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Review

A TAD Further: Exogenous Control of Gene Activation

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ABSTRACT Designer molecules that can be used to impose exogenous control on gene transcription, artificial transcription factors (ATFs), are highly desirable as mechanistic probes of gene regulation, as potential therapeutic agents, and as components of cell-based devices. Recently, several advances have been made in the design of ATFs that activate gene transcription (activator ATFs), including reports of small-molecule-based systems and ATFs that exhibit potent activity. However, the many open mechanistic questions about transcriptional activators, in particular, the structure and function of the transcriptional activation domain (TAD), have hindered rapid development of synthetic ATFs. A compelling need thus exists for chemical tools and insights toward a more detailed portrait of the dynamic process of gene activation.

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Natural transcriptional activators are essential players in the cascade of signaling events that lead to a gene being turned on. Responding to specific cues, activators search out particular genes or sets of genes within the nucleus of a cell and, once localized there, recruit the macromolecular machines that modify chromatin structure and initiate messenger RNA (mRNA) production (Figure 1, panel a) (*1*). Activator artificial transcription factors (activator ATFs) are non-natural replacements of these powerful proteins and are highly desirable tools for biomedical, biochemical, and biomanufacturing/synthetic biology applications. The ideal activator ATF would reconstitute all aspects of natural transcriptional activator function: signal-responsive up-regulation of selected genes to predetermined levels in a tissue-specific and time-sensitive manner. This is a daunting prospect because natural activators operate *via* a complex network of molecular recognition events and by a mechanism that is poorly understood at the molecular level. As with any molecule designed to perturb a natural system, effective delivery of an intact ATF to the specific tissue, the nucleus, and the promoter is also essential.

The evolution of activator ATFs from the first protein examples to those constructed from non-protein components has been the subject of several recent reviews (*1–9*). In this Review, we summarize the current model of the structure and function of natural transcriptional activators with particular emphasis on the many mechanistic questions still remaining that impact activator ATF design. Most of these questions surround the transcriptional activation domain (TAD) of these proteins. In addition, we highlight several recent advances in activator ATF design, as well as exciting applications for these and future generations of molecules.

Structure and Function of Natural Transcriptional Activators. *The Building Blocks.* Natural transcriptional activators are minimally composed of two domains or modules: a DNA binding domain (DBD) and a TAD (Figure 1, panel a) (*1*). The DBD provides much of the gene-targeting specificity of the activator because it is responsible for localizing the protein to a cognate sequence within genomic DNA. The TAD, in contrast, dictates the extent and timing of up-regulation mediated by the activator. Early in the dissection of the eukaryotic transcriptional activators, scientists unexpectedly found that the DBD could be readily separated from the activation domain with no loss of function of either module (*10*). A functional chimeric molecule could be generated, for example, by attaching a eukaryotic activation domain to a DBD from a different protein altogether (*1*). This was the earliest description of an engineered activator ATF and illustrated the power of this class of molecules as mechanistic probes. However, these early chimeric molecules could not be readily programmed to target any desired gene in a cell; that development came nearly a decade later.

