Cell, Vol. 96, 611–614, March 5, 1999, Copyright ©1999 by Cell Press

Generic Signals and Specific Outcomes: Signaling through Ca^{2+}, Calcineurin, and NF-AT

Minireview

Gerald R. Crabtree
Department of Pathology
Department of Development Biology
Stanford University Medical School
Stanford, California 94062

A surprising and yet vexing outcome of the rapid progress made in understanding signal transduction is the observation that while activation of specific receptors leads to highly specific biologic responses, these receptors seem to use ubiquitous signaling intermediates. One solution to this dilemma is that perhaps the specific signaling molecules are yet to be discovered. This notion is consistent with the early analysis of EST collections indicating that the mammalian genome encodes as many as 4,000 intracellular kinases and phosphatases and as many as 10,000 transcription factors (Henikoff et al., 1997). Thus, most mammalian signaling pathways have probably not yet been discovered. Due to the universal nature of Ca^{2+} signaling, the dilemma becomes tightly focused on the second messenger itself. Furthermore, within a specific biologic context calcium signaling is often sufficient for certain responses. Recent reviews have discussed the diverse roles of this ubiquitous signaling intermediate (for example, see Berridge et al., 1998). I will focus on recently discovered mechanisms that are likely to underlie the specificity of signaling through Ca^{2+}/calcineurin and the NF-AT transcription complex and discuss the implications for the development of drugs to modulate responses through this pathway. This signaling pathway (Figure 1) was defined a number of years ago (Flanagan et al., 1991; Liu et al., 1991) and has been recently reviewed (Rao et al., 1997). In this pathway, a rise in intracellular Ca^{2+} triggered by ligand binding to a cell membrane receptor leads to the activation of calcineurin's phosphatase activity, which dephosphorylates cytoplasmic NF-AT (NF-ATc) family members. Dephosphorylated NF-ATc family members enter the nucleus and cooperatively bind to DNA with AP-1, c-MAF, GATA4, and others that were originally named NF-ATn to designate the entire constellation of proteins that could supply this function (Flanagan et al., 1991). By this means, Ca^{2+}/calcineurin signaling is integrated with and made dependent on other signaling pathways such as ras, rac, and PKC (Figure 1).

Tissue-Specific Expression of NF-ATc Family Members: One Determinant of Specificity of Ca^{2+} Signals

Possibly the simplest mechanism for generating specificity is illustrated by recent studies on the role of calcineurin in the development and function of the heart. Heart muscle responds to a heavy workload by hypertrophy, which happens in humans in response to hypertension or after a series of myocardial infarctions. This hypertrophic response can be produced with constitutively active calcineurin or NF-ATc4/NF-AT3 overexpression, which in myocardium activates genes such as b type natriuretic peptide (BNP) (Molkentin et al., 1998). Cyclosporin A and FK506, two chemically different natural products that are highly specific calcineurin inhibitors (Liu et al.,1991) prevent hypertrophy of cultured cardiomyocytes in response to angiotensin II or phenylephrine. Furthermore, in several, but not all, murine models of cardiac hypertrophy induced by pathologic stimuli, the response is blocked by high doses of FK506 (Sussman et al., 1998). The latter observation is both additional evidence that calcineurin is involved in cardiac hypertrophy and a result of some therapeutic importance, since it implies that interference with this signaling pathway could help prevent myocardial hypertrophy in response to pathologic processes. The doses of FK506 required for prevention of hypertrophy are much higher than those required for immunosuppression and produce kidney damage, cautioning that cyclosporin or FK506 by themselves may not be useful to prevent hypertrophy in humans. However, NF-ATc family members have a

