitution method is salt gradient dialysis (SGD). Purified DNA and histone octamers are initially mixed at high ionic strength, usually 2 M NaCl, where DNA and histones hardly interact. The salt concentration is decreased gradually or in a stepwise way by dialysis or dilution, respectively, so that the electrostatic interactions between DNA and histones [32, 33] begin to drive nucleosome assembly. Due to the slow salt dilution, there is a sufficiently long period during which histones and DNA experience an ionic strength around ca. 1 M NaCl. This salt regime allows repeated nucleosome assembly and disassembly, i.e., the histone octamers, more precisely the $(H3-H4)_2$ tetramers, which assemble first on the DNA, can effectively equilibrate to their energetically preferred positions and avoid unfavourable sequences [34-36]. This procedure was extensively used to compare different DNA sequences for their relative nucleosome formation propensities and to select especially strong nucleosome positioning sequences, like the "Widom 601" sequence [29, 30, 36, 37]. The outcome of such measures is usually referred to as the "intrinsic" nucleosome formation propensity or affinity of a DNA sequence. Poly(dA:dT) tracts score low in this regard [29] and lead to low nucleosome occupancy, for example, in SGD with whole genomes [38-40].

It should be noted that SGD-derived intrinsic properties are measured under non-physiological conditions as the histones equilibrate to their positions at around 1 M salt and become kinetically stuck at lower salt concentrations, i.e., cannot re-equilibrate to other positions, even if these were the thermodynamically preferred ones at lower salt [30, 33, 35, 36]. Incubation of SGD-reconstituted nucleosomes at low salt but elevated temperature, like 40-60°C, allows histone octamers to move again along the DNA and to re-equilibrate to positions that are preferred under these conditions and that may be different from the positions equilibrated at high salt but lower temperature [41, 42]. This illustrates how intrinsic nucleosome positioning preferences depend on the thermodynamic conditions. Unfortunately, there is no technique available that would allow histone octamers to equilibrate to their intrinsically preferred positions at physiological ionic strength and temperature without inclusion of other non-histone factors, which may again tweak thermodynamic preferences. Therefore, we do not know the physiological purely intrinsic sequence preferences for nucleosome assembly. Intrinsic preferences measured by SGD or similar techniques, like equilibrium measurements at physiological buffer and temperature conditions but in the presence of the histone chaperone Nap1 [43], have to be taken as operational approximations of the *in vivo* situation [44].

Nonetheless, there is a striking correlation between nucleosome depletion over poly(dA:dT) tracts *in vivo* and in SGD-reconstituted chromatin *in vitro* [38-40]. This and alone this *correlation* has been (mis)taken as evidence that this nucleosome depletion *in vivo* is mechanistically due to poly(dA:dT) intrinsically disfavouring nucleosome assembly [3, 45, 46]. This mechanism is called "intrinsic" to signify that only the intrinsic properties of DNA and histone octamer in the nucleosome, without thermodynamic influence of other factors, determine such nucleosome depletion. By principle, correlation need not

reflect causality and in the following we delineate the evidence against this intrinsic nu- 101
cleosome depletion mechanism. 102

In vivo nucleosome depletion over poly(dA:dT) tracts is not universal 103

As canonical histones are among the most highly conserved proteins, it may be ex- 104
pected that the intrinsic nucleosome disfavouring properties of poly(dA:dT) tracts should 105
play out across species. Indeed, nucleosome depletion over poly(dA:dT) was observed in 106
SGD using histones from several species, e.g., fly or chicken histones [38, 40], and in many 107
species *in vivo* [1-3]. However, there is the notable exception of *S. pombe*. Here, nucleosome 108
depletion over poly(dA:dT) is far less pronounced than in *S. cerevisiae* and dA:dT-rich se- 109
quences are not enriched in linkers or nucleosome-depleted promoter regions but within 110
nucleosomes close to the dyad [47, 48]. Nucleosome depletion over poly(dA:dT) as based 111
on MNase-seq measurements for *S. pombe* was not confirmed by chemical mapping [48]. 112
For example, dA:dT-rich replication origins in *S. pombe* appeared nucleosome depleted in 113
MNase-based but not in chemical mapping. This is a case of misleading MNase sequence 114
bias. We wonder if we had ever heard of the intrinsic "genomic code for nucleosome po- 115
sitioning" [49] had *S. pombe* rather than *S. cerevisiae* been the main model species. 116

As another exception from a putative universal intrinsic mechanism, introduction of 117
a $(dA:dT)_{19}$ tract into a well-positioned nucleosome at the *S. cerevisiae PHO84* promoter 118
did not lead to removal of this nucleosome like otherwise seen upon promoter activation, 119
but a clear DNaseI footprint was maintained and nuclease accessibility increased only 120
moderately [50]. 121

The energetic penalty for incorporation of poly(dA:dT) into nucleosomes is not very 122
high 123

The manipulated *PHO84* promoter nucleosome is a counterexample regarding the 124
misconception that poly(dA:dT) tracts cannot be incorporated into nucleosomes at all. 125
There are many other examples *in vivo* where nucleosomes are positioned over 126
poly(dA:dT) tracts, for example by the ATP depedent *S. cerevisiae* remodeler ISW2 [51] or 127
at (dA:dT)-rich terminator regions in *S. cerevisiae*, which leads to MNase-sensitive nucle- 128
osomes [11]. Also *in vitro* reconstitution of nucleosomes onto poly(dA:dT)-containing se- 129
quences is well possible [52, 53], including even a crystal structure of a nucleosome with 130
a $(dA:dT)_{16}$ tract [54]. 131