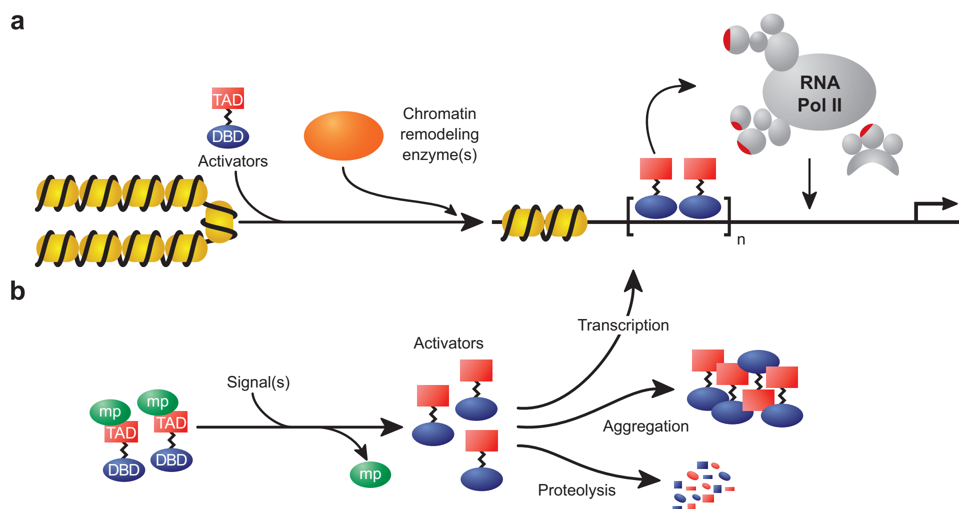

Figure 1. Schematic of transcriptional activation. a) Transcriptional activators minimally contain two domains: DBD (blue oval) and an activation domain (TAD, red square) (*1*). Activators up-regulate transcription upon binding to specific DNA sequences within genomic DNA and facilitating assembly at the targeted gene of the RNA polymerase II holoenzyme (the transcriptional machinery) through one or more protein binding events. b) The concentration of activators available to regulate transcription is controlled in part by masking protein (mp) interactions that provide signal-responsiveness and prevent aggregation and premature proteolysis (*1*).

The observation that the DBD and TAD could act independently led to investigations to define the structure, functional characteristics, and targets of each domain. The DBD proved to be far more tractable, and several new DNA binding folds were discovered, including zinc fingers and other folds unique to eukaryotes (*11, 12*). The DBDs defined the sequences targeted by transcription factors in the genome, so it was thought that understanding the folds of TADs would similarly illuminate the mechanism of TAD function (*1, 13, 14*). This triggered two decades of intensive analysis of TADs. The first surprise was that activation domains did not appear to form specific folds (*15*). An early combinatorial search to identify novel regions that would activate transcription in yeast was performed by fusing random segments of the bacterial genome to two different DBDs (*1, 16*). These chimeric proteins unexpectedly provided numerous peptides that would activate transcription. The only commonality between the different randomly generated TADs was that they had a preponderance of acidic residues (*16*). In contrast, a later combinatorial selection employing randomized octapeptides fused to the Gal4 DBD yielded highly hydrophobic peptides (*17*). Studies with natural TADs had by then defined hydrophobic residues as particularly important in transcriptional potency, and acidic residues were proposed to help solubilize the key hydrophobic residues (*18, 19*). However, no clear domain structure or strict motif, akin to those found for DBDs, emerged from the multitude of studies over two decades (*1*).

Searching for Structure. In solution, isolated TADs have generally been found to lack structure, although they often adopt secondary structure upon interaction with binding partners (*20–23*). The best-characterized examples of TAD structure are from co-complexes of TADs with inhibitory masking proteins that shield key residues of the TAD. These interactions are typically of higher affinity and specificity than those of TAD–transcriptional machinery interactions, and this likely

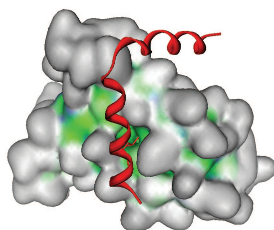

Figure 2. Solution structure of the KID(CREB)/KIX(CBP) complex. The activation domain of CREB (KID, red) bound to the KIX domain of CBP (PDB accession number 1KDX) is shown (*28*). A key leucine residue conserved is highlighted interacting with the hydrophobic binding surface of CBP. This surface is also used for interactions with other TADs.

explains why such complexes have been more straightforward to characterize. One of these is the complex between 15 residues of the p53 TAD bound to the mDM2 inhibitor protein in which the TAD forms an amphipathic helix with the hydrophobic residues buried in a hydrophobic cleft on mDM2 (*20*). The amphipathic helix motif is common among TADs, but other structural motifs have been identified. For example, a short peptide TAD from E2F binds in an extended conformation within a hydrophobic cleft of Rb, its natural inhibitor (*24*). In another example, Gal4 activation domain was proposed to fold as a β-hairpin when bound by its inhibitor Gal80 or even its targets in the transcriptional machinery (*25, 26*). A proline-scan analysis revealed that mutations that negatively affected interaction with its masking protein Gal80 did not affect the ability of the mutant TAD to stimulate expression of target genes *in vivo* (*27*). One implication of this study is that the secondary structural requirements for TAD–transcriptional machinery interactions may be fundamentally different than those for TAD–masking protein complexes.