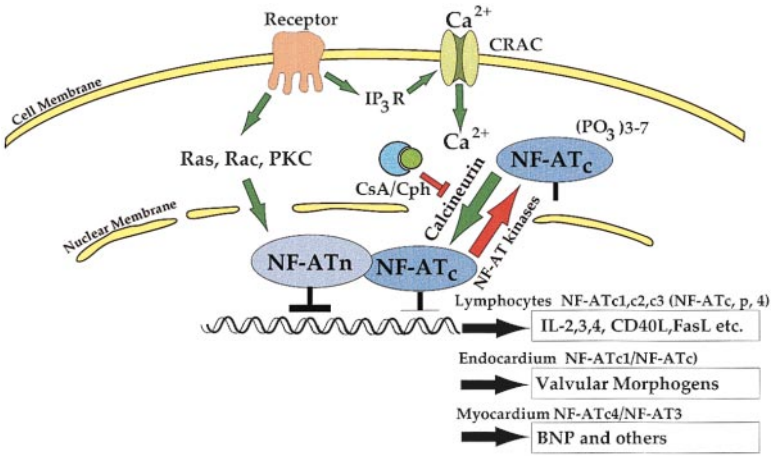


Figure 1. Signal Transduction by Ca^{2+}, Calcineurin, and NF-AT

Signaling pathways depicted are those found in lymphocytes, cardiac valves and cardiomyocytes. In each case the receptors that activate the pathway would be different (for example, the T cell receptor in the case of lymphocytes and the angiotensin receptor in the case of mycocardium). The red arrow indicates inhibition of the signaling pathway. NF-ATn is used to indicate transcription factors, such as AP1 and GATA4, and other yet-to-be-identified transcription factors that act together with nuclear NF-ATc to activate transcription. The term NF-AT kinases is used for the group of kinases that either oppose calcineurin's actions or actually regulate export of the protein. Abbreviations used: CsA, cyclosporin A; CpH, cyclophilin; PKC, protein kinase C. The names used for the cytoplasmic, calcium-dependent, cyclosporin-sensitive subunit (Flanagan et al. 1991) of the NF-ATc family of proteins are taken from the Human Genome Database nomenclature: NF-ATc1 = NF-ATc, NF-ATc2 = NF-ATp = NF-AT1, NF-ATc3 = NF-AT4 = NF-ATx, NF-ATc4 = NF-AT3.

number of different modes of regulation as outlined below and should provide many targets for pharmaceutical development. These considerations aside, the experiments of Molkentin et al. (1998) map out an important signaling pathway in myocardial cells leading from the angiotensin receptor to Ca^{2+} to calcineurin and finally to NF-ATc4/NF-AT3 resulting in an increase in muscle size (Figure 1).

Early in the development of the heart, but only one cell layer away from the myocardium, signaling through Ca^{2+} and calcineurin are used for a very different purpose: the morphogenesis of cardiac valves. NF-ATc1/NF-ATc, another NF-ATc family member and also a calcineurin target, is first expressed at about 9.5 days postconception as the bilateral heart primordia meet and a lumen forms (Ranger et al., 1998). Other NF-ATc family members do not appear to be present in valvular precursor cells. At this stage of development, the cells that line the lumen of the developing heart, but not the myocardial cells, uniformly express NF-ATc1/NF-ATc. However, over the next two days NF-ATc1/NF-ATc expression becomes localized only to sites of valve formation. At these sites NF-ATc1/NF-ATc is located in the nucleus, presumably reflecting calcineurin action (Flanagan et al., 1991), and is consistent with the finding that both cyclosporin A and FK506 block nuclear localization of NF-ATc1/NF-ATc in endocardial cells. In mice lacking NF-ATc1/NF-ATc, heart valves do not form and embryos die of congestive failure at day 11 (Ranger et al., 1998). By these criteria, calcineurin and NF-ATc1/NF-ATc are essential for vertebrate cardiac valve development. In summary, the point for understanding specificity of calcium signaling appears simple: signaling through Ca^{2+} and calcineurin activates one NF-ATc family member to give rise to the hypertrophic response in myocardium and activates another NF-ATc family member in endocardial cells to give rise to the morphogenesis of cardiac valves. However, the selective use of downstream transcriptional targets is not sufficient to account for all the specificity of Ca^{2+}/calcineurin signaling, since only two days after NF-ATc1/NF-ATc is used in heart valve development, the same protein will have a role in lymphocyte function and development. Clearly there must be other ways of discriminating calcium stimuli and permitting NF-ATc1/NF-ATc to assume different roles in different developmental responses. One way this occurs is by using different types of Ca^{2+} signals to regulate different NF-ATc family members.

