A general approach to the design of allosteric, transcription factor-regulated DNAzymes

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Here we explore a general strategy for the rational design of nucleic acid catalysts that can be allosterically activated by specific nucleic-acid binding proteins. To demonstrate this we have combined a catalytic DNAzyme sequence and the consensus sequence recognized by specific transcription factors to create a construct exhibiting two low-energy conformations: a more stable conformation lacking catalytic activity and lacking the transcription factor binding site, and a less stable conformation that is both catalytically active and competent to bind the transcription factor. The presence of the target transcription factor pushes the equilibrium between these states towards the latter conformation, concomitantly activating catalysis. To demonstrate this we have designed and characterized two peroxidase-like DNAzymes whose activities are triggered upon binding either TATA binding protein or the microphthalmia-associated transcription factor. Our approach augments the current tool kit for the allosteric control of DNAzymes and ribozymes and, because transcription factors control many key biological functions, could have important clinical and diagnostic applications.

Introduction

DNAzymes and ribozymes, naturally occurring or in vitro selected RNAs or DNAs that catalyze specific chemical reactions, couple the advantages of enzymes (e.g., high turnover and specificity) with those of nucleic acids (e.g., low cost, ready designability) and thus represent a promising set of tools for use in biosensing, synthetic biology and bionanotechnology. Catalytic nucleic acids have similarly emerged as a new class of gene silencing agents, leading to the development of, for example, new cancer therapies.

An advantage of nucleic acid catalysts is the ease with which they can be rationally redesigned to introduce allosteric regulation, a mechanism that allows the fine-scale regulation of their activity upon the binding of an effector, an effect that has proven of value in a range of applications, including synthetic biology and biosensing. Most commonly, the design of allostery has been achieved via fusion of the catalytic nucleic acid with a regulation domain for the binding of the effector. This could be a specific short oligonucleotide sequence or a small molecule, or protein target that binds an aptamer domain. This binding event induces a conformational change that, in turn, regulates the catalytic activity. Here we expand on this theme by demonstrating a new class of allosterically regulated, catalytic nucleic acids that employ nucleic-acid-binding proteins as their effectors.

As our test bed nucleic-acid catalyst we have employed a guanine-rich, horseradish peroxidase (HRP)-mimicking DNAzyme. This single-strand DNA adopts a G-quadruplex structure that, in the presence of the cofactor hemin, catalyzes the oxidation of the substrate 3,3'-tetramethylbenzidine (TMB) to give a colored product which is detectable by absorbance (λmax = 650 nm).

Fig. 1. Transcription factor-induced activation of a DNAzyme. Here we demonstrate a DNAzyme allosterically activated by specific transcription factors (TF-regulated DNAzyme). To do so we coupled two functional domains: (i) a catalytic DNAzyme domain (red sequence in the cartoon) and (ii) a double-stranded transcription factor (TF)-binding domain (green). A sequence element complementary to the sequence of the DNAzyme stabilizes an alternative conformation (left) that “sequesters” both domains in an inactive (i.e., non-catalytic and non-TF-binding, respectively) state. This off-state is in equilibrium with a second conformation, the on-state, in which both domains are functional. TF binding shifts this equilibrium towards the on-state, activating catalysis. Here we used the HRP-like G-quadruplex DNAzyme as our model catalytic domain. In the presence of hemin and hydrogen peroxide, this domain catalyzes the oxidation of the HRP substrate 3,3'-tetramethylbenzidine (TMB) to give a coloured product which is detectable by absorbance (λmax = 650 nm).
the oxidation of HRP-substrates. To re-engineer this DNAzyme to introduce transcription factor (TF)-regulated allostericity we combined this catalytic domain (red domain in Fig. 1) with a consensus sequence recognized by a specific TF (green domain in Fig. 1) in such a way that the fusion populates two low-energy conformations. In the more stable of these, termed the off-state, the catalytic domain and the double-stranded, TF-binding region are “sequestered” and thus inactive (Fig. 1, left). In the less stable conformation, termed the on-state, both the domains are in their functional forms. TF binding thus pushes the equilibrium between these conformations from the former, off-state, towards the latter, on-state,43 activating catalysis (Fig. 1, right).

