Validated Zinc Finger Protein Designs for All 16 GNN DNA Triplet Targets

Qiang Liu, ZhenQin Xia, and Casey C. Case‡

From Sangamo BioSciences Inc., Point Richmond Technical Center, Richmond, California 94804

The Cys2-His2-type zinc finger DNA-binding proteins can be engineered to bind specifically to many different DNA sequences. A single zinc finger typically binds to a 3–4-base pair DNA subsite. One strategy for design is to identify highly specific fingers that recognize each of the 64 possible DNA triplets. We started with a subgroup of the 64 triplets, the GNN-binding fingers. The GNN-binding fingers have been examined in several studies, but previous studies did not produce specific fingers for all of the 16 GNN triplets. These previous studies did not provide any information on the possible positional or context effects on the performance of these fingers. To identify the most specific design and take the possible positional effects into consideration, we did a large-scale site selection experiment on our GNN designs. From this study, we identified very specific fingers for 14 of the 16 GNN triplets, demonstrating for the first time a clear positional dependence for many of the de-
each of the 16 GNN triplets. The establishment of a GNN directory will allow us to better understand the interaction between the ZFP and DNA, as well as the positional effects of the finger. The highly specific ZFPs constructed using the GNN directory will enhance the practical application of the zinc finger technology.

EXPERIMENTAL PROCEDURES

Zinc Finger Protein Synthesis and Gel Mobility Shift Assays—ZFPs used in this study were designed based on the DNA binding codes and the zinc finger data base of Sangamo BioSciences Inc. The designed ZFPs were then assembled using the human transcription factor Sp1 (amino acids 532–624) (20) as the backbone. To create the synthetic genes encoding ZFPs, we developed a PCR-based assembly procedure that utilizes six overlapping oligonucleotides (21). The PCR products were cloned directly into the Tac promoter vector pMal-c2 (New England Biolabs) using the KpnI and BamHI restriction sites. The fusion maltose binding protein ZFPs were purified following the manufacturer’s procedures (New England Biolabs). Gel mobility shift assays were performed according to the conditions described (21).

Site Selection Experiment—A complete randomized double strand N14 library was synthesized by annealing SBLIB1B, one of the two primer oligos, SBLIB1F (5'-ATCGGACTCCTGTTCAATA-3') or SBLIB1B (5'-GATCGGATCCTGTTCAATA-3') to the N14 degenerated oligo (5'-ATCGGACTCCTGTTCAATA NNNNNNNNNNN ATTTG-CAATGCTGCTATG-3'). Then the annealed oligos were filled using a standard Klenow (New England Biolabs) filling reaction. End labeling with polynucleotide kinase used the following reaction: the filled-in oligo (5'-32P, 0.5 μCi) was incubated with 10 μl of T4 polynucleotide kinase, and 19 μl of γ-32P, 0.5 μl of T4 polynucleotide kinase, and 19 μl of H2O. After incubating at 37 °C for 30 min, the reaction volume was increased to 50 μl and purified by passing through a Pharmacia ProbeQuant G-50 column. 5 nmol of the labeled degenerate oligo library were incubated with ZFP protein (10× ZFP Kd) following the established gel mobility shift assay protocol (21). After exposure of the dried polyacrylamide gel to an x-ray film, the position of the shifted ZFP-DNA complex was determined. The same incubation and gel running conditions were used to do the site selection experiment, except a cold library at 5 nmol was used with the amount of protein about 4 times that of the ZFP Kd in the first-round site selection. Using the previously developed x-ray film or pre-stained rainbow protein marker, the shifted gel band that contains the bound oligos was cut out and pulverized by centrifuging in an Amicon column at 14,000 rpm for 10 min. DNA was eluted from the gel bits again using 10 μl of Tris-EDTA (1/10 strength) following the instructions of the Amicon Gel Extraction Kit (Millipore). We took 4 μl of the DNA elute and, using SBLIB1F and SBLIB1B primer sets, PCR-amplified the selected oligo pool (25 cycles). The PCR product (5 μl) was used to continue the second round of selections. With each round of selection, we decreased the protein amount by 2-fold. After 3–5 rounds of selection, the PCR-amplified products were cloned into the TOPO TA cloning vector (Invitrogen), and about 24 clones were picked and sequenced. All of the sequencing results were compiled and aligned to reflect the consensus sequence.