Not only can poly(dA:dT) tracts be nucleosomal *in vitro* and *in vivo*, the respective 132
energetic cost is actually not as high as initially assumed. The more extreme differences 133
between intrinsic affinities of histone octamers to DNA sequences as measured by com- 134
petitive SGD in the Widom group were ~5000fold, which corresponds to an energetic dif- 135
ference ($\Delta\Delta G = RT \ln 5000$) at 30°C of ca. 5 kcal/mol. This is not even half the energy of one 136
ATP hydrolysis reaction (ca. 12 kcal/mol under physiological conditions [55]). Nucleo- 137
some dynamics are mediated *in vivo* by ATP dependent chromatin remodeling enzymes 138
(remodelers) [56, 57] that (dis)assemble, reposition (slide) and reconfigure (histone ex- 139
change) histone octamers on DNA. For some remodelers a step size of 1-2 bp per ATP 140
hydrolysis was estimated [58-60], which amounts to hundreds of ATP hydrolyses for slid- 141
ing a nucleosome along DNA even over short distances. Therefore, we suggest that the 142
energetic differences of intrinsic sequence preferences for nucleosome formation are 143
merely ripples in a remodeler-dominated energy landscape and may be easily overcome 144
by ATP input. 145

Further, reassessement of energetic measurements arrived at quite smaller energetic 146
differences than before. If the 5S rDNA positioning sequence was taken as reference point, 147
then the Widom 601 strong positioning sequence showed a difference of 0.7 kcal/mol 148
(~3fold higher affinity) in the Nap1-assembly method [61], which is less than the originally 149
reported 2.9 kcal/mol (~124fold higher affinity) derived by competitive SGD. Comparison 150
of mononucleosomes containing poly(dA:dT) tracts taken from *S. cerevisiae* promoters 151
showed that they are less stable by at most 0.86 kcal/mol (~4fold lower affinity) relative to 152
5S rDNA-nucleosomes in competitive salt step reconstitutions [53], i.e., by less than 10% 153

of ATP hydrolysis energy. Not that the 5SrDNA reference itself is already a strong nucle- 154
osome positioning sequences. Therefore, the energetic differences between poly(dA:dT)- 155
containing sequences and the average genomic sequences will be even smaller. Nonethe- 156
less, this does not preclude that they matter in the end. 157

Nucleosome depletion over poly(dA:dT) tracts by the intrinsic mechanism *in vitro* in 158
SGD is much weaker than by the *in vivo* mechanism 159

    The caveat that MNase digestion bias may exaggerate the appearance of nucleosome 160
depletion (see above) was not controled for in SGD chromatin by MNase-independent 161
methods so far. As MNase-dependent nucleosome mapping has to employ limiting di- 162
gests, resulting occupancy values (peak heights, trough depths) depend on the chosen 163
digestion degree, which is difficult to control for or normalize for comparisons between 164
independent experiments [12, 17]. This obscures the comparison of nucleosome depletion 165
between different conditions, like *in vivo* vesus *in vitro* chromatin, by MNase-based meth- 166
ods. Further yet, SGD chromatin can be prepared at arbitrary nucleosome densities, which 167
also may affect the degree of nucleosome depletion over poly(dA:dT) tracts. 168

    Here, we addressed these so far unresolved issues by using published absolute occu- 169
pancy data obtained by MNase-independent DNA methylation footprinting (ODM-seq, 170
[17]). This allows direct comparison of occupancies across conditions. We compared on 171
the one hand ODM-seq data for *S. cerevisiae in vivo* chromatin, averaged over five repli- 172
cates, as well as for SGD reconstituted chromatin along the *S. cerevisiae* genome at two 173
different nucleosome densities with on the other hand published MNase-seq data of *in* 174
vivo chromatin and for two replicate sets of SGD chromatin at three different densities 175
each [62]. As ODM-seq reports nucleosome occupancy over the whole nucleosome length, 176
we plotted MNase-seq data as 147 bp extended dyads, too. 177