Results and discussion

As our first allosteric effector we employed microphthalmia-associated transcription factor (MITF), a DNA-binding protein associated with melanoma and renal cell carcinoma.46–48 We designed four variants of MITF-regulated DNAzymes, each presenting the same consensus binding sequence46–48 for the TF (Fig. 2A, green). The four differ, however, in the stability of their off-states (Fig. 2B). Specifically, by increasing the number of bases in the double-stranded stem used to stabilize the off-state we obtained variants with estimated free energies ranging from −30.5 to −57.7 kJ mol⁻¹. The stability of the on-state, in contrast, is effectively identical in all four variants (predicted stability = −26.9 kJ mol⁻¹), and thus this approach provides a means of tuning the switching equilibrium constant, Kᵣ, between the two states. This, in turn, provides a means of controlling the range of TF concentrations over which the DNAzyme is properly regulated.45,50

To avoid wasting the (rather expensive) TF, we first characterized our re-designed DNAzymes using a DNA strand (the “MITF-mimicking strand”) that binds to and thus stabilizes the on-state mimicking the target TF (Fig. 2A). As expected, the range of mimicking-strand concentrations over which activation is observed depends strongly on the switching equilibrium constant (Fig. 2C). Variant #1, for example, which exhibits the least stable off-state (predicted ΔG = −30.5 kJ mol⁻¹), is partially activated even in the absence of its effector. In contrast, variant #4, for which the predicted stability of the off-state conformation is the highest (ΔG = −57.7 kJ mol⁻¹), remains nearly inactive even at the highest effector concentrations we have employed (10 μM). The two variants between these extrema, in contrast, exhibit robustly regulated activation and show the optimal increase in activity upon increasing the effector concentration (Fig. 2D).

Because it is well regulated, we selected variant #2 to further characterize the extent to which the allosterically regulated catalyst is regulated by its specific TF (Fig. 3). For this variant we observe a monotonic increase in activation with increasing TF and an EC₅₀ (the effector concentration at which 50% activation is observed) of 375 ± 105 nM (Fig. 3, left). The activation fold of the DNAzyme activity in the presence of saturating concentration of the target protein is 1.9 which is in good agreement with previous strategies for the allosteric activation of the same G-quadruplex peroxidase-like DNAzyme.1,15,27,41

In contrast, the DNAzyme remains inactive when challenged with other, non-effector proteins. Incubation with 500 nM of the transcription factor TATA binding protein, for example, does not produce any significant cross-reactivity (Fig. 3, right).

To demonstrate the generality of our approach we designed a DNAzyme that is, instead, regulated by TATA binding protein (TBP), a TF present in virtually all eukaryotic cells.51 To do so, we engineered three variants differing in Kᵣ (Fig. 4A and B) that, upon switching to the on-state, exhibit the consensus binding sequence of TBP.50,51 We found that the resultant activation profiles (using again a TF-mimicking DNA strand) are consistent with the predicted energy gap between
the on- and off-states. From these results we selected variant 
#2, which exhibits an intermediate $K_s$ and thus robust activa-
tion, for further characterization. The 2.6-fold, TBP-
induced activation of this DNAzyme occurs with an EC$_{50}$ of
104 ± 12 nM (Fig. 4C) and, as was true for its MITF-activated
counterpart, the DNAzyme is insensitive to other, non-
targeted proteins (500 nM) (Fig. 4D).

**Conclusions**

In nature, the regulation of the activity of an enzyme is usually
achieved through allosteric regulation, in which a binding site
distal from the active site can recognize a molecule (an allosteric
effector) and lead to a conformational switch that affects the
enzyme’s affinity for the substrate. Through this mechanism the
activity of naturally occurring enzymes can be finely regulated
by a variety of allosteric effectors in a concentration-dependent
fashion. Alloster, also called “the second secret of life” by
Perutz, could thus be considered as the optimal strategy to
regulate the affinities and activities of biomolecules and bio-
receptors. Because of this, engineering alloster into artificial
systems could greatly improve the functionality of biomolecules
employed in biotechnologies. In response, we have demon-
strated here a general strategy for the design of nucleic acid
catalysts allosterically activated by specific transcription factors.