Luciferase Reporter Gene Assays—After characterization of the DNA binding specificities of EP2C, this ZFP was used to construct a stable Tet-inducible EP2C ZFP cell line (T-Rex-293T, Invitrogen) as described (21). To generate reporter constructs, three tandem copies of the various EP2C target sites were annealed and inserted in front of the SV40 promoter of the pG53 promoter vector (Promega) between the MluI and BglII sites. All of these reporter constructs were confirmed by DNA sequencing. Luciferase reporter assays were performed by co-transfection of luciferase reporter DNA (200 ng) and pCMV-Δgal (100 ng, used as an internal control) into the stable EP2C expression cells seeded in 6-well plates. The expression of EP2C was induced with doxycycline (0.05 μg/ml) 24 h after the transfection of reporter constructs. Cell lysates were harvested 40 h post-transfection, and the luciferase activities were measured by the Dual-Light luciferase and β-galactosidase reporter assay system (Tropix).

RESULTS

Site Selection Results Reflect Affinity Differences—To test how dependably the site selection results reflect the actual binding affinities of the ZFP to various related targets, we did site selection experiments on two three-finger ZFPs and later quantitative gel mobility shift assays to measure the binding affinities of some of these ZFP-target interactions (Fig. 1). The site selection results showed that most of our designs preferentially selected the intended target sequences, like fingers 1 and 3 of ZFP1 and ZFP2. The second finger of both ZFPs seemed to prefer an alternative sequence instead of the intended sequence (GTT instead of ZFP1 and GGA versus GGT for ZFP2).

To confirm the site selection results, we used gel shift assays to determine the binding affinities of ZFPs to some related individual targets suggested by the site selection results. We designed two mutant oligos for each of the ZFPs: Mt-1 and Mt-2 for ZFP1; Mt-3 and Mt-4 for ZFP2 (Fig. 1). Overall the binding affinities of the ZFPs to the various target sites supported the site selection data. For ZFP1, the selected consensus sequence, GAG GTG GAC, differs from the intended target sequence, GAG TCG GAC, by two bases. The consensus sequence binds 4 times more tightly to ZFP1 than the originally intended target sequence (Kf 12.5 versus 50 in Fig. 1). Besides this TCF finger, other ZFP1 finger designs were very specific for their intended targets. The F1 DRSNLTR was very specific for the GAC triplet. A cytosine to guanine (C — G) change caused the binding affinity to decrease at least 8-fold (Fig. 1). For ZFP2, we tested the degeneracy of the 3' base of the GGT triplet. The Kf values produced by gel shift experiments supported the selection results, indicating that ZFP2 F2 bound the 3' base adenine best and then thymine and cytosine with Kf (nM) 0.5, 1, and 1, respectively, as reflected by the occurrences of A3, T3, and C3 at each target sequence. The dissociation constants (Kf) of ZFP1 and ZFP2 to their cognate and mutant target sequences are given in nanomolar (nM) along each target sequence. The mutant sequences that differ from the cognate target sequences are shown by boldface, underlined letters.
the sixth base position in the site selection experiment (Fig. 1). Taken together, the strong correlation of site selection results with binding affinities of ZFPs to each of the variant target sequences prompted us to use the site selection method to systematically test all of our GNN finger designs.

A Complete GNN Binding Directory Validated by Site Selection Experiments—We expanded our selection efforts to validate all of our designs for GNN triplets and for each finger position. The goal was to establish a directory filled with highly specific zinc fingers for future rapid construction of sequence specific ZFPs. To take the potential positional effects into consideration, we felt it necessary to test each finger design at different positions. We successfully selected consensus sequences from more than 110 three-finger ZFPs with 4–6 rounds of site selection experiments. The selection results of all 16 GNN binders tested at all 3 positions are listed in Fig. 2. For each GNN triplet, we generally had 2–4 designs and tested all of these variant designs by site selection experiments. Many different designs worked equally well for each triplet, but in Fig. 2 we chose only one design for each triplet.