Although small in number ($<$15), structural studies of TAD–transcriptional machinery protein complexes have provided some insight into the conformational requirements for transcriptional activation. The amphipathic helix is the most commonly observed structural motif for TADs in complex with transcriptional machinery targets, with the hydrophobic face of the helix participating in the bulk of the contacts. The complex formed between the TAD of CREB and the transcriptional machinery protein CBP/p300, for example, shows that the phosphorylated TAD adopts an amphipathic helix for the binding event (Figure 2) (*28*). Similar amphipathic helices have been observed for the TADs of VP16 (*21, 29*), c-Myb (*30*), and ESX (*31*). In addition, the artificial TAD $Gal4_{dd}$ interacts with its target protein Gal11P as an amphipathic helix (*32*). One roadblock to more extensive structural studies of TAD–transcriptional machinery targets is that the relevant targets of most TADs have not yet been definitely identified; this issue is addressed more fully in the subsequent section.

The dearth of structural information about TAD–transcriptional machinery interactions has limited the ability of chemists to use traditional structure-based design approaches to develop activator ATFs that mimic the structure and thus function of natural counterparts. Clearly, additional structural information about TAD–transcriptional machinery complexes would facilitate design efforts from both a computational and a synthetic standpoint. Of particular interest will be comparisons of the same TAD in complex with several different target proteins; subtle or obvious differences in the structures could provide a mechanism by which specificity could be engineered into small-molecule or even peptidomimetic inhibitors.

The Elusive Binding Partners of Transcriptional Activators. In parallel with the search for domains and properties of TADs, much of the transcriptional community has devoted considerable effort to identifying the transcriptional machinery targets of different TADs (Figure 3). This has been a contentious research area. Early on, scientists assumed that ubiquitous TADs would interact with common components of the transcriptional machinery that were needed at every promoter and were present in every cell. Early efforts to identify targets of potent activation domains thus focused on general components of the transcriptional machinery, and the most celebrated target was the TATA box binding protein (TBP) that is required for RNA polymerase II-mediated transcription in eukaryotes (*33, 34*). Immediately following the demonstration that TADs could interact with TBP, a series of elegant experiments by Green and coworkers suggested that TFIIB rather than TBP was the key rate-limiting component that was recruited by TADs (*35, 36*). Subsequently, subunits of TFIIH (*37*), TFIIA (*38, 39*), and even RNA polymerase II were suggested as the primary targets of TADs (*40*).

The demonstration that transcriptional activators did not stimulate transcription when supplied with purified TBP, TFIIB, or other basal transcription factors such as TFIIH, TFIIA, and RNA polymerase II led to a second wave of target discovery. In this era, “coactivators” dominated the attention of most labs. Coactivators were defined as proteins or protein complexes that did not interact with DNA but served as a bridge between DNA-bound transcription factors that targeted gene-specific sequences and the general components of the transcriptional machinery (*1*). The most celebrated class of coactivators encompassed the TBP-associated factors (TAFs) that formed a stable complex with TBP. TAFs soon gained prominence in the field as the universal conduits through which the majority of the transcription activa-

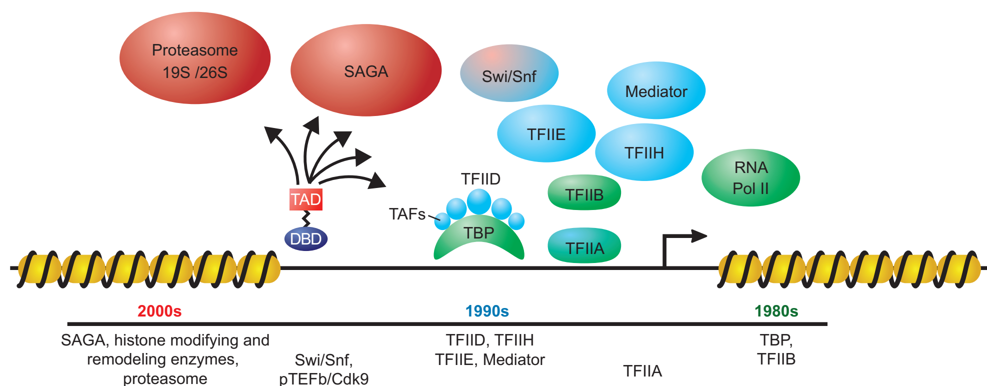

Figure 3. A timeline of transcriptional activator targets. During the past three decades, many transcriptional machinery complexes and individual proteins have been identified as key binding partners of natural transcriptional activators. Significant debate still exists about the relevance of most of the interactions.

tors function to stimulate gene transcription (*41–45*). Moreover, cell-type-specific and developmental-stage-specific TBPs and TAFs were identified and thought to be the solution for how different activators might function in different cellular milieus (*46–49*). However, experiments in yeast that inactivated individual TAFs showed that only a small fraction of genes (typically cell-cycle-specific genes) were dependent on TAFs, whereas the majority of the genes were not affected by TAF inactivation (*50, 51*). This led the field to refocus its attention on other coactivators that bridged gene-specific activators and the basal transcriptional machinery.

