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Small-molecule regulators that mimic transcription factors

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Abstract

Transcription factors (TFs) are responsible for decoding and expressing the information stored in the genome, which dictates cellular function. Creating artificial transcription factors (ATFs) that mimic endogenous TFs is a major goal at the interface of biology, chemistry, and molecular medicine. Such molecular tools will be essential for deciphering and manipulating transcriptional networks that lead to particular cellular states. In this minireview, the framework for the design of functional ATFs is presented and current challenges in the successful implementation of ATFs are discussed.

Keywords

chemical biology; synthetic biology; transcription factor mimics; gene regulation; cooperative binding; protein-DNA interactions

1. Introduction

Most cells in multicellular organisms carry the same genome, yet are able to produce a wide range of phenotypes which gives rise to sets of specialized cells that differ in morphology and function. This diversity is in part attributed to differences in tightly regulated gene expression patterns, with some genes being actively transcribed and others repressed. Transcription factor (TF) proteins are active participants in the regulation of specific geneexpression programs in response to cellular needs. Therefore, it is not surprising that the malfunctioning of TFs has been directly linked to many disease states [1]. This link has turned TFs into attractive therapeutic targets for treating a wide range of diseases, including cancer [2– 4].

In response to specific signal, TFs target particular genes within the genome. Once localized to the targeted genes, TFs recruit macromolecular machines to modify chromatin and initiate transcription [5]. Over several decades, much effort has been invested in the identification of

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the components of the transcriptional machinery targeted by TFs [6, 7]. Transcription factors have been shown to interact with RNA polymerase II, the general transcription factors (GTFs) [5], coactivators, such as components of the Mediator protein complex [8, 9], and TBP-associated factors [10, 11]. TFs also recruit nucleosome remodeling complexes such as the Swi/Snf complex and histone acetyltransferases, such as the SAGA complex [12, 13]. Components of the proteasome have also been identified as targets of transcriptional activators (Figure 1) [7].

Natural transcription factors can be minimally composed of two functional domains: a DNA-binding domain (DBD) and a regulatory domain (RD) [5]. The DBD determines which genes will be activated or repressed by selectively targeting specific DNA sequences within the cis-regulatory motifs associated with the target genes; the RD dictates whether to activate or repress transcription by recruiting components associated with the transcriptional machinery or the repression machinery, respectively. The magnitude of the response is encoded within the regulatory domain.

An important feature of natural TFs is that the DBD and the RD function independently from each other, as demonstrated by domain swapping experiments in yeast and other eukaryotes [14]. The modular nature of TFs highlights the possibility of exchanging the DBD and RD for synthetic counterparts to engineer artificial transcription factors (ATFs). Engineering replacements for the DBD and RD has been the most used strategy for creating TF mimics (Figure 1) [15].

The potential benefits of implementing ATF-based tools are extensive [16]. These molecular tools could be used to dissect genome-wide transcriptional cascades, yielding fundamental insights on developmental processes. Diseases based on malfunctioning transcription factors could be treated or prevented with ATFs. The metabolic pathways of an organism could be engineered to produce valuable compounds. ATFs would also be invaluable tools for the emerging field of synthetic biology, as they could be used to control synthetic cellular circuits [17].

2. DNA binding domains

The information contained within the DBD dictates which DNA sequence is targeted and therefore determines which genes are regulated by a given transcription factor. Similarly DBD confers specificity on a given ATF. Different types of binding domains have been employed in ATFs to target specific DNA sequences. Examples of DNA binding domains used for ATF construction include protein-based zinc fingers, oligonucleotides and oligonucleotide analogs, as well as synthetic small molecules (Figure 2).