    Classical *in vivo* +1 nucleosome-aligned composite plots showed the typical *in vivo* 178
pattern of regularly spaced nucleosomal arrays downstream and the prominent nucleo- 179
some-free region (NFR) over the promoter region just upstream of the +1 nucleosome in 180
both the ODM-seq and the MNase-seq data (Figure 1a-c). However, nucleosome depletion 181
over promoters was less pronounced in SGD chromatin if measured by MNase-seq (Fig- 182
ure 1b-c) and even less if measured by ODM-seq (Figure 1a). Nucleosome density had 183
hardly an effect on the degree of nucleosome depletion in all cases. The corresponding 184
traces of the three densities were virtually identical in the MNase-seq data. The differences 185
between the replicates of MNase-seq data for SGD chromatin likely reflected the differ- 186
ences in MNase digestion degrees, which are notoriously diffcult to control exactly. As 187
expected and shown before [17], absolute occupancy levels scaled with nucleosome den- 188
sity in the ODM-seq data (Figure 1a). The comparison of nucleosome densities in Figure 189
1a showed that the high nucleosome density in our genome-wide reconstition system 190
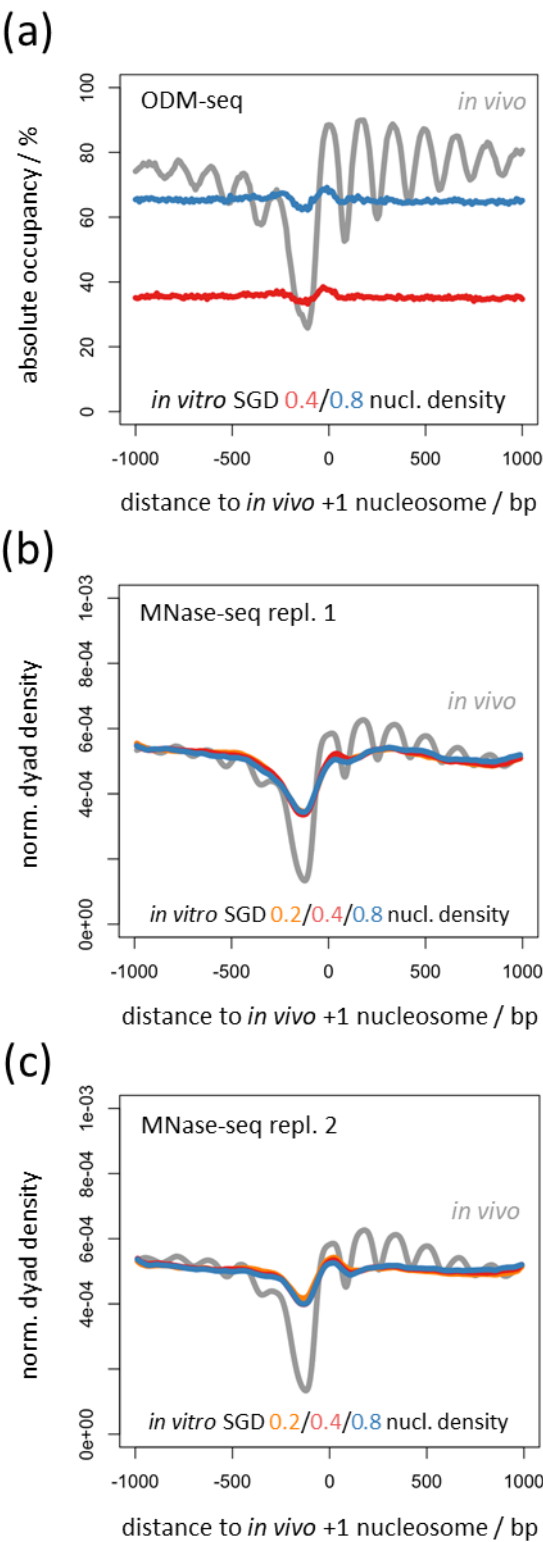almost reached the *in vivo* density. 191

Figure 1. Monitoring nucleosome depletion over poly(dA:dT) tracts by DNA methylation foot-   193
printing (ODM-seq) shows that intrinsic nucleosome depletion in SGD chromatin is even less pro-   194
nounced relative to the *in vivo* depletion in *S. cerevisiae* than previously seen by MNase-seq [38, 39,   195
63]. (**a**) Composite plots of absolute occupancy (ODM-seq data from [17]) for *S. cerevisiae* wild type   196
cells (*in vivo*, grey, GSE141051) and for 0.4/0.8 nucleosome density reconstituted by SGD on an *S.*   197
cerevisiae genomic library (*in vitro* SGD 0.4/0.8 nucl. density, red/blue, GSM4193222/GSM4193216,   198
respectively) aligned at *in vivo* +1 nucleosome positions (annotation as in [64]). (**b-c**) Composite   199
plots as in panel (a) but for two independent replicates of MNase-seq data from [62] for *S. cere-*   200
visiae wild type cells (*in vivo*, grey, GSM4175394) and for 0.2/0.4/0.8 nucleosome density reconsti-   201

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tuted on an *S. cerevisiae* genomic library by SGD (*in vitro* SGD 0.2/0.4/0.8 nucl. density, or-   202
ange/red/blue, replicate 1: GSM4175428/GSM4175429/GSM4175430, replicate 2:   203
GSM4175803/GSM4175804/GSM4175805, respectively). MNase-seq data were plotted as 147 bp   204
extended dyads. Nucleosome density is quantified as described in [17, 62].   205