Discrimination of Ca^{2+} Signals and the Regulated Import and Export of NF-ATc Family Members

Perhaps the most interesting aspect of Ca^{2+} signaling is its capacity to encode information in the frequency, duration, and spatial distribution of Ca^{2+} spikes. Lymphocytes, for example, require a prolonged Ca^{2+} stimulus for activation of immune response genes (Goldsmith and Weiss, 1988) but respond to brief Ca^{2+} stimuli in other ways. Recent work indicates that this prolonged Ca^{2+} stimulus is actually a sustained series of Ca^{2+} spikes rather than a constant high level of Ca^{2+} (reviewed in Berridge et al., 1998). Prolonged Ca^{2+} signaling in lymphocytes requires the function of the Ca^{2+} release–activated Ca^{2+} (CRAC) channel, which opens in response to depletion of intracellular stores through the IP3 receptor. Somatic cell mutants defective for the regulation of the CRAC channel generate brief pulses of Ca^{2+} that are not sustained. In these somatic cell mutants, NF-ATc family members are not maintained in the nucleus and hence are unable to activate NF-AT-dependent transcription of immune response genes (Timmerman et al., 1996). Consistent with these data, when Ca^{2+} signaling is terminated with EDTA, cyclosporin, or FK506 or simply by washing away Ca^{2+} from the media, NF-ATc family members rapidly exit the nucleus and transcription of IL-2 and presumably other immune response genes ceases (Timmerman et al., 1996; Rao et al., 1997). Artificially maintaining NF-ATc1/NF-ATc in the nucleus renders transcription of the endogenous IL-2 gene relatively Ca^{2+}-independent and cyclosporin-resistant (Timmerman et al., 1996). These results demonstrate that NF-AT activity requires the sustained Ca^{2+} stimulus provided by the CRAC channel and that brief Ca^{2+} signals are discriminated from sustained signals by the rapid export of NF-ATc family members from the nucleus. This mechanism prevents brief Ca^{2+} pulses, such as those involved in cell locomotion and adhesion, from activating NF-AT-dependent genes. These experiments underscore the importance of the mechanisms that shuttle NF-ATc family members in and out of the nucleus in distinguishing Ca^{2+} signals.

The control of the nuclear entry and exit of NF-ATc proteins is only partially understood. NF-ATc family members are present in the cytoplasm in a transcriptionally active form (Flanagan et al., 1991) and enter the nucleus when Ca^{2+}-activated calcineurin dephosphorylates critical serines in the N terminus of the protein (reviewed by Rao et al., 1997). Dephosphorylation appears to expose or activate two nuclear localization sequences in NF-ATc1/NF-ATc, either of which is competent for the import of a heterologous cytoplasmic protein (Beals et al., 1997b) (Figure 2). These serines are located within a serine-rich region and in a repeated motif termed the SP repeat (SPXXSPXXSPXXXXD/ED/E), which is also present in a number of other proteins (Figure 2). Exit from the nucleus appears to depend on: (1) an NES, which is competent for exporting heterologous nuclear proteins to the cytoplasm (Klemm et al., 1997); and (2) the same group of serines that are essential for import (Beals et al., 1997a). Phosphorylation of these serines in NF-ATc1/NF-ATc occurs by a two-step mechanism involving one or more priming kinases that are necessary for phosphorylation by GSK3 (Beals et al., 1997b). For another family member, NF-ATc3/NF-AT4, two kinases, JNK (Chow et al., 1997) and the combination of MEKK1 and Casein kinase 1α (Zhu et al., 1998), have been identified that appear to oppose calcineurin's actions. Hence, current observations are consistent with two possible mechanisms for the nuclear export of NF-ATc family members. One possibility is that an export kinase somehow exposes the NES, which has only been identified in NF-ATc1/NF-ATc, resulting in nuclear exit. Alternatively, the NES could be constitutively active and only the activity of the NLS is regulated. The latter possibility seems inconsistent with the observation that NF-ATc proteins are found in the nucleus for several hours after an activating stimulus (Flanagan et al., 1991). Nevertheless, these results predict that each NF-ATc family member might have a distinctive mechanism of nuclear localization, a prediction consistent with recent results in muscle that show selective entry of NF-ATc3/NF-AT4 in response to Ca^{2+} stimuli (Abbott et al., 1998).