The **zinc finger** (ZF) domain is one of the most represented DBD in the human genome [18, 19]. A zinc finger module is composed of 30 amino acids assembled in a ββα fold stabilized by a zinc ion. Each ZF recognizes and binds to three base pairs in the target DNA (Figure 2). ZF modules can be strung together to recognize larger unique sequences in the genome. For example, three consecutive ZFs target a 9 bp sequence, and a polydactyl ZF consisting of six ZFs targets an 18 bp sequence [20]. The complexity of sequences that can be recognized by ZFs has been expanded through a variety of strategies, including structure-guided methods,

phage display screens, and the bacterial one-hybrid system [21–23]. The most successful artificial ZF modules target sequences containing GNN triplets [20, 24]. A detailed protocol for the modular construction of ZF libraries was recently published by the Barbas group [25]. Zinc fingers have been widely employed as DNA-binding domains in the construction of ATFs [26]. However, it has been shown that in some cases the binding sequences of individual ZFs are not completely separable and that DNA binding is influenced by the neighboring ZFs as well [27, 28].

Two recent reports described the DNA recognition "code" of the **transcription activator-like (TAL) effectors** of bacteria from the genus *Xanthomonas* [29, 30]. TAL effectors are DNA binding proteins from plant pathogenic bacteria [31]. Members of the TAL effectors family posses a characteristic central domain of tandem repeats of 34 amino acids. In each repeat, the amino acids located in positions 12 and 13 are hypervariable and referred to as the repeat-variable diresidue (RVD). The DNA binding specificity of TAL effectors is determined by the tandem repeat region [32]. Specifically, a one-to-one correspondence was found between the identity of the RVD and target DNA [29, 30].

The deciphering of the DNA binding code of TAL effectors highlights the possibility of engineering TAL effectors with custom DNA sequence specificity. However, the molecular details on how the repeat domain of TAL effectors recognizes targeted DNA are currently lacking. Although more work is needed to support the generality of the proposed DNA binding code, TAL effectors could potentially be utilized as DBD in designing transcription factor mimics.

DBDs have also been constructed from oligonucleotides [33, 34] as well as oligonucleotide analogs, such as **locked-nucleic acids** (LNAs) [35] and **peptide nucleic acids** (PNAs) [36]. These molecules recognize and bind to DNA by forming a triple helix DNA strand (referred to as **triplex-forming oligos** (TFO)), or by strand invasion of double-stranded DNA [37]. An ATF consisting of a triplex-forming oligonucleotide DBD linked to a minimal VP16 peptide AD was first reported by Kuznetsova *et al*. [38]. This work was later extended by Young and colleagues to create TFO-based ATF that induced the expression of a reporter gene in tissue culture cells [39].

The most effective small-molecule DBDs to date are based on N-methylpyrrole and N-methylimidazole polyamides (PA). These molecules bind in the minor groove of dsDNA [40]. When engineered to form hairpins, PAs are capable of binding to targeted DNA sequences, based on a set of pairing rules, with nanomolar affinity [28, 41–43]. Due to this high affinity, a PA can modify gene expression by competitively inhibiting binding of endogenous TFs [44–47]. An artificial activation domain (AD) attached to a hairpin PA was shown to activate transcription *in vitro* [48, 49]. Applications using PA-based ATFs are often limited due to poor cell permeability of PAs; research efforts aimed at improving the cellular permeability of PA-based compounds are ongoing [50–53].

For an ATF to work properly, its DNA binding domain must find and bind to the targeted DNA sequence in the cellular context. In the cell, the accessibility of an ATF binding site is in part dictated by its chromatin state. However, genome-wide maps of nucelosome

positions have highlighted that the regions near transcription start sites are often depleted of nucleosomes [54– 56]. These nucleosome-free regions are potential binding sites for ATFs. In addition, studies have shown that polyamide DBDs are able to bind to targeted DNA sequences in nucleosome particles [57] and nuclear chromatin [58]. In addition, strategies have been designed to alter the accessibility of DNA binding site by chromatin modification. Snowden et al fused a ZF DNA binding domain to histone modifying enzymes to (i.e., a histone deacetylase and a histone methyltransferae) [59, 60]. More recently a DNA methylase enzyme was fused to TFO DNA binding domain and shown to specifically methylate the targeted promoter in a reporter plasmid [61].