The classical plot type in Figure 1 shows the nucleosome depletion effect of   206
poly(dA:dT) sequences only indirectly via their enrichment in *S. cerevisiae* promoters. This   207
analysis is convoluted as there are promoters without poly(dA:dT) tracts and effects of   208
binding competition with transcription factors and others shown to contribute to nucleo-   209
some depletion at promoters in addition to the assumed intrinsic mechanism over   210
poly(dA:dT) tracts [2, 65-67]. To address the poly(dA:dT)-driven nucleosome depletion   211
more directly, we binned all genomic poly(dA) tracts into three length categories (5 to 9,   212
10 to 14 and more than 15 dA in a row) and prepared composite plots of again the same   213
ODM-seq and MNase-seq data as in Figure 1, but aligned at the centers of these poly(dA)   214
tracts (Figure 2a-f). This clearly showed how nucleosome depletion over poly(dA) tracts,   215
in vivo and for all nucleosome densities *in vitro,* increased with tract length. Note that   216
composite plot traces became more rugged with decreasing number of instances as gen-   217
erally true. Depletion *in vitro* was very similar as *in vivo* if monitored by MNase-seq, which   218
is the observation that suggested the intrinsic nucleosome depletion mechanism in the   219
first place. However, this correlation was much less pronounced in ODM-seq data. This   220
shows that the intrinsic nucleosome depletion over poly(dA:dT) tracts is inflated by the   221
MNase digestion sequence bias, which preferentially removes nucleosomes that contain   222
(dA:dT)-rich sequences. Nonetheless, long poly(dA) tracts showed clear nucleosome de-   223
pletion *in vitro* also by ODM-seq, which confirms that the intrinsic depletion over   224
poly(dA:dT) tracts can be real. It is just much weaker than the *in vivo* depletion.   225
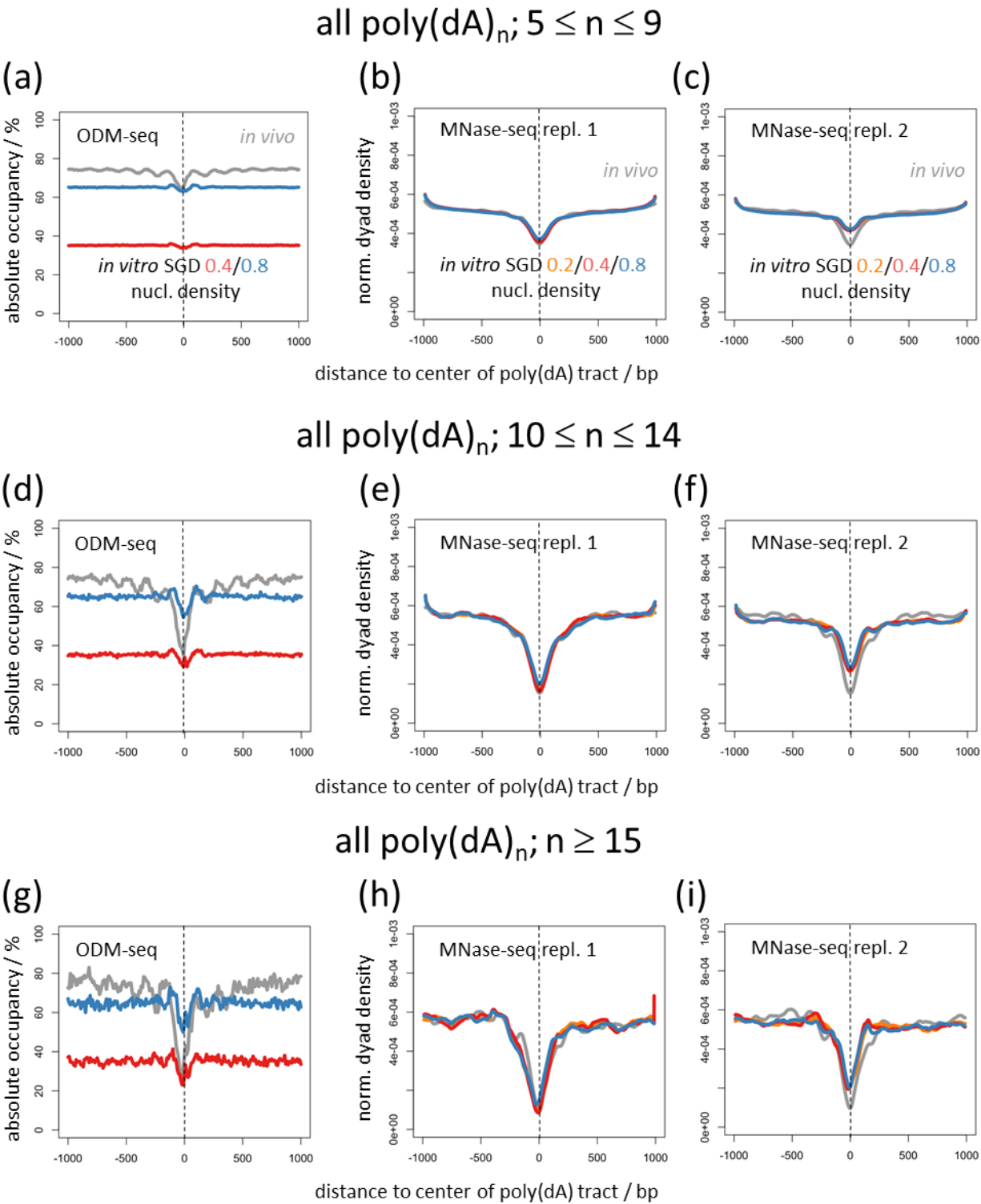
Figure 2. Nucleosome depletion over poly(dA;dT) tracts scales with tract length and seems similar in extent *in vivo* versus *in vivo* if monitored by MNase-seq but not by ODM-seq. Composite plots of same data as in Figure 1, but aligned at all poly(dA:dT) tracts in the *S. cerevisiae* genome of (**a-c**) five to nine (75,745 instances), (**d-f**) 10 to 14 (1,963 instances) and (**g-i**) 15 and more (303 instances) bp lengths. Strand orientation was taken into account by flipping the orientation for poly(dA) tracts on the opposite strand. Vertical dashed lines mark the alignment points.