Parallel genetic and biochemical experiments led to the identification of a complex of proteins that could "mediate" the function of activators in purified systems (*52–54*). The components of the complex were called the Srb/Mediator proteins (*55, 56*). These proteins formed a stable complex that interacted with RNA polymerase II as well as with TADs (*52, 53*). Several detailed genetic, biochemical, and biophysical studies suggested that TADs interact with a subset of the components of the Mediator complex (*1*). In yeast, these appeared to include Srb4, Srb10, Med15(Gal11), and Med2, among others (*57–61*) in metazoan systems, such as DRIP/TRAP220 (*62–64*), and subunits of the PC2, ARC, and CRSP coactivator complexes (*65, 66*). Recent experiments suggest that different components of the Mediator complex may be targeted by different TADs in human cells (*64, 67*).

The focus on the Mediator complex as the main target of TADs was so great through the mid-1990s through early 2000 that a genetically and biochemically identified TAD target (Sug1) was initially deemed a component of the Mediator/RNA polymerase II holoenzyme complex (*68*). When it was discovered that this protein was in fact a component of the proteasome, the idea that Sug1 was a *bona fide* target was questioned (*69*). In the meantime, several groups had begun to explore whether chromatin-remodeling and -modifying enzymes were targeted to particular genes by TADs, prompted by the complexity of transcription activation in the context of nucleosomes and chromatin (*66, 70–80*). Early *in vitro* experiments yielded tantalizing results suggesting that TADs could recruit ATP-dependent nucleosome remodeling enzymes to promoters and that remodeling would greatly enhance stimulation of transcription from chromatinized templates (*70, 77, 81, 82*). Subsequently, scientists found that TADs also recruit the SAGA complex, which contains a subset of TAFs (but no TBP) as well as enzymes capable of covalently modifying histones (*75*). Thus, proteins such as CBP and other enzymes that could act on histones as well as components of the transcriptional machinery became the most actively examined TAD targets (*83–85*). A key contribution from this area was the identification of Rpd3/HDAC1 as a histone deacetylase linked to repression, thus encouraging further examination of the link between histone acetylation and gene activation (*86*).

Considerable evidence supporting an ordered recruitment of proteins to a promoter by one or more transcriptional activators has been provided by chromatin immunoprecipitation experiments in living cells. A particularly

elegant study in *Saccharomyces cerevisiae* showed that an activator at the HO locus brings in an ATP-dependent nucleosome remodeling enzyme complex, the Swi/Snf complex (*87*). This complex acts on the chromatin and enables the binding of a second cell-cycle-specific transcriptional activator that in turn recruits the histone-modifying SAGA complex. The SAGA complex is needed for subsequent recruitment by Mediator followed by RNA polymerase II (*87, 88*). A similar ordered recruitment has been reported in mammalian systems as well. However, these studies also show evidence of "cycles" of the activator and the transcriptional machinery binding at promoters (*89, 90*). Such cycling has been proposed to occur because of proteolysis of the activator upon transcriptional activation (*91*), perhaps by interactions with Sug1 and the associated proteasome. This model has recently been challenged by the observation that the recruitment of the ATPase-rich 19S subcomplex of the proteasome is important in transcription elongation and not proteolysis of the TAD (*92–94*). Along similar lines, Yamamoto and coworkers have reported that large chaperone complexes are needed for efficient transcription by glucocorticoid receptor (*95, 96*). The implication is that molecular chaperones targeted by TADs increase the efficiency of RNA polymerase II release from the stable pre-initiation complex that assembles at promoters prior to transcription (*95*).