3. Regulatory domains

3.1. Activation domains

Most of the activation domains used in ATFs are derived from peptide sequences inspired by the architecture of natural activation domains [7]. Natural ADs are usually composed of unstructured peptides with potential to form amphipathic helices. Based on the peptide sequences of ADs and on structures of natural ADs bound to their protein partners, it appears that many ADs form an amphipathic α-helix upon interaction with the transcriptional machinery, with the hydrophobic face of the helix contacting the binding partner [62–64]. However, extended conformations with a buried hydrophobic surface are also observed [65, 66]. Potent short peptides that function as ADs have been indentified from screening libraries of random peptides [67, 68] and from peptide libraries that targeted components of the transcriptional machinery [69, 70]. Peptide-based ADs have the disadvantage of short lifetime *in vivo*, likely due to the unstructured nature of the peptide that alerts the cellular surveillance machinery (e.g. proteases) to degrade the peptide. Nevertheless, recent studies have highlighted that the potency of peptide-based ADs can be enhanced by engineering intramolecular interactions between the AD and the DBD (see section 6.2) [71, 72].

Proteolysis of the AD can be avoided by using peptoids or small molecules (Figure 3). A novel regulatory module was found by screening a combinatorial library of ~100,000 **peptoids** for binding to the KIX domain of the mammalian co-activator CBP. The most promising peptoid from the screen was delivered into cells as a dexamethasone conjugate that bound to a glucocorticoid receptor-Gal4 chimera DBD. This ATF activated transcription of a reporter luciferase gene in HeLa cells [73].

In addition to peptides, **RNA** can activate transcription when conjugated to a DBD. In an adaptation of the three-hybrid system used to screen for RNA ligands that interacted with the yeast protein Snp1, it was observed that some RNA sequences activated transcription [74]. This finding was further supported by research from Saha *et al*., where a library of RNA hairpins with a randomized 10 nucleotides loop conjugated to a DBD was screened for transcriptional activation [75]. The authors found a consensus sequence of 6 nucleotides in the hairpin loop that activates transcription (Figure 3). RNA-based ADs have also been designed through *in vivo* evolution in yeast [76]. *In vivo* evolution can also be used to select for RNA-DBD conjugates that repress transcription [77].

An early small molecule AD was reported by Minter *et al*. [78]. **Isoxazolidine** derivatives displaying functional groups commonly found in natural ADs were synthesized (Figure 3). The functional groups were chosen to mimic the amphipathic character of natural ADs. These isoxazolidines, when tethered to a DBD, activated transcription both *in vitro* [78] and *in vivo* [79]. It was later shown that isoxazolidine ADs can bind to different components of the transcriptional machinery. Specifically, isoxazolidines have been shown to interact with the KIX domain of the co-activator CREB binding protein (CBP), TRRAP/Tra1 (a component of the SAGA complex), and the components of Mediator complex, Med15/Gal11 and MED23/Sur2 [80].

Another small molecule AD was discovered by targeting specific components of the transcriptional machinery. **Wrenchnolol**, a “wrench-shaped” molecule, previously shown to bind tightly to the transcriptional coactivator MED23/Sur2 [81], show modest transcription activation *in vitro* when conjugated to a hairpin polyamide DBD [82] (Figure 3). The function of the wrenchnolol-based ATF was further extended to modulate transcription activation in cells [83].

In principle, molecules that interact with the transcriptional machinery may function as ADs by increasing the local distribution of the machinery and its functional engagement at the targeted promoter.

3.2. Repressor domains

In addition to transcription activation, ATFs can be designed to repress the transcription of targeted genes. Repressor domains have been used less frequently in ATFs than their activator counterparts. Early attempts at artificial repressor modules revealed peptides enriched in positively charged residues [84]. Much of the recent work on repressor domains has been based on peptides derived from natural repressors (e.g., Kruppel-associated box (KRAB) domain) [15]. The Barbas group achieved transcriptional repression of the protooncogene erbB-2 by fusing a zinc finger DBD to the natural repressor domains KRAB, ERD repressor domain, or mSIN3 interaction domain (SID) [85]. The advantage of using repressor domains lies in the ability to actively repress gene expression, rather than doing so by competitive inhibition, as in the case of TF displacement [44], or by nucleic acid decoys [86, 87]. In principle, the repression domain bypasses the need for competitively displacing endogenous transcription factors and would use the cellular repression machinery to down regulate targeted genes.