The GNG Finger Designs—Most of our designs for the GNG-type fingers were very specific, and the same or very similar designs worked for all finger positions (Fig. 2). The common helix motif RSDLXLR (position −1 to +6) worked for all four GNG triplets, with Asn−3 for the middle base adenine, His−1 for guanine, Ala+3 for thymine, and Asp+3 for cytosine. For the +5 position, we tested Ala, Thr, Ser, and Gln; they all showed similar specificity profiles, and these residues seemed to be interchangeable. Also, contrary to previous report (19), we found that the two natural GCG fingers RSDDELTR and RSDELKR, from zif268, were not GCG-specific. They selected found that the two natural GCG fingers RSDELTR and RS-/H11001

To improve the specificity of the natural GCG finger, we made change drastically increased the finger’s specificity for the mid-

Fig. 2 we chose only one design for each triplet.

The GNG Finger Designs—Most of our designs for the GNG-type fingers were very specific, and the same or very similar designs worked for all finger positions (Fig. 2). The common helix motif RSDLXLR (position −1 to +6) worked for all four GNG triplets, with Asn−3 for the middle base adenine, His−1 for guanine, Ala+3 for thymine, and Asp+3 for cytosine. For the +5 position, we tested Ala, Thr, Ser, and Gln; they all showed similar specificity profiles, and these residues seemed to be interchangeable. Also, contrary to previous report (19), we found that the two natural GCG fingers RSDDELTR and RSDELKR, from zif268, were not GCG-specific. They selected found that the two natural GCG fingers RSDELTR and RS+/H11001

The Strong Positional Dependence of GCA, GAT, GGT, GAA, and GCC Fingers—We used the code-based design QSGDLTR to specify the GCA triplet at three positions (F1, F2, and F3) and tested specificities of this design at 3 positions. To our surprise, the QSGDLTR worked for GCA triplet at only the F2 and F3 positions but not at the F1 position (Fig. 3A). We had tested this design at the F1 position in three different proteins, and each time it selected GC(T/N). Gln−1 Gly−2 Ser−3 Arg−6 had been selected previously from a randomized F1 library using GCA as the target and had been shown to bind GCA well (8). The variant design, QSGSLTR with an Asp−3 to Ser−3 change, was then used at the F1 position in three different proteins. This Asp−3 to Ser−3 change did significantly enhance the overall F1 specificity, and the QSGSLTR specified all three bases of the GCA triplet at F1 (Fig. 2). To see how the QSGSLTR design behaves at F2 position, we did a side-by-side comparison experiment with two ZFPs harboring these two designs, QSGDLTR and QSGSLTR at F2; F1 and F3 were the same for these two ZFPs. The data showed that when used at F2 position, QSGSLTR specified GTC instead of GCA (data not shown). Thus it seemed that to specify a GCA triplet, the design QSGSLTR can only be used at the F1 position, whereas the QSGDLTR can only be used at F2 and F3 positions (Fig. 2). Ser−3 had been selected previously to bind middle base thymine at the F2 position by phage display (6). This is the first evidence that different fingers are needed to specify the same triplet while at different positions.

Positional effects also existed for the GAT and GTT fingers. The code-based design QSSNLAR for GAT only selected GAT at the F1 position but not at the F2 and F3 positions (Fig. 3B). Instead, QSSNLAR selected GAA at the F2 and F3 positions. When a previously selected finger, TSGNLVR (18), was used at F2 and F3 positions, it did select GAT at both F2 and F3 (Fig. 3B). Similar to the GAT situation, QSSHLTR, a code-based design for GGT, selected GGT at the F1 position but not at F2 and F3; At F2 and F3 positions, it specified GGA instead (Fig. 3C). For GGT at F2 and F3, we used TSGHLVR, another finger selected from the F2 libraries (18). Finger TSGHLVR was shown to be highly specific for GGT at the F2 and F3 positions. However, the TSGHLVR was not specific for the GGT at F1 (Fig. 3C). These results indicate that many fingers are position-dependent, and a finger selected from an F2 library may only work at the F2 or F3 position. There is evidence suggesting that fingers for GAA and GCC triplets were also position-dependent (Fig. 2).

Evaluation of ZFPs by in Vivo Functional Assays—To ask whether there is a correlation between the functionality and the affinity of a ZFP, we used cell-based transient reporter gene assays to analyze the functionality of one of the designed ZFPs. EP2C binds to a target sequence, GCGGTTGCT, with a 2 nM dissociation constant. Site selection results indicated that the overall specificity for this protein is reasonably good, except for the F3 position. This is the first evidence that different fingers are needed to specify the same triplet while at different positions.