A Key Dilemma. This is only a partial list of some of the prominent targets of TADs, and it illustrates a key dilemma and the outstanding mechanistic questions in the transcription field. It is highly unlikely that all targets that have been identified over the past 25 years are in fact targeted by TADs at each promoter. Whether TADs interact with only one target at a promoter remains a source of controversy. It is possible that at specific promoters particular targets are more relevant, that TADs are capable of delivering a variety of targets/complexes to the promoter, and that the physiological response (transcription) is dependent on whichever target is rate-limiting at that specific promoter. Also intriguing is a model that posits that "transcription factories" exist that contain most, if not all, of the components of the transcriptional machinery and that TADs effectively target genes to these factories (*97, 98*). Once the gene is recruited, the entire transcriptional machinery "tool kit" is available to the gene, and it can choose what it needs to effectively stimulate transcription.

The identification of the physiologically relevant protein targets of activators would have profound implications both for the general mechanistic understanding of transcriptional regulation and for the design and discovery of molecules that perturb (positively or negatively) the transcription process. One of the few well-characterized cases of an activator–target pair is that between the activator CREB and the coactivator CBP/p300 (*28, 99*). As a validated target of an important transcriptional activator, CBP/p300 has been the subject of several screens to provide peptide and peptoid ligands that, when linked to a DBD, function as transcriptional activators, presumably through this binding interaction (*100–103*). Expanding the number of confirmed activator targets would enormously open up the screening possibilities and discovery opportunities. In addition, because activator–target interactions are likely to vary across tissue types and genes and with cell-cycle timing, the identification of key activator targets in each of these circumstances would pave the way for activator ATFs that would function only in those environments.

Redundancy and Promiscuity in Activator Binding Interactions. One of the challenges associated with mechanistic investigations of transcription initiation is the apparent redundancy built into the system. For example, so-called activator-bypass experiments in which transcriptional machinery proteins are fused to DBDs and assayed for their transcriptional ability have demonstrated that a single interaction with one of several complexes (*e.g.*, Mediator, TFIID, or SAGA) suffices to activate transcription (*1*). Further, as described earlier, most TADs exhibit a promiscuous binding profile *in vitro*, interacting with a range of transcriptional

KEYWORDS

Artificial transcription factor (ATF): An ATF is a designer molecule that seeks out specific genes or groups of genes and directly regulates them either positively or negatively. An ATF typically contains at least two functional domains, a DNA binding domain and a regulatory domain.

Activator ATF: An activator ATF up-regulates specific genes or sets of genes by binding to a particular sequence of DNA and interacting with one or more components of the transcriptional machinery. Molecules that indirectly affect gene activation, for example, by stimulating signal transduction cascades or altering DNA structure, are thus not activator ATFs.

Transcriptional activator: These natural transcription factors are key players in the cascade of events that lead to gene activation. Minimally composed of a DNA binding domain and a transcriptional activation domain, activators function in a signal-responsive fashion to regulate the timing and extent of gene-specific activation.

DNA binding domain (DBD): One of the two key domains of an activator ATF, the DBD provides the gene-targeting specificity of the molecule.

Transcriptional activation domain (TAD): One of the two key domains of an activator ATF, the TAD dictates the timing and extent of transcriptional up-regulation through binding interactions with one or more components of the transcriptional machinery.

Coactivator: A protein that interacts with the transcriptional activation domain of a DNA-bound transcriptional activator and participates in the gene-activation process.

Box 1. Applications of ATFs

The biomedical applications for activator ATFs arise from the observation that altered transcription patterns are associated with disease states as either a cause or an effect (*137, 138*). ATFs are excellent probes for deciphering the mechanistic details of disease as well as candidates for therapeutic agents that could be used to correct errors in gene transcription (*139*). Table 1 lists a subset of human diseases in which transcriptional misregulation has been demonstrated to play a fundamental causal role. Overexpression of the transcriptional repressor REST/NRSF in medulloblastoma, for example, leads to the suppression of genes critical for the proper differentiation of neuronal cells (*140, 141*). However, up-regulation of the REST/NRSF-controlled genes by a protein-based ATF induces tumor cell apoptosis, an indication of the power of transcription-based therapeutics (*140*). Further, ATFs could be used in conjunction with gene therapy strategies to provide fine control over production of the introduced protein (*142*). Although the examples shown in Table 1 are all human cancers, applications for a wide range of other human diseases can also be envisioned, including metabolic and genetic disorders.