We wondered, if the much more pronounced discrepancy between nucleosome depletion *in vivo* versus *in vitro* over promoter regions (Figure 1) versus over poly(dA:dT) tracts (Figure 2) was due to some special feature of poly(dA:dT) tracts in promoters. Or,

if it would actually be decreased if we focused only on promoters with poly(dA:dT) tracts, as the discrepancy may stem mainly from promoter regions without poly(dA:dT) tracts, which were encompassed in Figure 1 and where nucleosome depletion was due to some poly(dA:dT)-independent mechanism like factor binding competition. Therefore, we compared nucleosome depletion at all poly(dA:dT) tracts subdivided in promoter versus non-promoter regions (Figure 3). In ODM-seq data the disrepancy between *in vivo* and *in vitro* depletion was still especially pronounced at promoters (Figure 3a) and there was only a small increase in intrinsic nucleosome depletion in SGD chromatin over promoter versus non-promoter poly(dA:dT) tracts (Figure 3a versus 3b). In contrast, MNase-seq data showed similar relative differences between *in vivo* and *in vitro* depletion over poly(dA:dT) tracts in promoter versus non-promoter regions for both replicates, i.e., depletion in promoters was more pronounced to similar relative extents *in vivo* and *in vitro* (Figure 3c-f). Promoters are known to be especially enriched in poly(dA:dT) tracts, both in tract number as well as in average tract length. This affected nucleosome depletion *in vivo* as well as apparent intrinsic nucleosome depletion *in vitro* if monitored by MNase, probably due to the MNase-bias, but had a much smaller effect on intrinsic depletion as monitored by ODM-seq.
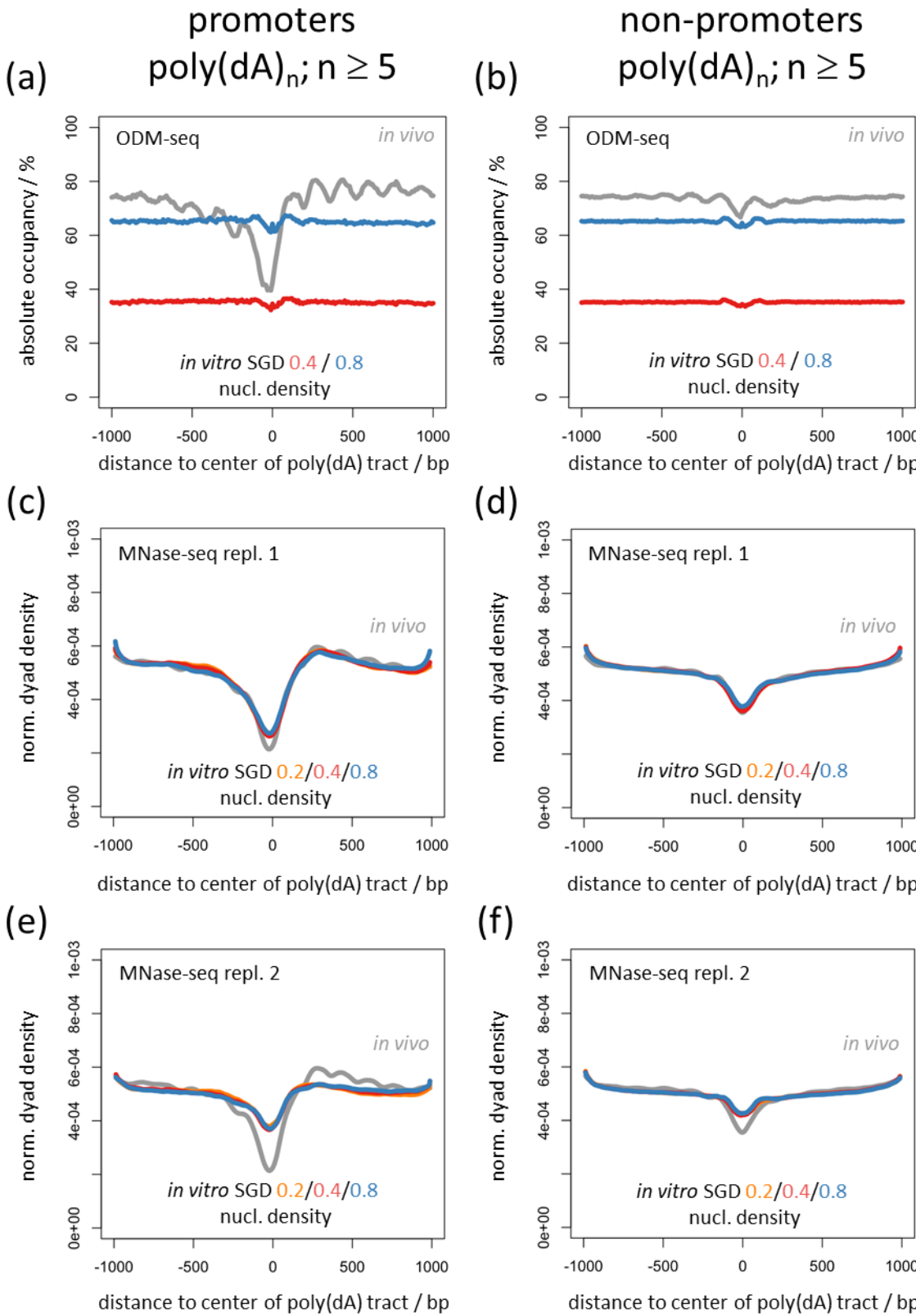
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Figure 3. The difference between nucleosome depletion over poly(dA:dT) tracts *in vivo* versus *in vitro* is much more pronounced in promoter versus non-promoter regions if monitored by ODM-seq than by MNase-seq. Composite plots of the same data as in Figure 1, but aligned at all poly(dA:dT) tracts with five or more bp lengths (**a, c, e**) within (8,802 instances) or (**b, d, f**) outside (69,300 instances) of promoters (0 to -250 bp from transcription start sites [68]). Strand orientation was taken into account by flipping the orientation for poly(dA) tracts on the opposite strand.