4. Controlling the activity of ATFs

A desirable characteristic of a TF mimic would be the ability to externally regulate its function at desired times and locations. Also, the utility of an ATF would be greatly increased by coupling its function to endogenous signaling cascades. A first step toward this goal relied on the use of the ligand-binding domain (LBD) of nuclear receptors. Fusing the LBD to a zinc finger ATF allowed control of the ATF activity by external delivery of its hormone ligand [88]. In this example, the LBDs of the estrogen and progesterone receptors were used. In a more direct and elegant approach, the ZF DBD itself was engineered to dock

a small molecule “prosthetic”. The resulting ATF required the small molecule ligand for DNA binding activity and transcriptional activity in cells [89].

A similar strategy was used for an RNA-based activation domain. The AD was rendered ligand-dependent by including an aptamer sequence in the AD that recognized tetramethylrosamine (TMR). In the absence of TMR, the ATF promoted transcription of a reporter gene in *Saccharomyces cerevisiae*. Conversely, in the presence of TMR, the conformational changes of the TMR-bound aptamer resulted in an inactive conformation of the RNA activation domain [90].

Using a novel approach to control ATF activity, Hauschild et al develop a temperature controlled ATF [91]. A hairpin PA was conjugated to a peptide that interacted with the endogenous transcription factor Exd. This PA-peptide conjugate efficiently recruited Exd to bind to DNA. The activity of the PA-peptide conjugate was made sensitive to temperature by optimizing the length of the linker connecting the PA and the peptide hook. One of the linkers tested efficiently recruited Exd to its DNA binding site between 4°C and 23°C, but above 30°C the ATF was no longer functional [91].

5. Current challenge: Improving cellular uptake

For ATFs to be more broadly effective, they must go through the cell membrane, enter the nucleus, find the targeted sequence in the genome and recruit the cellular machinery for either transcription activation or repression. Through all of these steps, the ATF must circumvent various surveillance mechanisms of the cell. Therefore, it is not surprising that efficient delivery of ATFs remains an obstacle.

One approach towards improving cellular uptake of molecules is the use of cell penetrating peptides (CPPs) [92, 93]. CPPs are often derived from proteins that naturally translocate across cell membranes, such as the Tat protein from HIV [94] and the homeodomain Antennapedia (AtnHD) from *Drosophila* [95]. Also, molecules enriched in arginine, inspired by the Tat protein, have been developed as synthetic CPPs [96, 97]. In an interesting application of this strategy, a zinc finger ATF that upregulates VEGF-A was rendered cell-permeable by conjugating it to a 10-residue fragment from the HIV Tat protein [98, 99]. In addition, Mascareñas and colleagues improved the nuclear localization of a tripyrrole DNA binding molecule by conjugating the compound to an arginine octapeptide [100].

In another approach, the Dervan lab has improved nuclear localization of PAs by conjugating isophthalic acid derivates to the C-terminus of the PA [51, 53]. Conversely, the Kodadek lab improved nuclear localization by using an ethylene diamine turn for the hairpin PA in an ATF [101]. It should be pointed out that in the latter example, the hairpin PA is conjugated to a lipophylic steroid, which may enhance the permeability of the PA through hydrophobic membranes. In fact, covalent attachment of a steroid to a PNA has been shown to increase cellular uptake [102]. Improving the cellular uptake of TF mimics remains an active area of research.

6. Future directions

6.1. Cooperativity

Attempts to target unique sites in the genome have relied on expanding the number of DNA binding modules. For example, polydactyl ZFs have been engineered to target DNA sites of 18 bp. In the case of PAs, tandem hairpins were synthesized to target larger sites [103]. While reasonable, both examples were accompanied by a significant drop in selectivity due to increased non-specific binding [24, 103]. Natural transcription factors overcome the difficulties associated with finding unique targets in large genomes by forming non-covalent complexes through cooperative binding at the DNA target site [5]. Binding sites for multiple activators are commonly found in the gene promoter regions of higher eukaryotes. In principle, incorporation of cooperative binding between ATFs and other transcription factors should enhance the functionality of ATFs [104].