ATFs also have many non-biomedical applications. For biomanufacturing applications, ATFs that up-regulate transcription to prescribed levels in a signal-responsive fashion are highly desirable either to directly improve the amount of protein product or to increase the concentrations of biosynthetic enzymes in order to further boost product yields (*126, 143*). In synthetic biology, transcriptional networks are key building blocks used to construct cell-based devices and networks, and ATFs that function in a predictable and orthogonal fashion relative to natural regulators would be particularly valuable additions for such applications (*144*). This is especially true for the still-nascent field of eukaryote-based networks, because the complexity of the transcription process increases significantly from prokaryote to eukaryote.

machinery proteins (*1, 104*). As further evidence of redundancy, a single transcriptional machinery protein often interacts with multiple activators. For example, the Mediator component Med15(Gal11) interacts *in vitro* with natural TADs derived from Gal4, Gcn4, and VP16, among others, and also binds to the non-natural TAD XL_{Y} (*59, 105–109*). Similarly, the binding partners of the mammalian coactivator CBP include CREB, c-Myb, p53, and Hif1α (*110*). The result of this functional redundancy is that disruption of a single or even several interactions can often be compensated for, at least partially, by other interactions that may or may not be physiologically relevant. Genetic strategies such as mutagenesis or deletion to validate activator–target interactions *in vivo* or in cells have been challenging to interpret because of the often pleiotropic effects observed upon alteration of critical transcription proteins. Future chemical approaches such as the use of small-molecule inhibitors of particular TAD–protein interactions will thus be enormously useful in parsing the relative importance of those interactions in a cellular context.

Putting It All Together: Activator ATFs. *Module Replacement.* The basic function of a transcriptional activator can be reconstituted by replacing the key modules of the natural protein with synthetic or non-natural counterparts (*1–4*). An activator ATF designed to activate a particular gene can be constructed by choosing a DBD that will bind to the promoter region of that gene and linking it, covalently or noncovalently, to a TAD. The evolution of this design strategy and the non-natural domains employed have been described in several recent reviews (*2–5, 7, 9, 111*). The domain that has proven the most tractable to replace is the DBD. The advent of powerful strategies for the creation of designer proteins such as zinc fingers, nucleic-acid-derived molecules, and polyamides to target particular DNA sequences has enabled the construction of activator ATFs with novel DNA targeting properties for applications *in vitro*, in cell culture, and *in vivo* (*1, 111, 112*). However, the vast majority of activator ATFs have employed a TAD sequence adapted from a natural activator or a peptide that mimics natural TADs. Efforts to identify nonpeptidic TADs have been far less successful.

A TAD Challenging. Peptide-based TADs, although widely used, do have significant limitations, such as cellular and nuclear permeability and potential immunogenicity issues. Proteolytic degradation of a peptide TAD or

TABLE 1. Some malfunctioning transcription factors implicated in the onset of cancer

Transcription factor	Event	Type of cancer	References
AR	Mutations in the AR gene that attenuate its ligand specificity to include anti-androgens or other endogenous hormones, making them androgen-independent and resistant to androgen ablation therapy	Advanced prostate cancer	(*145–147*)
E2F	Loss-of-function mutations in its masking protein, the retinoblastoma tumor suppressor protein	Retinoblastoma, osteosarcomas, breast carcinomas	(*148–151*)
ERα	Increased mRNA expression of the ERα gene from the distal promoter B	Early stages of breast cancer	(*152, 153*)
HIF-1α	Stabilized in the hypoxic conditions of solid tumors, although its contribution to tumor progression is cell-type- and context-dependent A mutation in the von Hippel Lindau protein results in decreased ubiquitination and proteasomal degradation (and therefore accumulation) of HIF-1α	Breast, cervical, colon, prostate, clear cell renal carcinoma	(*154–159*)
c-JUN	Phosphorylation by constitutively active Jun N-terminal kinase (JNK) produced by oncogenic Ras signaling	Chronic myelogenous leukemia, small-cell lung cancer	(*160–163*)
c-MYC	Overexpression by gene amplification or protein stabilization Overexpression by juxtaposition of the immunoglobin heavy-chain enhancer to the c-*myc* gene in the case of Burkitt's lymphoma Stabilization from degradation by abnormal phosphorylation of Thr58 and Ser62 in the case of acute lymphoblastic leukemia	Burkitt's lymphoma, pediatric acute lymphoblastic leukemia, medulloblastoma, breast cancer	(*152, 164–167*)
NFκB	Constitutively activated due to decreased levels of its cytoplasmic sequestering protein IκBα	Pediatric acute lymphoblastic leukemia, renal cell carcinoma, retinoblastoma, melanoma	(*168–171*)
p53	Mutations within the p53 gene that are predominantly somatic, but can also be germ-line Overexpression of its masking protein MDM2	Colon, breast, leukemias/lymphomas, lung, esophageal, sarcomas, Li–Fraumeni syndrome	(*150, 152, 172–178*)
RARα	Chromosomal translocation of the RARα gene to produce X-RARα and RARα-X fusion genes, in which the X gene can be PML, PLZF, NPM, NuMA, or STAT 5B	Acute promyelocytic leukemia	(*179–181*)
REST	Overexpression	Medulloblastoma	(*182*)
STAT3	Constitutively activated by oncogenic tyrosine kinases that are either intrinsic to the receptor itself (*e.g.*, EGF receptor and PDGF receptor), or associated with the receptor (*e.g.*, Janus and Src-family kinases)	Head and neck, multiple myeloma, leukemias/lymphomas, breast	(*183–187*)