Positional effects also existed for the GAT and GTT fingers. The code-based design QSSNLAR for GAT only selected GAT at the F1 position but not at the F2 and F3 positions (Fig. 3B). Instead, QSSNLAR selected GAA at the F2 and F3 positions. When a previously selected finger, TSGNLVR (18), was used at F2 and F3 positions, it did select GAT at both F2 and F3 (Fig. 3B). Similar to the GAT situation, QSSHLTR, a code-based design for GGT, selected GGT at the F1 position but not at F2 and F3; At F2 and F3 positions, it specified GGA instead (Fig. 3C). For GGT at F2 and F3, we used TSGHLVR, another finger selected from the F2 libraries (18). Finger TSGHLVR was shown to be highly specific for GGT at the F2 and F3 positions. However, the TSGHLVR was not specific for the GGT at F1 (Fig. 3C). These results indicate that many fingers are position-dependent, and a finger selected from an F2 library may only work at the F2 or F3 position. There is evidence suggesting that fingers for GAA and GCC triplets were also position-dependent (Fig. 2).

Evaluation of ZFPs by in Vivo Functional Assays—To ask whether there is a correlation between the functionality and the affinity of a ZFP, we used cell-based transient reporter gene assays to analyze the functionality of one of the designed ZFPs. EP2C binds to a target sequence, GCGGTTGCT, with a 2 nM dissociation constant. Site selection results indicated that the overall specificity for this protein is reasonably good, except for the F3 position. This is the first evidence that different fingers are needed to specify the same triplet while at different positions.
Recently several laboratories have stated that there is no universal recognition code for directing zinc finger-DNA binding (11, 22). It has been suggested that the combination of selection and rational design is needed to produce specific fingers (12). The design codes do not address the potential positional dependence of fingers and unanticipated protein-DNA interactions. There are several selection schemes that have been developed to select three-finger ZFPs. The first one is the simple parallel pre-selection of individual zinc fingers and the subsequent stitching together of these selected fingers to produce a three-finger ZFP (12, 18, 23). The second scheme

![Table and Diagram]

**Fig. 2. Summary of the 16 GNN finger designs.** Tested designs are presented for each of the 16 GNN triplets at each position. The α-helix sequence of each finger is presented from the 1 to 6 position, and target triplets are listed in 5’ to 3’ order underneath each finger. The selected consensus sequences are aligned beneath their target sequences.

**DISCUSSION**

Recently several laboratories have stated that the there is no universal recognition code for directing zinc finger-DNA binding (11, 22). It has been suggested that the combination of selection and rational design is needed to produce specific fingers (12). The design codes do not address the potential positional dependence of fingers and unanticipated protein-DNA interactions. There are several selection schemes that have been developed to select three-finger ZFPs. The first one is the simple parallel pre-selection of individual zinc fingers and the subsequent stitching together of these selected fingers to produce a three-finger ZFP (12, 18, 23). The second scheme
ments from a completely randomized oligonucleotide pool, the DNA binding profiles of the ZFPs can be reflected by the occurrence of the selected nucleotides at each base position. Gel shift experiments supported the selection results, showing that the occurrence of nucleotides at each target position represents the binding affinities of these bases to the tested ZFPs (Fig. 1). Depending on how the site selection experiments are performed (number of selection cycles, stringency, etc.), site selection results can include the consensus sequence and many less optimal interactions; for example, ZFP1 selected the ninth base of the target with C11, G2, and A1, and the gel shift confirmed that a C to G change did decrease the binding affinity to ZFP1 by more than 8-fold (Fig. 1, ZFP1).

In contrast to the previous findings, we found that RS-DHLTR is very specific for the GGG triplet, although it does have some cross-recognition to the GAG triplets but to a much lesser degree than the reported data based on enzyme-linked immunosorbent assays (18). We used Ala3 to specify the middle base thymine in the GTG triplet and found that Ala3 was more specific than Ser3 in specifying thymine. Ser3 had difficulties in discriminating the middle thymine from cytosine (12, 18). Our designs for the four GNG triplets stand out as very specific for their targets, as they can specify all three bases of their target DNA (27) can also influence the modularity of a finger. In addition to the interchangeability at the +5 position for many amino acid residues, we noticed that the charged residue, Arg5, could decrease the specificities of the involved fingers.