The development of allosterically regulated DNAzymes or
ribozymes through both rational design and combinatorial
selection strategies has been the subject of several studies
starting from the seminal and fundamental work of Breaker and
coworkers regarding the design and development of allosteric
ribozymes. In the past ten years several efforts have been
devoted to the development of nucleic acid catalysts that
can be activated or regulated by different effectors including
short nucleic acid sequences and aptamer targets. Allosteric
nucleic acid catalysts have thus been demonstrated to be
useful tools not only for biosensing purposes but also as
controllable therapeutic agents for gene therapy strategies or
or as molecular tools for controlling gene expression.

Despite this, to the best of our knowledge, there is no report of a
rational design of DNAzymes allosterically regulated by tran-
scription factors (TF). We demonstrated here a general strategy
to obtain TF-responsive DNAzymes by joining a TF consensus
sequence with a G-quadruplex peroxidase-like DNAzyme
domain. Our approach is based on the rational design of a
conformational switching probe that flips between a non-
binding inactive conformation to a second binding-competent
active conformation in the presence of the specific TF. Because
of this, we can regulate the TF concentration range at which
activation of the DNAzyme is observed. We can thus control in a
fine-tuned fashion the activity of our TF-regulated DNAzyme not
only using different concentrations of the specific TF, but also
using variants with different stabilities of the non-binding
conformation, over completely different TF concentration
ranges.

Compared to similar strategies reported earlier on the allo-
steric activation of catalytic nucleic acids, our approach appears
very versatile. The consensus site recognized by each TF almost
invariably consists of a specific double stranded DNA sequence
with a length ranging from 6 to 12 base pairs, rendering the
rational design of the necessary conformational switch quite
straightforward. We also note that our approach can have
important biological and clinical implications. While there is
only a limited number of molecular targets recognizable via the
use of aptamers, more than 10% of the ca. 25 000 human genes

![Fig. 3](image-url) (Left) Our MITF-regulated DNAzyme (variant #2) is activated by
its target TF in a dose-dependent fashion and is insensitive to other,
non-targeted proteins (right). For the specificity test (right) we have
used a concentration of target MITF and of non-specific proteins of
500 nM. In the $y$-axis of the right panel the difference (Absorbance)
between the absorbance value obtained in the presence of the target
and that obtained in a blank solution has been used.

![Fig. 4](image-url) (A) To demonstrate the generality of our strategy we designed
variants containing the consensus binding site for TATA binding
protein (TBP). (B) Using a DNA strand mimicking the action of
the target TF (see Fig. 2a), we find that, as expected, increasing the off-
state stability shifts the activation profile to higher effector concen-
trations. (C) The intermediate variant (variant #2) was selected for
further tests with the TF showing the activation of the TF-regulated
DNAzyme as the concentration of TBP increases (EC$_{50}$ = 104 ± 12 nM).
(D) This DNAzyme, too, exhibits good specificity against other non-
specific proteins. Here, we have used a concentration of target TBP
and of non-specific proteins of 500 nM. In the $y$-axis of panel D the
difference (Absorbance) between the absorbance value obtained in
the presence of the target and that obtained in a blank solution has
been used.
encode DNA-binding proteins suitable for the regulatory role presented here, the majority of which function as transcription factors thus controlling crucial biological mechanisms.\(^{37}\)

Despite the limited activation fold achieved with our strategy, we note that this is comparable to other examples of allosterically activated DNAzymes based on the use of the peroxidase-like G-quadruplex.\(^{1,15,27-44}\) Because other examples of DNAzymes and ribozymes activated by different targets show larger activation folds we believe that this difference might be due to the low efficiency and high background signal characteristics of peroxidase-like DNAzyme in comparison to other DNAzymes.\(^{1,27,42-44}\) Finally, our strategy could be applied in the design of RNA/DNA chimeras to produce TF-regulated ribozymes and RNA-binding-protein-regulated DNAzymes that could have potential applications in several fields. Engineering allosteric catalytic nucleic acids that respond to TFs could in fact lead to novel molecular tools for gene expression regulation, gene regulatory mechanisms or novel riboregulators that can allow the development of genetic circuits in the growing fields of synthetic biology and bioengineering.\(^{58-60}\)

### Experimental section

Unmodified oligonucleotides were synthesized and purified (desalt purification) either by IBA GmBH (Göttingen, Germany) or Sigma-Aldrich (St. Louis, Missouri) and used without further purification. The sequences of the constructs are as follows.

