Crystallization of the Ets1–Runx1–CBF β –DNA complex formed on the TCR α gene enhancer

Masaaki Shiina,^a Keisuke Hamada,^a Taiko Inoue-Bungo,^b Mariko Shimamura,^b Shiho Baba,^b Ko Sato^a and Kazuhiro Ogata^{a*}

^aDepartment of Biochemistry, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan, and ^bDepartment of Biochemistry, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan

Correspondence e-mail:
ogata@med.yokohama-cu.ac.jp

Received 13 June 2014

Accepted 13 August 2014

Gene transcription is regulated in part through the assembly of multiple transcription factors (TFs) on gene enhancers. To enable examination of the mechanism underlying the formation of these complexes and their response to a phosphorylation signal, two kinds of higher-order TF–DNA assemblies were crystallized composed of an unmodified or phosphorylated Ets1 fragment, a Runx1(L94K) fragment and a CBF β fragment on the T-cell receptor (TCR) α gene enhancer. Within these complexes, the Ets1 and Runx1 fragments contain intrinsically disordered regulatory regions as well as their DNA-binding domains. Crystals of the complex containing unmodified Ets1 belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 78.7$, $b = 102.1$, $c = 195.0$ Å, and diffracted X-rays to a resolution of 2.35 Å, and those containing phosphorylated Ets1 belonged to the same space group, with unit-cell parameters $a = 78.6$, $b = 101.7$, $c = 194.7$ Å, and diffracted X-rays to a similar resolution. To facilitate crystallization, a Runx1 residue involved in a hydrophobic patch that was predicted to be engaged in crystal packing based on the previously reported structures of Runx1-containing crystals was mutated.

1. Introduction

Intrinsically disordered (ID) regions of proteins, which lack a defined fold, are commonly found in eukaryotes and are thought to be involved in a wide variety of biological processes (Dyson & Wright, 2005; Mittag *et al.*, 2010). The conformations of ID regions fluctuate in solution, but they assume a stable fold upon binding to their biological targets. In addition, the molecular function of ID regions within proteins is often regulated through post-translational modification. Thus, structural analysis of protein complexes that include ID regions is important for understanding the regulatory mechanisms that govern protein function. Because fluctuating regions within molecules disturb crystallization processes, proteins containing ID regions need to be crystallized as complexes with their interacting partner molecules so as to suppress the fluctuation of the proteins to be crystallized.

Nuclear proteins, including transcription factors (TFs), contain significant amounts of ID regions that are involved in a variety of regulatory functions. These regions also frequently undergo site-specific chemical modification in response to cell signals, resulting in further modulation of the function of the protein. We have been focusing on TF binding to the T-cell antigen receptor (TCR) α gene enhancer because two functionally well characterized TFs with ID regions, v-ets erythroblastosis virus E26 oncogene homologue 1 (Ets1) and runt-related transcription factor 1 (Runx1), cooperatively associate with this enhancer and their ID regions are involved in their functional regulation. In particular, phosphorylation of the ID region in Ets1 by Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) reportedly exerts a repressive effect on Ets1–DNA binding.

Ets1 belongs to the Ets family of TFs. It contains a conserved DNA-binding domain called the Ets domain and is involved in a variety of important biological processes including haematopoiesis, angiogenesis, apoptosis and tumour progression and invasion (Dittmer, 2003). The DNA-binding activity of Ets1 is known to be autoinhibited by a region called the inhibitory module, which flanks the Ets domain (Fig. 1; Lim *et al.*, 1992; Skalicky *et al.*, 1996; Jonsen *et*

et al., 1996; Wasylyk *et al.*, 1992; Hagman & Grosschedl, 1992). Moreover, Ets1–DNA binding can be further inhibited by CaMKII-mediated phosphorylation in the N-terminal ID region flanking the inhibitory module of Ets1 (Fig. 1; Mittag *et al.*, 2010). This reduces Ets1–DNA binding activity 50-fold to 1000-fold by stabilizing the inhibitory module in a folded state (Cowley & Graves, 2000; Lee *et al.*, 2008; Pufall *et al.*, 2005; Rabault & Ghysdael, 1994).