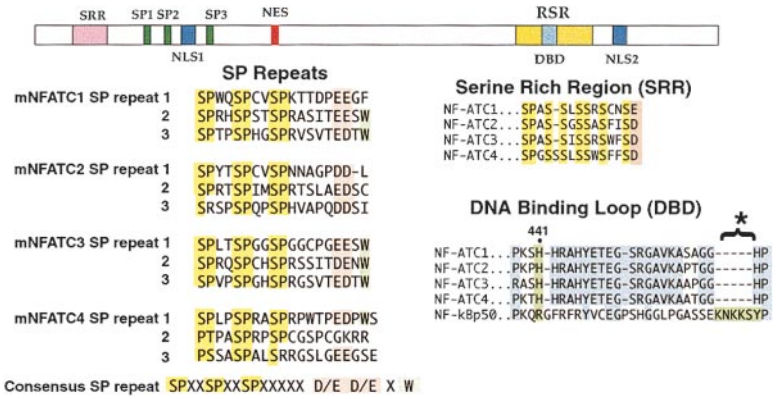


Figure 2. Features of NF-ATc Family Members

The SP repeats and serine-rich regions are shown for four of the known murine NF-ATc family members. The serine-rich region has also been called the A region (Zhu et al. 1998). A calcineurin interaction domain is also present within the N termini of NF-ATc family members (Rao et al., 1997; Zhu et al., 1998). The DNA-binding loop (DBD) within the Rel similarity region (RSR) of the NF-ATc family members is compared with NF-κB p50. Histidine 441 in NF-ATc1/NF-ATc—which when mutated to arginine, as in NF-κB p50, leads to enhanced binding—is shown in green. In addition, a critical DNA-binding loop, which is present in NF-κB p50 but not present in any NF-ATc family members, is also shown in green.

The realization that calcium signaling often depends on information encoded in the frequency, duration and spatial distribution of Ca^{2+} spikes has sparked attempts to artifically reproduce these variations. Two new approaches developed by the Tsien and Lewis laboratories have provided powerful evidence for the importance of frequency of Ca^{2+} pulses as well as ways to produce and measure them (reviewed by Berridge et al., 1998). By releasing pulses of IP3 from a caged precursor or by controlling Ca^{2+} with a rapid perfusion apparatus, both groups showed that NF-AT-dependent transcription had an optimal Ca^{2+} frequency. As might be expected from the early studies described above, brief isolated Ca^{2+} spikes were ineffective, probably due to the rapid, regulated export of NF-ATc family members from the nucleus. These new technical developments will likely be of importance in understanding the functions of signaling through Ca^{2+} and calcineurin in excitable cell types. For example, one recent report has demonstrated that different types of neuron-induced Ca^{2+} signals mediated through calcineurin and NF-ATc proteins lead to the differentiation of slow-twitch versus fast-twitch myofibers (Chin et al., 1998). The ability to artifically induce Ca^{2+} signals of different amplitude, frequency, and duration should allow rigorous investigation of the mechanism underlying the encoding properties of Ca^{2+} signals.

Cellular Context as a Determinant of Specificity: A Lesson from the Structure of NF-AT Complexes

The importance of cellular context in generating specificity from generic signaling pathways comes from recent studies in which one receptor has been removed by mutation and another, sometimes unrelated receptor expressed in its place. Surprisingly, a relatively unrelated receptor can trigger events characteristic of the recipient cell type and not the receptor itself. The first of these experiments was carried out in cell lines in which the T cell receptor (TCR) was replaced with the muscarinic receptor (Goldsmith et al., 1989). Recent versions of this experiment have used erythropoietin-to-prolactin receptor switches or bone morphogenetic protein receptor family member switches in mice. For the specific case of the TCR-to-muscarinic receptor switch, calcium signals generated by either receptor were sufficient to elicit immune response gene activation in lymphocytes (Goldsmith et al., 1989). The reciprocal experiment was not performed, but the anticipated result would be that the TCR and T cell–specific signaling molecules would induce muscarinic receptor responses if expressed in the appropriate cell type. These results indicate that the specificity of generic signals is determined by cellular context, raising the question of what defines cellular context.