**MITF variant #1:** 5’-CCACGTG CGGCCT TACGTG TCTTGGGT AAGGCC GGTGTTGGG-3’.

**MITF variant #2:** 5’-CCACGTG CCGCCT TACGTG TCTTGGGT AAGGCC GGTGTTGGG-3’.

**MITF variant #3:** 5’-CCACGTG CCGCCT TACGTG TCTTGGGT AAGGCC GGTGTTGGG-3’.

**MITF variant #4:** 5’-CCACGTG CCGCCTA TACGTG TCTTGG GTAGGGCCGGT TTGGG-3’.

**TBP variant #1:** 5’-CTATATAAA GCCTT TTTATATA TCTTGGGT AAGGCC GGTGTTGGG-3’.

**TBP variant #2:** 5’-CTATATAAA GCCTT TTTATATA TCTTGGGT AAGGCC GGTGTTGGG-3’.

**TBP variant #3:** 5’-CTATATAAA GCCGCCTA TTTATATA TCTTGGGT AAGGCC GGTGTTGGG-3’.

In the sequences above the underlined bases represent the stem portion of the non-binding conformation (off-state), while the italic bases represent the TF recognition element of the binding conformation (on-state) (see Fig. 1).

Mimicking strand oligonucleotides were purchased either from IBA GmBH (Göttingen, Germany) or from Sigma-Aldrich (St. Louis, Missouri) and below their sequences are reported:

**MITF-mimicking strand:** 5’-AGACAC GTGAAGG-3’.

**TBP-mimicking strand:** 5’-AGATATA TAAAAGG-3’.

The mimicking strand sequences bind the TF-regulated DNAzyme sequences in a manner that induces the TF-regulated DNAzyme to adopt its catalytically active conformation.

Microphthalmia-associated transcription factor (MITF) was a gift from Prof. Colin Goding’s group at the University of Oxford (UK). It is constituted by residues 198–302 of the human MITF-M (melanocyte specific), which encompasses the DNA-binding domain bHLH-LZ.\(^{48}\)

TATA-binding protein was obtained by expression of recombinant, His-tagged proteins in *Escherichia coli*, as described previously.\(^{61}\)

All experiments with TFs and mimicking strands were conducted with a DNAzyme concentration of 50 nM in a 25 mM HEPES buffer containing 20 mM KCl, 200 mM NaCl and 1% DMSO at pH 7.4. Absorbance at 650 nm was measured 40 min after the addition of the DNAzyme substrates, 3,3’,5,5’-tetramethylbenzidine (TMB) liquid substrate, supersensitive, for ELISA (ready-to-use solution with H₂O₂ purchased from Sigma-Aldrich, St. Louis, Missouri) using either a Bio-Rad Model 550 Microplate Reader or a Tecan Infinite M1000 PRO.

Activation curves were measured in microtiter plates. In every well, a concentration of the TF-regulated DNAzyme of 50 nM was used in a total volume of 150 μl and different concentrations of the proper mimicking strand or protein were added and allowed to react for 1 h at 37 °C. After this, the DNAzyme cofactor hemin was added at a final concentration of 500 nM and incubated for 1 h at 37 °C. Finally, 150 μl of the DNAzyme substrates TMB and H₂O₂ were added and the absorbance was measured at 650 nm after 40 min of incubation. The absorbance of the off-state was set relative to 0 for all normalized figures, while the absorbance obtained in the presence of a saturating target concentration was set relative to 1. The curves were fitted with the following equation:

\[
A_T = A_o + \frac{[T](A_T - A_o)}{[T] + EC_{50}}
\]

where \(A_T\) is the absorbance in the presence of a different concentration of target; \(A_o\) is the background absorbance; \([T]\) is the concentration of the TF or the mimicking strand; \(A_T\) is the absorbance in the presence of a saturating concentration of target; and \(EC_{50}\) is the target concentration at which the activation is half of the maximum activation.

### Acknowledgements

This work was supported by the European Research Council, ERC (project no. 336493) (F.R.), by the Associazione Italiana per la Ricerca sul Cancro, AIRC (project no. 14420) (F.R.) and by the Int. Research Staff Exchange Scheme (IRSES). Prof. Goding and his group at the University of Oxford (UK) are acknowledged for kindly providing the protein MITF.