a peptidic ATF may also significantly impact cellular function (*113*). TADs constructed from small molecules or other synthetic materials are thus highly desirable (Box 1). In one of the earliest examples, activator ATFs with TAD of the same sequence but composed of either L or D (non-natural) amino acids were found to activate transcription to similar levels *in vitro* (*113*). However, in cell culture, only the D-amino-acid-containing ATF functioned as an activator; the observed difference in activity was presumed to originate in the differences in proteolytic resistance of the natural and unnatural TADs (*113*). Building on this theme, researchers isolated a peptoid-based TAD from a binding screen against a portion of the coactivator CBP/p300. They observed it to be a robust activator in human cell culture (*101*), perhaps at least in part because of the resistance of peptoids to proteolysis (*114*). Somewhat unexpectedly, even RNA aptamers have been shown to function as TADs, opening the door for the potential use of modified/stable nucleic acids as future activator ATFs (*115–117*).

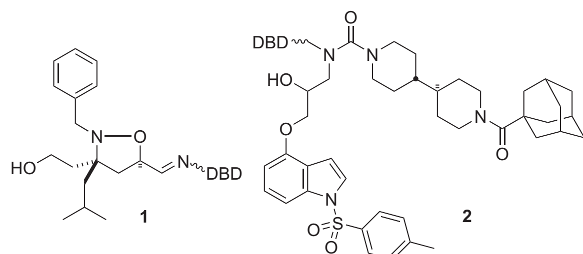

Figure 4. Small-molecule activation domains. Only two small molecules that reconstitute activation domain function have been reported: amphipathic isoxazolidine 1 **(and related structures) (*118, 119*) and the more hydrophobic wrenchnolol (**2**) (*120, 121*).**

The first example of a small-molecule TAD, the isoxazolidine shown in Figure 4, was based upon the observation that a hydrophobic surface formed by a few key residues in natural TADs is essential for activation (*118, 119*). Thus, isoxazolidine **1** containing isoleucine- and phenylalanine-like substituents functions as a TAD *in vitro* with activity comparable to that of a TAD derived from a natural protein, VP16. Consistent with the demonstrations that many different amphipathic peptide sequences function as TADs, changing the positioning of the functional groups in **1** does not significantly impact function (*119*). A second small-molecule TAD is wrenchnolol (**2**, Figure 4). In this example, the NMR structure of the TAD of ESX bound to the coactivator Sur2 was used to identify a molecular scaffold that would position key functional groups analogous to ESX; a screen of a focused library was then used to identify wrenchnolol (*120*). This molecule is an inhibitor of the ESX–Sur2 interaction (IC_{50} = 10 μM) (*120*) and, when attached to a synthetic DBD, up-regulates transcription 3.5-fold *in vitro* (*121*).