A new class of ATFs that incorporates cooperative binding was developed by Arndt et al. The ATF functions by nucleating the assembly of natural transcription factors on promoters/enhancers [105]. In this particular case, a synthetic mimic of the Hox family of transcription factors was generated. Hox proteins are developmental regulators, and have poor DNA affinity and sequence specificity on their own. However, cooperative binding with partner proteins, such as Exd, can increase their DNA binding affinity and specificity [106]. A polyamide was conjugated to a dipeptide derived from the Hox protein known to interact with Exd. The Hox mimic was able to cooperatively interact with Exd and bind to specific DNA sequences with nanomolar affinity [105, 107].

The principle of cooperative binding to DNA was incorporated in the ATF designed by the Ptashne and Dervan groups [48]. Specifically, the dimerization domain of Gcn4, a yeast transcription factor, was incorporated into a polyamide-based ATF [48]. Similarly, cooperative binding to DNA was incorporated in a DBD designed by Blanco *et al*. [108]. In this work, a tripyrrole-cyclodextrin (CD) conjugate that binds to an A/T rich DNA region was used to recruit a 23 amino acid peptide derived from the basic region of a bzip TF conjugated to an adamantane group. In the absence of the tripyrrole-CD conjugate, the BR peptide does not bind to DNA. This work adds to previous studies by Schepartz and coworkers, that achieved the dimerization of bZip DNA binding domains by incorporating transition-metal binding groups into the DBDs [109].

6.2. “Molecular blinking” of activation domains

An unusually potent AD, P201, was discovered by screening random sequences of 8 amino acid residues attached to Gal4(1–100), a DNA binding domain [68]. Like natural TFs, P201 also targets Gal11/Med15, a component of the transcriptional machinery [110]. This small artificial AD is as potent as the natural AD, Gal4, and it is far stronger than artificial ADs of similar size [68]. Mechanistic studies of this potent ATF identified that hydrophobic intramolecular interactions between the dimerization domain of the DBD Gal4(1–100) and the AD were crucial for the potency of the ATF [71]. Mutations which disrupted or further stabilized this interaction significantly lowered the activity of the ATF.

It was hypothesized that this *transient* intramolecular interaction between domains "masks" the unstructured peptide AD from unproductive interactions that could lead to ATF degradation (Figure 4). In support of this "molecular blinking" hypothesis, the cellular potency of natural and unnatural peptide ADs was found to significantly increase when this "masking" interaction was engineered into the ATF [72]. The potency of the ATF decreased when the interaction between the AD and DBD was disrupted. This was completely unexpected, and it revealed that a fine balance between "exposure" and "masking" significantly affected the potency of the ATF. Engineering such new properties will aid in the development of potent ATFs.

7. Conclusion

During the last decade, many advances have been made in the design of ATFs, with some ATFs currently undergoing clinical trials [111]. However, some obstacles must be overcome in order to realize the full potential of ATFs. Spatial and temporal control of the ATF activity and incorporating the ATF into cell signaling pathways are also highly desirable goals. Surmounting these challenges will require the collaboration of chemists, biologists, computational scientists, and bioengineers.