Positional dependence existed for many of the non-GNG fingers. For example, QSGDLTR could not specify the GCA triplet when presented at the F1 position (Fig. 3A). The Asp3 could still specify the middle C, but it seems to have prevented the Gln1 from making a specific interaction with the 3′ base adenine. To specify GCA at the F1 position, we replaced the Asp5 with a smaller Ser3 residue, as the Ser5 had also been selected to bind base cytosine at F1 previously (8). We surmised that the smaller Ser5 could also specify the middle base cytosine while at the same time not interfering with the Gln1 → Ala interaction at the N-terminal tip of the ZFP. As expected, Ser5 maintained the finger’s specificity to the middle base cytosine, while in the meantime allowing the Gln1 to specify the 3′ base adenine. In three different proteins, the site selection results consistently showed the design QSGSLTR specified GCA triplet at the F1 position. Another potential change we could make was to use Asn4 to replace the Gln1, instead of making the Asp5 to Ser5 change, to try to improve the finger’s specificity for GCA. Asn had been shown to make base-specific contact with the middle base adenine when at the +3 position (6). This is the first time it has been demonstrated that two different fingers are required to specify the same triplet while at different positions. The positional effects demonstrated for GCA, GAT, and GGT fingers. The shaded area indicates that F2: QSGGLTR selected GCT instead of the intended target, GCA. B, positional effects of GAT fingers. The shaded area indicates that F2: QSSNLAR and F3: QSSNLAR selected GAA most often and not the intended target sequence, GAT; and F1: TSGNLVR selected GNN. C, positional effects of GGT fingers. The shaded area indicates that F2: QSSHLTR and F3: QSSHLAR both selected GGA and not the intended target sequence, GGT; and F1: TSGHLVR selected GGN.

is the serial zinc finger selection, selecting one finger at a time (17). The third is the recently published parallel pre-selection of two halves of a three-finger ZFP followed by further selection of the recombined three-finger ZFP (24). Although these selection schemes have generated many good fingers, some of the selections also produced nonspecific fingers, for example, the 16 P2 GNN finger selections. Not all of the 16 GNN targets produced specific fingers. The selection did not produce any specific finger for the GTG and GCG triplets (18). Selection can be slow, typically taking 2 weeks to finish the whole process from pre-made libraries. These factors argue for the building of a directory or archive which then could allow the assembly of ZFPs to any target site.

Alternative methods can be used to measure the specificities of ZFPs, such as binding site signatures (7) and the recently published DNA microarrays (25). To validate and further improve our designs, we chose the site selection method as the way to evaluate our finger designs. By selecting DNA frag-
Although we came out with very specific fingers for most of our GNN triplets, there were still a few triplets we could not specify all three bases. Our designs for the GTT triplet could only specify GTN. Many different designs including the TSGSLTR, TSGALTR, TTSALTR, and QSSALTR were tried in order to improve the specificities for GTT triplet, but all gave similar or worse specificity profiles than those presented in Fig. 2. For these triplets, unconventional designs might be needed, and this might be better accomplished using selection methods.

The in vivo functionality of a ZFP is closely related to its in vitro DNA binding affinities. In vivo, there are a lot of factors that could influence a ZFP function, such as the ZFP concentration, location, and accessibility to its target DNA in chromatin. Here, we demonstrated that the specificity of these ZFPs, their binding affinity to various target sequences, is one of the main factors in deciding the function of a ZFP in vivo. The luciferase activities in Fig. 4B showed that inside cells the EP2C-VP16 activates the cognate 2C0, GCG target as well as the one-base change 2C2, GTG target to a similar level. This was in striking agreement with the gel shift data showing the EP2C bound these two targets with equal affinity. Overall, the luciferase activities closely mirrored the binding affinities of the various target sequences to the ZFP EP2C.

We have identified very specific zinc fingers for 14 of 16 GNN triplets through a design and site selection process. During this process, we found that many GNN fingers were position-de-
dependent, and different fingers were needed to specify the same target triplet at different finger positions. We also found that the existing design code was not applicable to all of the context situations. Many code-based designs could be used only at certain finger positions. Building a zinc finger directory brings us a step closer to solving these complex issues. Our future goal is to expand this finger directory to include specific fingers for the maximum number of all 64 triplets. The cell-based assays demonstrated that the ZFP \textit{in vivo} functionality is highly dependent on its binding affinity to the target sequence. Highly specific ZFPs are naturally expected to function better inside of cells. The highly specific ZFPs will be useful tools in the study of gene functions and will find broad usage in human therapeutics and plant engineering.