Together, we confirm that there is poly(dA)-dependent nucleosome depletion by an intrinsic mechanism *in vitro*, and that there is a correlation with depletion over poly(dA) *in vivo*. However, the effects *in vitro* are so much weaker than *in vivo* if monitored by absolute occupancy in an MNase-independent way and especially in promoter regions enriched in poly(dA:dT) tracts (Figure 3a), that we wonder if anyone had ever drawn mechanistic conclusions from this correlation if MNase had not been the principal mapping tool.

Nucleosome depletion over poly(dA:dT) tracts is not an intrinsic default state but actively maintained

If poly(dA:dT) tracts excluded nucleosomes by a merely intrinsic mechanism, one may expect that they do so without further maintenance. Indeed, most poly(dA:dT) tracts in *S. cerervisiae* promoters are constitutively nucleosome-free and there is no viable mutant known where this is not the case [69, 70]. However, if the essential ATP dependent chromatin remodeling complex RSC and/or a certain class of essential sequence specific DNA binding proteins with binding sites in promoters, so called general regulatory factors (GRFs), like Reb1, Abf1 or Rap1, are ablated in conditional mutants, these NFRs start to fill up with nucleosomes [70-79]. This argues that promoter NFRs over poly(dA:dT) tracts are actively maintained by these factors and we suggest that this is the main reason why these factors are essential. Other non-essential remodelers, like the INO80 or the ISW2 complex, also participate in shaping promoter NFRs as NFR borders are affected in their respective deletion mutants [70, 74, 80, 81] and in *in vitro* reconstitutions [39, 62, 64]. Further, NFRs with or without poly(dA:dT) tracts are assembled into nucleosomes in the wake of replication and only later become nucleosome-free again [82-84].

The *S. cerevisiae* RSC remodeling complex preferentially evicts nucleosomes from poly(dA:dT) sequences

Active nucleosome depletion over poly(dA:dT) tracts could result from removing nucleosomes either in *cis* along the DNA (sliding) or in *trans* (disassembly). Turnover experiments in *S. cerevisiae* monitoring differentially labled histones demonstrated nucleosome removal in *trans*, especially at promoter NFRs [85-88]. Nucleosome disassembly in *trans* is catalyzed by only a subset of ATP dependent remodelers. Remodelers are classified according to sequence similarity among their ATPase motor subunits into four major families, the SWI/SNF, INO80, CHD and ISWI remodelers [56, 57, 89]. Only members of the SWI/SNF family show nucleosome disassembly activity *in vitro*. *S. cerevisiae* contains two SWI/SNF type remodelers, the SWI/SNF complex, after which the family was named, and the RSC complex [89]. Both are involved in promoter NFR or NDR (nucleosome depleted regions, see comment on terminology below) formation *in vivo*, but only RSC has an essential and pervasive role [70-79, 90]. Only RSC but not SWI/SNF was able to reconstitute *in vivo*-like NFRs in the context of a RSC-depleted whole cell extract *in vitro* [91] and RSC showed a preference for remodeling promoter versus genic nucleosomes in *ex vivo* mini-chromosome circles [92]. This suggested that RSC may recognize some promoter feature. Indeed, RSC preferentially evicted nucleosomes from poly(dA:dT) tract-containing DNA in mononucleosome assays *in vitro* [53].

Directional nucleosome eviction from poly(dA:dT) tracts by RSC argues against the intrinsic but for an active nucleosome depletion mechanism

The above arguments already point towards an active and targeted role for RSC in clearing nucleosomes from poly(dA:dT) tracts. Nonetheless, they could still be regarded as circumstancial and at least qualitatively compatible with the suggested intrinsic mechanism of nucleosome depletion. RSC may just implement the intrinsic sequence preferences. *S. pombe* in general [48] and *S. cerevisiae* promoters with ISW2-dependent nucleosome positioning [51] in particular may be examples where some other mechanism overrides intrinsic biophysics. However, there are more arguments that are based on bias and directionality of the poly(dA:dT)-effect and clearly argue against the intrinsic mechanism.