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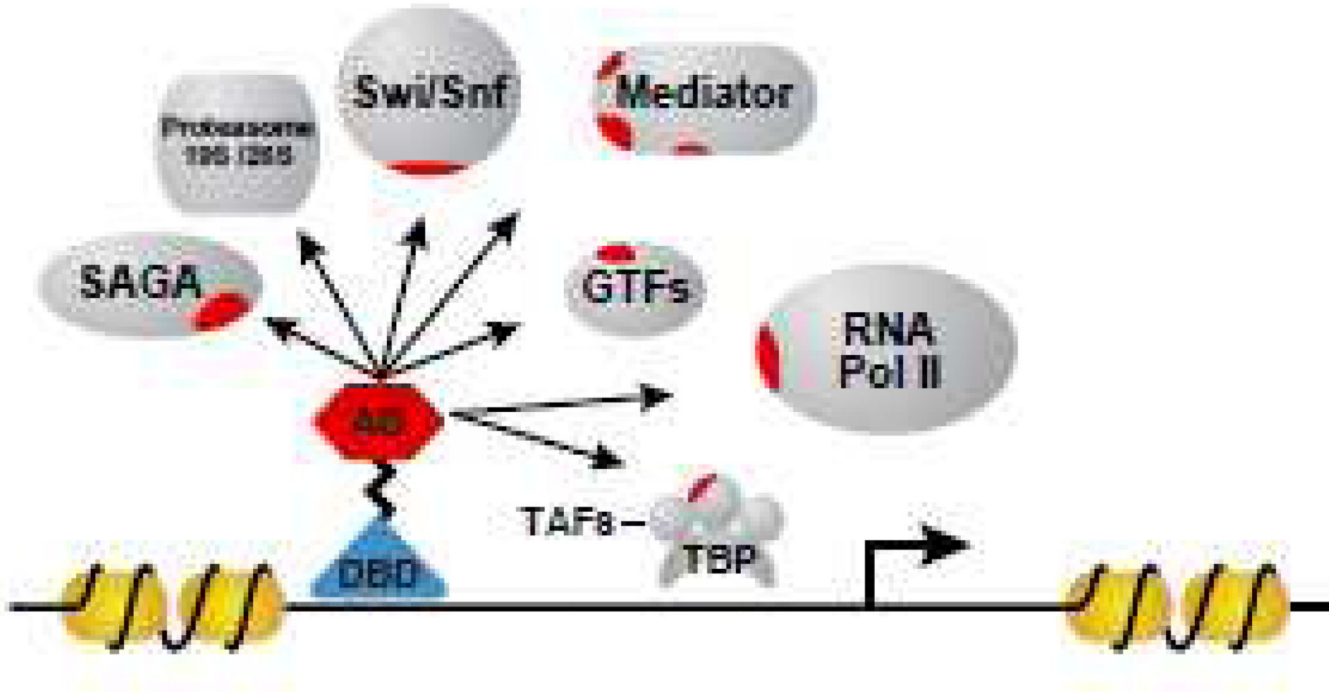

Figure 1. Transcription activation by transcription factors

TFs are minimally composed of a DNA binding domain (DBD) and an activator domain (AD). The DBD recognize and binds to a DNA sequence to activate the targeted gene(s). The AD recruits the transcriptional machinery components through interactions with RNA polymerase II (RNApol), general transcription factors, (GTFs), (TBP)-associated factors (TAFs), the Mediator complex, chromatin remodeling complexes such as SAGA and Swi/Snf complexes and/or the 19S and 26S components of the proteasome.