### Notes and references

1. I. Willner, B. Shlyahovsky, M. Zayats and B. Willner, *Chem. Soc. Rev.*, 2008, 37(6), 1153–1165.
2. R. R. Breaker and G. F. Joyce, *Trends Biotechnol.*, 1994, 12, 268–275.
3. R. R. Breaker and G. F. Joyce, *Chem. Biol.*, 1994, 1(4), 223–229.
4. M. Illangasekare, G. Sanchez, T. Nickles and M. Yarus, *Science*, 1995, 267(5198), 643–647.
5. A. Jenne and M. Famulok, *Chem. Biol.*, 1998, 5(1), 23–34.
6 J. B. Murray, A. A. Seyhan, N. G. Walter, J. M. Burke and W. G. Scott, Chem. Biol., 1998, 5(10), 587–595.
7 M. Illangasekare and M. A. Yarus, RNA, 1999, 5(11), 1482–1489.
8 J. Tang and R. R. Breaker, Proc. Natl. Acad. Sci. U. S. A., 2000, 97(11), 5784–5789.
9 J. Rogers and G. F. Joyce, RNA, 2001, 7, 395–404.
10 J. W. Liu, A. K. Brown, X. L. Meng, D. M. Cropek, J. D. Istok, D. B. Watson and Y. A. Lu, Proc. Natl. Acad. Sci. U. S. A., 2007, 104, 2056–2061.
11 J. Liu and Y. Lu, J. Am. Chem. Soc., 2007, 129, 9838–9839.
12 R. Orbach, F. Remacle, R. D. Levine and I. Willner, Chem. Sci., 2014, 5, 1074–1081.
13 Y. Xiao, V. Pavlov, T. Niazov, A. Dishon, M. Kotler and I. Willner, J. Am. Chem. Soc., 2004, 126, 7430–7431.
14 V. Pavlov, Y. Xiao, R. Gill, A. Dishon, M. Kotler and I. Willner, Anal. Chem., 2004, 76, 2152–2156.
15 T. Li, E. Wang and S. Dong, Chem. Commun., 2008, 3654–3656.
16 R. Orbach, F. Wang, O. Lioubashevski, R. D. Levine, F. Remacle and I. Willner, Chem. Sci., 2014, 5, 3381–3387.
17 X. Liu, R. Freeman, E. Golub and I. Willner, ACS Nano, 2011, 5(9), 7648–7655.
18 D. Balke, C. Wichert, B. Appel and S. Müller, Appl. Microbiol. Biotechnol., 2014, 98(8), 3389–3399.
19 T. A. Lincoln and G. F. Joyce, Science, 2009, 323, 1229–1232.
20 B. J. Lam and G. F. Joyce, J. Am. Chem. Soc., 2011, 133(9), 3191–3197.
21 G. Eckhoff, V. Codrea, A. D. Ellington and X. Chen, J. Syst. Chem., 2010, 1, 13.
22 B. Shlyahovsky, Y. Li, O. Lioubashevski, J. Elbaz and I. Willner, ACS Nano, 2009, 3(7), 1831–1843.
23 A. U. Khan, Clin. Chim. Acta, 2006, 367, 20–27.
24 Y. Isaka, Curr. Opin. Mol. Ther., 2007, 9, 132–136.
25 C. R. Dass, P. F. M. Choong and L. M. Khanchigian, Mol. Cancer Ther., 2008, 7, 243.
26 Y. Qu, L. Zhang, M. Mao, F. Zhao, X. Huang, C. Yang, Y. Xiong and D. Mu, Cancer Gene Ther., 2008, 15, 517–525.
27 J. L. Vinkenborg, N. Karnowski and M. Famulok, Nat. Chem. Biol., 2011, 7(8), 519–527.
28 M. Wieland and H. S. Hartig, Angew. Chem., Int. Ed., 2008, 47, 2604–2607.
29 M. A. Aleman-Garcia, R. Orbach and I. Willner, Chem.—Eur. J., 2014, 20, 5619–5624.
30 B. Klausler, J. Atanasov, L. K. Siewert and J. S. Hartig, ACS Synth. Biol., DOI: 10.1021/sb500626p.
31 P. Ketzer, J. K. Kaufmann, S. Engelhardt, S. Bossow, C. von Kalles, J. S. Hartig, G. Ungerechts and D. M. Nettelbeck, Proc. Natl. Acad. Sci. U. S. A., 2014, 111(5), E554–E562.