Recent work from the Verdine and Harrison laboratories have provided a structural basis by which Ca^{2+}/calcineurin signals propagated by NF-ATc family members might be shaped by cellular context (Chen et al., 1998; Zhou et al., 1998). Studies in the 1980s had shown that a Ca^{2+} signal had to be coincident with a PKC or ras signal to activate NF-AT-dependent transcription. Hence NF-AT-dependent transcription appeared to integrate influences from these two pathways. This ability was later shown to be due to the fact that the cytosolic components of the NF-AT transcription complex are not able to bind most of its recognition sites at physiologic concentrations by themselves, but require a newly synthesized, nuclear subunit that could be supplied by a nuclear fraction from a PKC- or ras-stimulated cell (Flanagan et al., 1991). Evidence indicates that several different proteins can cooperate with NF-ATc family members, including AP1 (Jain et al., 1992), GATA4 (Molkentin et al., 1998), and others. The NF-ATc proteins have a rel-type DNA binding domain lacking sequences in NF-κB p50 (Figure 2) that interact with DNA. In addition, a critical arginine involved in DNA binding by NF-κB, is subsituted with a histidine in NF-ATc family members (Figure 2). Remarkably, mutation of this single residue (H441-to-R in NF-ATc1/NF-ATc) to resemble NF-κB p50 gives nearly a 100-fold increase in DNA binding affinity (Zhou et al., 1998). Although this substitution has not been tested in other family members, the conservation of the DNA-binding loop (Figure 2) strongly indicates that cytoplasmic NF-AT family members have evolved to interact weakly with DNA and to require a partner for high-affinity DNA binding at most sites. Since complexes induced through other signaling pathways such as AP1 or cell type–specific proteins such as MEF, c-Maf, or GATA4 can provide the partner, this means that NF-ATc-dependent transcription will reflect the cellular context in which Ca^{2+} signals are delivered.

One interesting aspect of this result relates to the fact that targeted gene disruptions of different NF-ATc family members have unexpectedly resulted in enhanced expression of early activation genes in lymphocytes (Rao

et al., 1997; Oukka et al., 1998). This finding is surprising because it suggests that NF-ATc may have an inhibitory role in addition to its known ability to activate transcription. However, targeted disruptions of the genes encoding activators such as IL-2, IL-4, JNK, and IL-2 receptor α and β chains have also resulted in hyperproliferation and/or autoimmunity. Hence, hyperproliferation in NF-ATc mutants could reflect roles of NF-ATc family members in earlier developmental events or be the result of compensatory mechanisms brought into play to make up for the loss of an activator. However, a more intriguing possibility, suggested by the structural studies and the work from the Rao and Glimcher laboratories, is that NF-ATc proteins can pair with a negative regulator to produce an inhibitor in certain biologic settings. Such a pleotropic role for NF-ATc family members is also suggested by the recent observation that cyclosporin activates the TGFβ gene in a variety of cell types including malignant cells, a finding that is likely to have important ramifications for the basis of cyclosporin-induced malignancy (Hojo et al., 1999). The activation of TGFβ by a calcineurin inhibitor suggest an important repressive role of signaling through calcineurin and NF-AT in these cells.

Drug Specificity: How Does a Ubiquitous Signaling Intermediate Become a Therapeutic Target?

Why are drugs such as cyclosporin A and FK506 effective and specific immunosuppressants when their target, calcineurin is present in all tissues? A counterintuitive answer to this question arises from the unusual mechanism of action of these drugs (Liu et al., 1991). At therapeutic concentrations, cyclosporin and FK506 bind to their intracellular receptors, cyclophilin and FKBP, respectively, forming inhibitory complexes that bind calcineurin and block its phosphatase activity. Since the levels of calcineurin vary widely between different cell types (Klee, 1998 and references therein) low concentrations of these drugs would be expected to have a far greater effect on cells with lower cytoplasmic concentrations of calcineurin than cells with higher concentrations of calcineurin. In accord with this hypothesis, varying the concentration of either calcineurin, FKBP, or cyclophilin by transfection shifts the dose–response curve to cyclosporin and FK506 in the directions predicted by the laws of mass action. For example, transfection of calcineurin A and B chains renders cells resistant to cyclosporin. Based on published affinities and intracellular concentrations of FKBP, simple calculations predict that 10 nM FK506 would be expected to inhibit about 70% of calcineurin activity in lymphocytes, which have about 5000 molecules of calcineurin per cell, but only 4% of the calcineurin activity of hippocampal cells or cardiac muscle cells, which have about 200,000 molecules of calcineurin per cell (Klee, 1998 and references therein). These considerations are compatible with the finding that higher concentrations of cyclosporin or FK506, often in the range of 1 μM as opposed to 10 nM for lymphocytes, are required for exploring the actions of calcineurin in memory (Winder et al., 1998) and myocardial function (Molkentin et al., 1998; Sussman et al., 1998). Furthermore, in certain cell types the levels of calcineurin may actually exceed the concentration of FKBP or cyclophilin, making them resistant to even the highest drug concentrations.