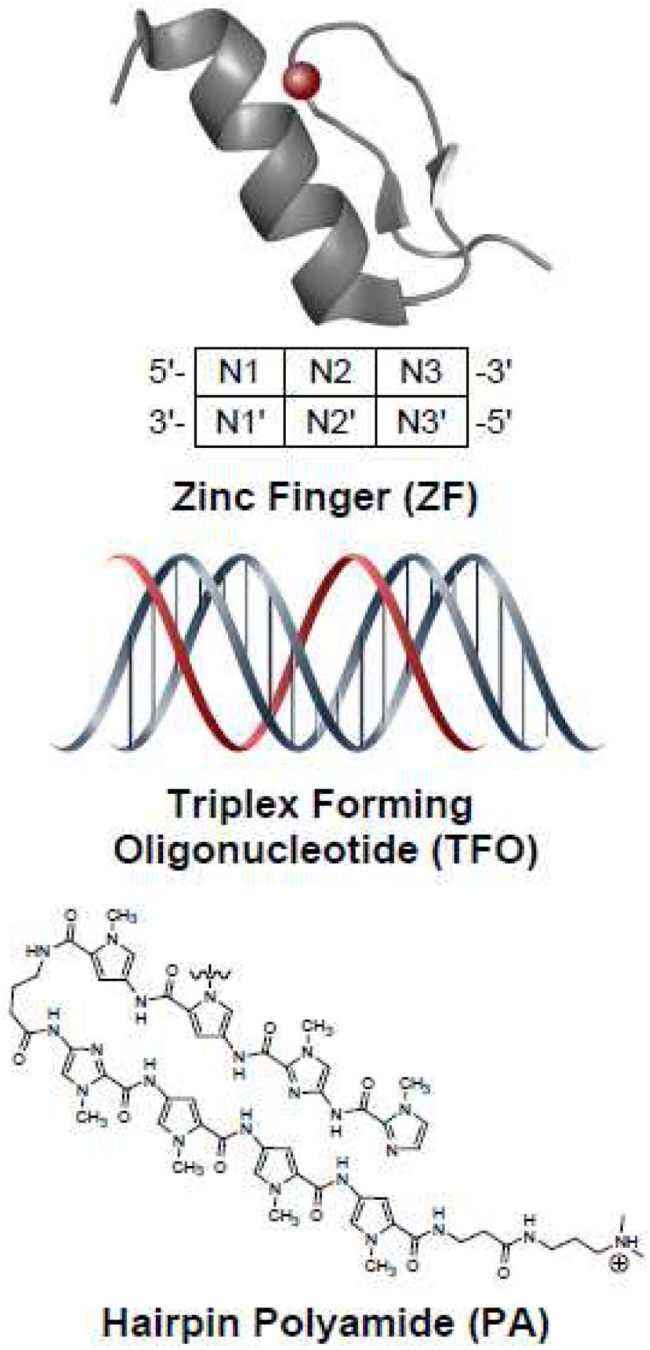

Figure 2. DNA binding domains commonly used in ATFs

Zinc fingers (ZF) recognize and bind to 3bp (N1-3) in dsDNA; triplex forming oligonucleotides (TFOs); hairpin polyamides (PAs).