S. cerevisiae promoters show a biased distribution of poly(dA:dT) tracts such that poly(dT) is often upstream of poly(dA) on the strand that is continuous with the coding strand [39, 93, 94]. Such bias would not be expected to evolve in the context of a purely intrinsic mechanism. If *in vivo* nucleosome occupancy data is aligned at the center of poly(dA) tracts (Figures 2 and 3; [93]) or at the midpoint between pairwise combinations of 5'-poly(dA)-poly(dT), 5'-poly(dA)-poly(dA) or 5'-poly(dT)-poly(dA) tracts on the same strand [93], the NFRs are formed asymmetrically with a 5' or 3' offset relative to poly(dA) or poly(dT), respectively. De Boer and Hughes [93] noted that the 5'-poly(dT)-poly(dA) pairwise arrangement thereby leads to mutual reinforcement of nucleosome displacement (from both sides towards the center of the paired tracts) and the strongest NFRs, which may explain why this evolved as the most common promoter organization in *S. cerevisiae* [93, 94]. The same asymmetric NFR formation was also seen in mouse and human cells as well as in genome-wide chromatin reconstitutions with whole cell extracts and ATP [93], arguing for a role of remodelers. Again, such asymmetry or strandedness of the poly(dA:dT)-effect would not be expected for a purely intrinsic mechanism. For intrinsic nucleosome exclusion it should not matter which strand has the dA and which the dT bases. Indeed, SGD chromatin does not show a 5' offset of NFR formation over poly(dA) tracts (Figures 2 and 3; [93]), which also controls that this 5' offset is not due to a biased distribution of poly(dA:dT) tracts in the vicinity of the alignment point. Even though the 5' offset was seen before in MNase-seq data [93], we note that it is mainly seen in ODM-seq but not in MNase-seq data (Figure 2a, d, g and Figure 3a, b versus the other panels in Figures 2 and 3).

The directional nucleosome depletion over poly(dA) tracts suggested that promoter sequences evolved so that they fit to a nucleosome depletion mechanism that reads out poly(dA:dT) tracts in a directional way, possibly via remodelers. Wu and Li [94] almost prophetically suggested (verbatim quote) "It is possible that G:C-capped poly(dA:dT) tracts may mark an initiation point for the remodeling activity of ATP dependent chromatin remodelers such as RSC." Indeed, this mechanism (without taking dG:dC-capping into account) was directly demonstrated in our genome-wide chromatin reconstitution system using purified components only [39]. SGD reconstitution of nucleosomes onto a genomic plasmid library representing essentially the whole *S. cerevisiae* genome pre-formed promoter NFRs according to the intrinsic nucleosome exclusion properties of poly(dA:dT) tracts. Incubation of this SGD chromatin with purified RSC and ATP widened these NFRs substantially and in a directional way leading to a 5'-offset relative to poly(dA) in promoters. Only RSC showed this directional nucleosome displacement, not the SWI/SNF, ISW1a, INO80 or ISW2 remodelers, which also controls against an MNase-bias effect. Subsequent *in vivo* analyses that took poly(dA) tract directionality into account confirmed the corresponding directional effects on NFRs upon RSC ablation [75]. Finally, RSC was literally "caught in the act" while disassembling nucleosomes from *S. cerevisiae* promoter regions. RSC complexes were extracted from yeast nuclei by the CUT&RUN technique and probed for histone content by anti-histone immunoprecipitation [95]. They were indeed associated with histones, especially the H2A histone variant H2A.Z, as well as with GRFs. This finding also resolved the debate around "fragile nucleosomes" (FN). FNs were operationally defined as particles of roughly nucleosome size that were detected in MNase-seq only at mild but not at more extensive MNase digestion degrees in yeast promoters [96]. Their nucleosomal nature was questioned as, for example, neither ChIP-exo nor chemical mapping detected histone-DNA contacts in these regions [11, 14-16]. In the end, FNs apparently correspond to RSC complexes that are in the process of disassembling nucleosomes from (dA:dT)-rich regions. This underscores again that it is not the intrinsic default state of such regions to be nucleosome-free, but that there is a constitutive nucleosome assembly system at work, as also seen in the wake of replication, that is constantly and actively counteracted by the disassembly activity of RSC.

Remodelers are information processing hubs that turn DNA sequence information into nucleosome organization

Together, this strongly argues that poly(dA:dT) tracts are specifically recognized by RSC so that RSC actively, preferentially and directionally displaces nucleosomes from these tracts. To be clear, this mechanism probably still entails the special intrinsic properties of poly(dA:dT) tracts as RSC reads them out while translocating along its tracking strand in the 3'-5' direction [97]. Therefore we still agree that the undebated intrinsic *properties* of poly(dA:dT) do play a role in nucleosome depletion *in vivo*. However, this depletion does not occur by an intrinsic *mechanism*, which entails only the interactions of DNA and histones as determinants of the resulting nucleosome organization. Such intrinsic mechanism seems unlikely in the light of the arguments given above and would definitely not show a directional effect with regard to the strandedness of poly(dA) tracts. Instead, the intrinsic poly(dA:dT) properties are actively implemented by extrinsic factors, for example, the RSC remodeler in *S. cerevisiae*.

In general, *in vitro* reconstitutions with SGD chromatin and purified remodelers demonstrated that remodelers like *S. cerevisiae* Chd1, RSC and INO80 are information processing hubs for nucleosome organization, with organization encompassing positioning as well as disassembly of nucleosomes. This means that they are able to read out DNA sequence and other information, e.g., distance to barriers like GRFs, and process this information together with their own input into remodeler-specific nucleosome organization [39, 64, 98]. The remodeler-specific information was conceptualized as a "remodeler ruler" [62, 99]. Especially for the INO80 complex, the modules that make up the ruler and are involved in sensing and relaying DNA sequence information, in particular DNA shape features [100], were recently defined in structural terms [62, 64]. Also recently, the concept of intrinsic DNA bendability was revisited by a novel genome-scale cyclizibility assay for nucleosome-sized DNA fragments [101]. Also here, DNA sequence-intrinsic properties (bendability) correlated with *in vivo* nucleosome organization in *S. cerevisiae*, i.e., low bendability in NFRs and linkers versus high bendability in nucleosomes, so that a "DNA mechanical code" was suggested. Importantly, also these authors did not consider this an updated version of the purely intrinsic nucleosome positioning mechanism, but show, also with INO80 as example, that these differential DNA mechanics features are read out not just by the histone octamer but also by a remodeler.