Although the mechanisms outlined above are only a few of possibly many that impose biologic and pharmacologic specificity on generic calcium signals, they provide concrete examples of ways that generic signals can be channeled into specific responses. Similar mechanisms for achieving specificity are likely to apply to the MAP kinase, JNK, and other signaling pathways that appear to be ubiquitous and also interface with the Ca^{2+}/calcineurin/NF-AT pathway.

Selected Reading

Abbott, K.L., Friday, B.B., Thaloor, D., Murphy, T.J., and Pavlath G.K. (1998). Mol. Biol. Cell *9*, 2905–2916.

Beals, C.R., Clipstone, N.A., Ho, S.N., and Crabtree, G.R. (1997a). Genes Dev. *11*, 824–834.

Beals, C.R., Sheridan, C.M., Turck, C.W., Gardner, P., and Crabtree, G.R. (1997b). Science *275*, 1930–1933.

Berridge, M.J., Bootman, M.D., and Lipp, P. (1998). Nature *395*, 645–648.

Chen, L., Glover, J.N., Hogan, P.G., Rao, A., and Harrison, S.C. (1998). Nature *392*, 42–48.

Chin, E.R., Olson, E.N., Richardson, J.A., Quan, Y., Humphries, C., Shelton, J.M., Wu, H., Zhu, W., Bassel-Duby, R., and Williams, R.S. (1998). Genes Dev. *12*, 2499–2509.

Chow, C.W., Rincon, M., Cavanagh, J., Dickens, M., and Davis, R.J. (1997). Science *278*, 1638–1641.

Flanagan, W.M., Corthesy, B., Bram, R.J., and Crabtree, G.R. (1991). Nature *352*, 803–807.

Goldsmith, M.A., and Weiss, A. (1988). Science *240*, 1029–1031.

Goldsmith, M.A., Desai, D.M., Schultz, T., and Weiss, A. (1989). J. Biol. Chem. *264*, 17190–17197.

Henikoff, S., Greene, E.A., Pietrokovski, S., Bork, P., Attwood, T.K., and Hood, L. (1997). Science *278*, 609–614.

Hojo, M., Morimoto, T., Malucci, M., Asano, T., Morimoto, K., Lagman, M., Shimbo, T., and Suthanthiran, M. (1999). Nature *397*, 530–534.

Jain, J., McCaffrey, P.G., Valge-Archer, V.E., and Rao, A. (1992). Nature *356*, 801–804.

Klee, C.B. (1998) J. Biol. Chem. *273*, 13367–13370.

Klemm, J.D., Beals, C.R., and Crabtree, G.R. (1997). Curr. Biol *7*, 638–644.

Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991). Cell *66*, 807–815.

Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R., and Olson, E.N. (1998). Cell *93*, 215–228.

Oukka, M., Ho, I.C., de la Brousse, F.C., Hoey, T., Grusby, M.J., Glimcher, L.H. (1998). Immunity *3*, 295–304.

Ranger, A.M., Grusby, M.J., Hodge, M.R., Gravallese, E.M., de la Brousse, F.C., Hoey, T., Mickanin, C., Baldwin, H. S., and Glimcher, L.H. (1998). Nature *392*, 186–190.

Rao, A., Luo, C., and Hogan, P.G. (1997). Annu. Rev. Immunol. *15*, 707–747.

Sussman, M.A., Lim, H.W., Gude, N., Taigen, T., Olson, E.N., Robbins. J., Colbert, M.C., Gualberto, A., Wieczorek, D.F., and Molkentin, J.D. (1998). Science *281*, 1690–1693.

Timmerman, L.A., Clipstone, N.A., Ho, S.N., Northrop, J.P., and Crabtree, G.R. (1996). Nature *383*, 837–840.

Winder, D.G., Mansuy, I.M., Osman, M., Moallem, T.M., and Kandel, E.R. (1998). Cell *92*, 25–37.

Zhou, P., Sun, L.J., Dotsch, V., Wagner, G., Verdine, G.L. (1998). Cell *92*, 687–696.

Zhu, J., Shibasaki, F., Price, R., Guillemot, J.C., Yano, T., Dotsch, V., Wagner, G., Ferrara, P., and McKeon, F. (1998). Cell *93*, 851–861.