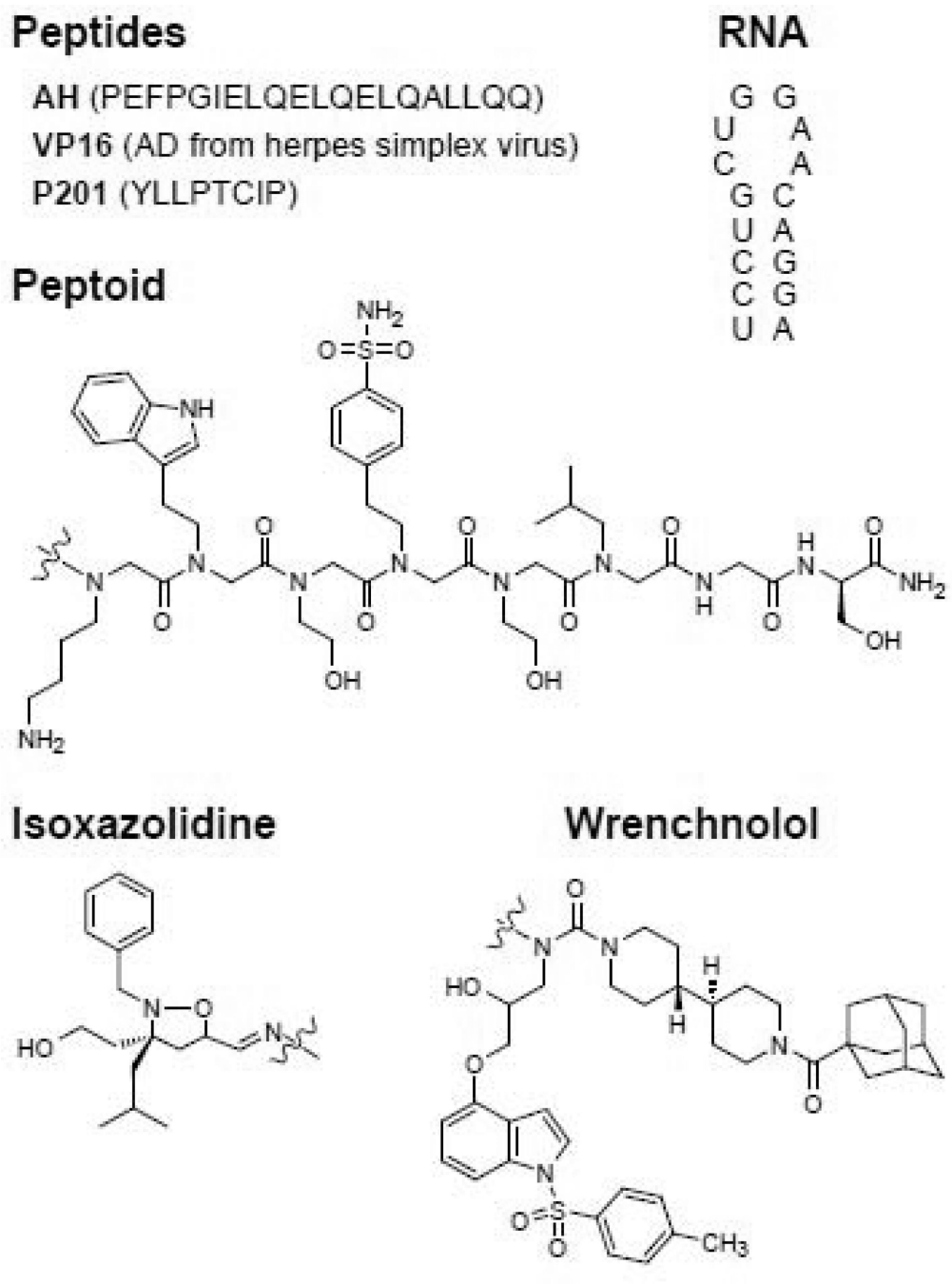

Figure 3. Activation domains commonly used in ATFs

Peptides: amphipathic helix (AH) [62], VP16 from herpes simples virus [112], and P201 [68]. Peptoid [73]. RNA [75] Smallmolecule: isoxazolidine [78] and wrenchnolol [82].

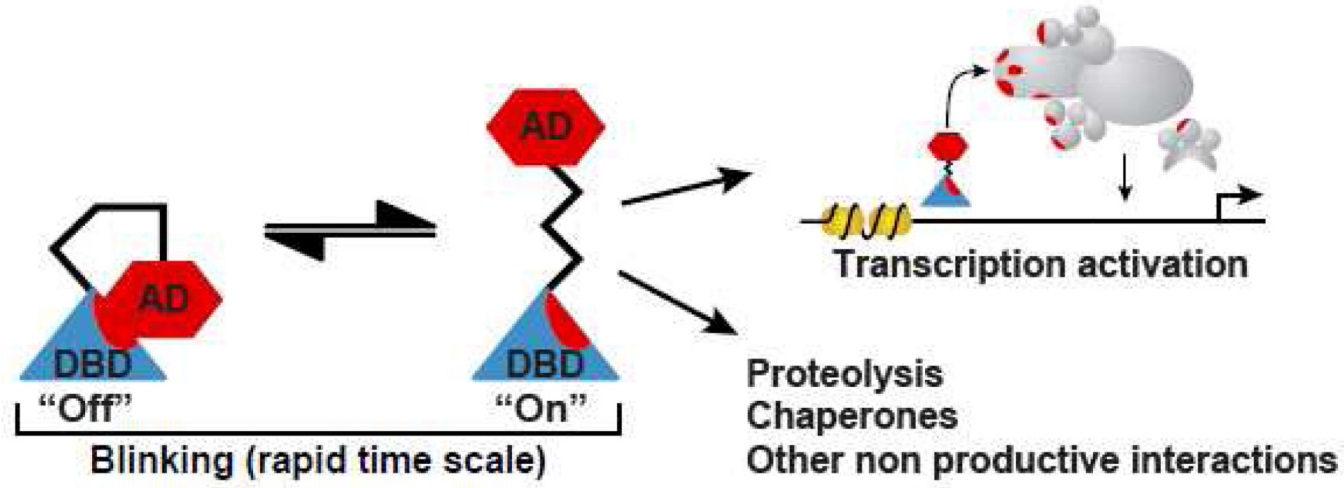

Figure 4. Model of ATF blinking

In the blinking model, the ATF is in rapid equilibrium between an 'off' (masked) state and an 'on' (exposed). The interconversion between these two states is mediated by intermolecular interactions between the DBD and the AD. In the 'off' state, the ATF is masked from the cellular milieu that could lead to degradation; whereas in the 'on' state the AD is transiently exposed and is able to recruit the transcriptional machinery.