32 S. Ausländer, P. Stücheli, C. Rehm, D. Ausländer, J. S. Hartig and M. A. Fussenegger, Nat. Methods, 2014, 11(11), 1154–1160.
33 J. S. Hartig, S. H. Najafi-Shoushtari, I. Grüne, A. Yan, A. D. Ellington and M. Famulok, Nat. Biotechnol., 2002, 20(7), 717–722.
34 D. Y. Wang and D. A. Sen, J. Mol. Biol., 2001, 310, 723–734.
35 D. Y. Wang, B. H. Y. Lai, A. R. Feldman and D. A. Sen, Nucleic Acids Res., 2002, 30, 1735–1742.
36 D. Y. Wang, B. H. Y. Lai and D. A. Sen, J. Mol. Biol., 2002, 318, 33–43.
37 J. Tang and R. R. Breaker, Chem. Biol., 1997, 4(6), 453–459.
38 M. Levy and A. D. Ellington, Chem. Biol., 2002, 9(4), 417–426.
39 W. Chiuaman and Y. Li, PLoS One, 2007, 2(11), e1224.
40 B. J. Lam and G. F. Joyce, Nat. Biotechnol., 2009, 27, 288–292.
41 C. Teller, S. Shimron and I. Willner, Anal. Chem., 2009, 81, 9114–9119.
42 M. Famulok, J. S. Hartig and G. Mayer, Chem. Rev., 2007, 107(9), 3715–3743.
43 P. Travascio, Y. Li and D. A. Sen, Chem. Biol., 1998, 5(9), 505–517.
44 P. Travascio, P. K. Witting, A. G. Mauk and D. A. Sen, J. Am. Chem. Soc., 2001, 123, 1337–1348.
45 A. Vallée-Bélisle, F. Ricci and K. W. Plaxco, Proc. Natl. Acad. Sci. U. S. A., 2009, 106(33), 13802–13807.
46 [a] C. Bertolotto, F. Lesueur, S. Giuliano, T. Strub, M. de Lichy, K. Bille, P. Dessen, B. d’Hayer, H. Mohamdi, et al., Nature, 2011, 480(7375), 94–98; [b] J. Vachetin and J. Borovansky, Exp. Dermatol., 2010, 19, 617–627.
47 C. R. Goding, Pigm. Cell Melanoma Res., 2014, 27, 7–8.
48 I. Aksan and C. R. Goding, Mol. Cell. Biol., 1998, 18(12), 6930–6938.
49 M. Zeker, Nucleic Acids Res., 2003, 31(13), 3406–3415.
50 A. Vallée-Bélisle, A. J. Bonham, N. O. Reich, F. Ricci and K. W. Plaxco, J. Am. Chem. Soc., 2011, 133(35), 13836–13839.
51 [a] N. Hernandez, Genes Dev., 1993, 7, 1291–1308; [b] A. Bareket-Samish, I. Cohen and T. E. Haran, J. Mol. Biol., 2000, 299(4), 965–977.
52 M. Perutz, Mechanisms of Cooperativity and Allosteric Regulation in Proteins, Cambridge University Press, Cambridge, U.K., 1990.
53 G. A. Soukup and R. R. Breaker, Curr. Opin. Struct. Biol., 2000, 10(3), 318–325.
54 G. M. Emilsson and R. R. Breaker, Cell. Mol. Life Sci., 2002, 59(4), 596–607.
55 R. R. Breaker, Curr. Opin. Biotechnol., 2002, 13(1), 31–39.
56 F. Wang, R. Orbach and I. Willner, Chem.—Eur. J., 2012, 18, 16030–16036.
57 M. M. Babu, N. M. Lascombe, L. Aravind, M. Gerstein and S. A. Teichmann, Curr. Opin. Struct. Biol., 2004, 14, 283–291.
58 A. A. Green, P. A. Silver, J. J. Collins and P. Yin, Cell, 2014, 159(4), 925–939.
59 A. S. Khalil, T. K. Lu, C. J. Bashor, C. L. Ramirez, N. C. Pyenson, J. K. Joung and J. J. Collins, Cell, 2012, 150(3), 647–658.
60 A. S. Khalil and J. J. Collins, Nat. Rev. Genet., 2010, 11(5), 367–379.
61 J. J. Stewart and L. A. Stargell, J. Biol. Chem., 2001, 276, 30078–30084.