All this fits to the earlier concept that different remodelers generate different nucleosome positioning on the same DNA sequence ("remodeler code" [102]) as they read out sequence information [103]. The DNA sequence information corresponds less so to classical sequence motifs but rather to the more redundantly implemented DNA shape and mechanics features. It should be noted that the classical intrinsic sequence preferences for nucleosome positioning as compared in competitive SGD reconstitutions represent rather static properties restricted to the contribution of DNA and histones. In contrast, remodelers may read out more dynamic DNA properties during twisting and bulging DNA in the context of the histone octamer and in the course of their ATP dependent remodeling mechanism [60, 104]. Thereby remodelers add their own information that feeds into the resulting nucleosome organization and are not just "enzymes" that lubricate the equilibration of positions solely determined by the intrinsic interactions of DNA with histone octamers [105].

Species-specific strategies for nucleosome depletion

Nucleosome organization by remodeler-specific input offers a versatile and regulatable mechanism that explains *in vivo* observations beyond the best studied *S. cerevisiae* model. The at first confounding sequence features of nucleosome organization in *S. pombe* may simply mean that *S. pombe* remodelers evolved different ways of reading sequence features. For example, the *S. pombe* RSC complex lacks the Rsc3/30 subunits [106] that confer DNA binding interfaces for *S. cerevisiae* RSC [72]. Also other yeasts use poly(dA:dT) to varying degrees in their promoter organizations [2]. Further, poly(dA:dT) tracts are enriched in human promoters [107] but may not play the main role for nucleosome depletion there. Rather, human promoters that contain CpG islands, i.e., are rich in (dG:dC) corre-

spond to the constitutively open poly(dA:dT)-rich *S. cerevisiae* promoters [108]. The en- [420]
richment of (dG:dC), i.e., "nucleosome favouring" sequences in the intrinsic SGD-derived [421]
sense, is typical for promoters of human and other multicellular species [109, 110]. In con- [422]
trast to *S. cerevisiae* where the vast majority of the genome is constitutively expressed and [423]
most promoters developed an "open door policy" [111], only a minor genome fraction is [424]
expressed in each individual cell of a multicellular organism. Therefore, it appears that [425]
mechanisms for constitutive nucleosome depletion are less often hard-wired by directly [426]
remodeler-recognized sequences, but rather that regulatable mechanisms of indirect re- [427]
modeler-recruitment, e.g., via transcription factor binding sites or by recruitment via his- [428]
tone-modifcations, open promoter chromatin on demand. Recently, an in-depth *in vivo* [429]
analysis of promoter nucleosome organization in two different human cell types, each [430]
with and without cell-type-specific gene induction stimuli, demonstrated cell type-spe- [431]
cific, induction-specific and even very transient promoter opening [112]. [432]

Afterthought on the terminology of nucleosome depletion [433]

For more than two decades, low nucleosome occupancy regions were mostly called [434]
nuclease hypersensitive sites or, more specifically, DNaseI hypersensitive sites [113-115]. [435]
This terminology is still in use and has the advantage of stating exactly the experimental [436]
observation, i.e., these regions are hypersensitive to nuclease digestion, and is therefore [437]
always appropriate as long as it fits the observation. Together with the first genome-scale [438]
mapping of nucleosome positions [20], Oliver Rando introduced the term nucleosome- [439]
free region (NFR). This term brings with it an interpretation of the observation, i.e., the [440]
low mapping signal is due to the absence of nucleosomes. Of note, this term encompasses [441]
two statments that are subject to debate: what is a nucleosome and does "free" really cor- [442]
respond to zero or just low occupancy? Accordingly, the designation as NFR has received [443]
criticism, for example, in the context of the discussion about FNs or non-canonical nucle- [444]
osome particles in promoter regions [96, 116]. Many colleagues feel uneasy about the NFR [445]
term as it conveys a too extreme case of zero occupancy and they therefore prefer the more [446]
gradual connotation that comes with the term nucleosome depleted region (NDR), which [447]
is, to our perception, more pervasively used nowadays. Then again, there is the mecha- [448]
nistic NFR-versus-NDR-distinction by Frank Pugh, who underscores that at least in *S.* [449]
cerevisiae there are truely nucleosome-free promoter NFRs as measured by ChIP-exo and [450]
chemical mapping [14, 69, 117]. In contrast, NDRs in Pugh's terminology correspond to [451]
regulated regions, e.g., *S. cerevisiae* promoters, which are covered with nucleosomes in [452]
their repressed state and depleted of nucleosomes in the course of promoter activation. [453]
We follow the Pugh terminology here, but realize that this mechanistic distinction of NFR [454]
versus NDR seems to be less heard or understood than the quantitative distinction and is [455]
used less often. We are aware that observation and interpretation are epistemiologically [456]
entangled. In general, we advocate to first use observational terms and then to add the [457]
interpretation by qualifyers. (Nuclease) hypersensitive site is an operationally defined [458]
term that is probably easy to agree on. Such hypersensitive sites can then by qualified and [459]
quantified as constitutive, regulated, nucleosome-free or else. [460]

[461]

Conflicts of Interest: **The authors declare no conflict of interest.** [472]

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