Acknowledgments—We thank Hong Zhong for technical assistance, S. Kaye Spratt and Yolanda Santiago for providing the EP2C expression stable cell line, Becky Vanderslice for providing protocols for site selection experiments, and Yen Choo for comments on the manuscript.

REFERENCES

1. Berg, J. M., and Shi, Y. (1996) \textit{Science} 271, 1081–1085
2. Pabo, C. O., Peisach, E., and Grant, R. A. (2001) \textit{Annu. Rev. Biochem.} 70, 313–340
3. Christy, B. A., Lau, L. F., and Nathans, D. (1988) \textit{Proc. Natl. Acad. Sci. U. S. A.} 85, 7657–7661
4. Berg, J. M. (1992) \textit{Proc. Natl. Acad. Sci. U. S. A.} 89, 1109–1111
5. Shi, Y., and Berg, J. M. (1995) \textit{Chem. Biol.} 2, 83–89
6. Choo, Y., and Klug, A. (1994) \textit{Proc. Natl. Acad. Sci. U. S. A.} 91, 11163–11167
7. Choo, Y., and Klug, A. (1994) \textit{Proc. Natl. Acad. Sci. U. S. A.} 91, 11168–11172
8. Rebar, E. J., and Pabo, C. O. (1994) \textit{Science} 263, 671–673
9. Jamieson, A. C., Kim, S. H., and Wells, J. A. (1994) \textit{Biochemistry} 33, 5689–5695
10. Wu, H., Yang, W. P., and Barbas, C. F., III (1995) \textit{Proc. Natl. Acad. Sci. U. S. A.} 92(2), 344–348
11. Wolfe, S. A., Greisman, H. A., Ramm, E. I., and Pabo, C. O. (1999) \textit{J. Mol. Biol.} 285, 1917–1934
12. Dreier, B., Segal, D. J., and Barbas, C. F., III (2000) \textit{J. Mol. Biol.} 303(4), 489–502
13. Elrod-Erickson, M., Roud, M. A., Nekludova, L., and Pabo, C. O. (1996) \textit{Structure} 4, 1171–1180
14. Pavletich, N. P., and Pabo, C. O. (1991) \textit{Science} 252, 809–817
15. Isalan, M., Choo, Y., and Klug, A. (1997) \textit{Proc. Natl. Acad. Sci. U. S. A.} 94, 5617–5621
16. Isalan, M., Klug, A., and Choo, Y. (1998) \textit{Biochemistry} 37, 12026–12033
17. Greisman, H. A., and Pabo, C. O. (1997) \textit{Science} 275, 657–661
18. Segal, D. J., Dreier, B., Beerrl, R. R., and Barbas, C. F., 3rd. (1999) \textit{Proc. Natl. Acad. Sci. U. S. A.} 96(6), 2758–2763
19. Swirnoff, A. H., and Milbrant, J. (1995) \textit{Mol. Cell. Biol.} 15, 2275–2287
20. Kadonaga, J. T., Carner, K. R., Masiarz, F. R., and Tjian, R. (1987) \textit{Cell} 51, 1079–1090
21. Zhang, L., Spratt, S. K., Liu, Q., Johnstone, B., Qi, H., Raschke, E. E., Jamieson, A. C., Rebar, E. J., Wolfe, A. P., and Case, C. C. (2000) \textit{J. Biol. Chem.} 275, 33850–33860
22. Pabo, C. O., and Nekludova, L. (2000) \textit{J. Mol. Biol.} 301, 597–624
23. Choo, Y., Sanchez-Garcia, I., and Klug, A. (1994) \textit{Nature} 372, 642–645
24. Isalan, M., Klug, A., and Choo, Y. (2001) \textit{Nat. Biotechnol.} 19, 656–660
25. Bulyk, M. L., Huang, X., Choo, Y., and Church, G. M. (2001) \textit{Proc. Natl. Acad. Sci. U. S. A.} 98, 7158–7163
26. Desjarlais, J. R., and Berg, J. M. (1993) \textit{Proc. Natl. Acad. Sci. U. S. A.} 90, 2256–2260
27. Fairall, L., Schwabe, J. W., Chapman, L., Finch, J. T., and Rhodes, D. (1993) \textit{Nature} 366, 483–487