Beyond Modular Replacement: Enhancing Functionality. Although an activator ATF composed of the two essential modules of a natural activator, DBD and TAD, will successfully up-regulate transcription, these minimal molecules lack much of the functionality, such as signal-responsiveness, of their natural counterparts. The activity of many natural transcriptional activators is regulated either directly or indirectly by small molecules. For example, receptors such as the estrogen receptor are transcriptionally inactive until bound to their cognate steroid, at which time they translocate to the nucleus and participate in transcriptional stimulation (*122, 123*). The transcriptional activator thus functions in a temporally controlled and environmentally sensitive manner. This model has provided inspiration for the development of a variety of small-molecule-responsive ATFs (*124–126*). Inclusion of receptor ligand binding domains, for example, into an ATF design confers small-molecule control over the resulting fusion protein (*126*). In a variation of the three-hybrid technique (*127*), a chemical dimerizer was used to noncovalently tether the TAD to the DBD, thereby reconstituting activator function in cells (*128*); modifications of this strategy have been widely employed (*129, 130*). More recently, an orthologous small-molecule modulator of an ATF was obtained by screening for molecules that could rescue the activity of a modified zinc-finger-based ATF, and a unique ATF–small-molecule pair was identified (*131*). In this example, the activator ATF bound to DNA very weakly (K_D = 5.7 μM) until 2-(4′-quinoline)benzimidazole (**3**, Figure 5) was introduced (K_D = 350 nM), and this led to an ~18-fold increase in observed gene expression in human cell lines (*131*).

A similar concept was recently applied to ATFs containing a protein DBD and an RNA TAD (*132*). In this case, an RNA sequence known to interact with tetramethylrosamine was added to the TAD. The resulting construct was 10-fold more active in the presence of tetramethylrosamine (**4**) in *S. cerevisiae*, with the activity enhancement likely arising from a conformational change upon ligand binding (Figure 5) (*132*). In a fourth example, differences in conformational entropy were exploited to develop molecules that function as protein–DNA dimerizers at low temperatures but are inactivated at slightly higher temperatures; these temperature-sensitive chemical dimerizers can be used to regulate protein–DNA interactions in organisms such as *Drosophila* that live at 16–20 °C without adversely affecting organisms such as humans with physiological temperatures of 37 °C (*133*). The external control provided by these examples may prove quite advantageous for cell and eventual organism studies, particularly if delivery and concomitant immunogenicity challenges can be addressed.

Future Directions. The recent breakthroughs in the design and creation of activator ATFs have occurred through the combination of chemical synthesis and biological insight. However, the current suite of activator ATFs still lacks several characteristics that are likely

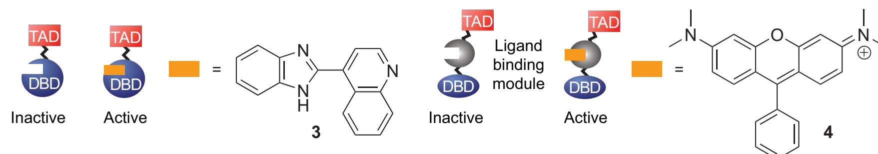

Figure 5. Small-molecule-activated ATFs. The incorporation of ligand binding domains into activator ATFs provides external control over function. In the absence of small-molecule ligand, the activator either cannot bind to DNA or is in an undesirable conformation for functional contacts with the transcriptional machinery ("inactive"). Upon addition of the ligand, however, activity is restored. Two recent examples are 2-(4′-quinoline)benzimidazole (3) (*131*) and tetramethylrosamine (4) (*132*).

to be important for ultimate therapeutic utility and other applications. In contrast to natural activators, for example, activator ATFs often lack tissue- or organism-specificity, and cellular delivery remains a challenge for both small-molecule- and protein-based activator ATFs. In addition, in cells, ATFs rarely up-regulate transcription as well as natural activators but instead exhibit more modest activity (*4, 7*). There are exceptions (*17, 101, 134*). For example, it was recently demonstrated that several artificial TADs as well as sequences derived from natural activators could be converted into TADs with robust cellular function when a binding site for the TAD was designed into the activator ATF (*134*). This intramolecular binding interaction appears to play the role of a masking interaction, perhaps shielding the TADs from aggregation and/or premature degradation and thus enhancing their activity. Further, as with any exogenous age, the immunogenicity of ATFs may prove to be a significant concern, particularly in the case of protein-based ATFs that are introduced *via* gene therapy (*135*). All of these issues must be addressed before activator ATFs can realize their potential as therapeutic agents in addition to their already important role as mechanistic probes. Advances on these fronts will require a higher-resolution picture of the protein–protein interactions in which natural activators and activator ATFs participate in terms of binding specificity, affinity, and kinetics. Further, evidence is emerging that interactions in which ATFs participate that are outside of the transcriptional machinery may contribute to functional potency as well as intracellular delivery (*134, 136*). Thus, efforts to dissect the complete molecular recognition profile of transcriptional activators will provide significant insight into activator ATF design. Chemical approaches to studying transcription mechanisms will likely play a critical role in these efforts.

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