Runx1 belongs to the Runx family of TFs and contains a conserved DNA-binding domain called the Runt domain, which also mediates heterodimerization with a non-DNA-binding partner, core-binding factor subunit β (CBF β ; Ogawa *et al.*, 1993), thereby enhancing Runx1–DNA binding (Tahirov *et al.*, 2001). Runx1 is known to recognize the TCR α/β gene enhancers in cooperation with Ets1 (Wotton *et al.*, 1994; Giese *et al.*, 1995) and to be involved in T-cell development and function. The Runx1 C-terminal ID region flanking the Runt domain has been reported to negatively regulate Runx1–DNA binding (Kim *et al.*, 1999).

It has been proposed that the cooperative DNA binding of Ets1 and Runx1 entails Runx1 counteracting the autoinhibition of Ets1–DNA binding through protein–protein interactions involving their ID regions (Fig. 1; Goetz *et al.*, 2000; Gu *et al.*, 2000; Kim *et al.*, 1999). Recently, Shrivastava and coworkers published crystal structures of the Ets1–Runx1–DNA complex (Shrivastava *et al.*, 2014). They argued that the C-terminal region of the Runt domain interacts with Ets1 *via* a missing flexible loop corresponding to the Runx1 region (178–189). However, this loop appears to be too short to connect the indicated sites within a complex, suggesting that the Ets1-interacting region of Runx1 would be derived from Runx1 of another complex in the crystal rather than from that within the same complex. Also, no functional or structural information is yet available for the effect of Ets1 phosphorylation on the cooperative DNA binding of Ets1 and Runx1. Considering the current situation, further structural studies are required to reveal the exact mechanisms of the cooperativity between Runx1 and Ets1.

Here, we report a crystallization strategy for a higher-order TF–DNA complex composed of Ets1 and Runx1 fragments containing their ID regulatory regions, CBF β and the TCR α gene enhancer. We also crystallized a phosphorylated form of the complex in which the ID region of Ets1 was phosphorylated by CaMKII.

2. Materials and methods

2.1. Macromolecule production

Fragments of mouse wild-type and L94K mutant Runx1 (amino acid residues 60–263) were overexpressed in *Escherichia coli* BL21 (DE3) cells using a T7 expression system. The cells were grown in LB medium containing 200 mg ml^{−1} ampicillin at 310 K until the optical density of the culture at 600 nm (OD₆₀₀) reached 0.8. Protein expression was then induced by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to the culture medium to a final concentration of 1 mM and the cells were further grown with mild shaking at 293 K. Note that cultures subjected to more vigorous shaking yielded smaller amounts of the Runx1 fragment under our experimental conditions. After induction for 12–14 h, the cells were harvested by centrifugation at 5000g for 15 min at 277 K, and the resultant pellet was stored at 193 K. All subsequent purification procedures were performed at 277 K, and all chromatography steps except for the phosphocellulose P11 column (Whatman) step were carried out using an ÄKTAexplorer 10S (GE Healthcare). After each chromatography step, the purity of the preparation was assessed using SDS–PAGE.

Frozen cells were thawed and resuspended in buffer A (50 mM Tris–HCl pH 8.0, 0.5 mM EDTA, 100 mM NaCl) with the addition of 0.125 mM phenylmethanesulfonyl fluoride (PMSF) and 10 mM dithiothreitol (DTT). After lysing the cell suspension using a French press, the lysates were centrifuged at 20 000g for 90 min to remove the cell debris. The supernatant was loaded onto a P11 column pre-equilibrated with buffer A; the column was then washed with buffer A and the Runx1 fragment was eluted in a linear gradient of 100 mM to 2.0 M NaCl in buffer A. After adding DTT to the eluate to a final concentration of 10 mM, the sample was diluted with 5 mM potassium phosphate buffer (KPB) pH 6.5 to reduce the NaCl concentration to 50 mM and was loaded onto a HiTrap CM-Sepharose FF column (GE Healthcare) pre-equilibrated with buffer B (50 mM KPB pH 6.5, 0.5 mM EDTA). The Runx1 fragment was then eluted with a linear gradient of 50–500 mM KPB in buffer B and the Runx1-containing fractions were collected. Buffer C (50 mM Tris–HCl pH 7.4, 0.5 mM EDTA, 3.0 M ammonium sulfate) was then slowly added to the pooled Runx1-containing fractions until the ammonium sulfate concentration reached 1.0 M. The resultant solution was loaded onto

Figure 1

Functional maps of Ets1 and Runx1 with indications of the polypeptide fragments used in crystallization, regions reportedly responsible for cooperative DNA binding between Ets1 and Runx1, and sites of CaMKII-catalyzed phosphorylation in Ets1. PNT, point domain; ETS, Ets domain; Runt, Runt homology domain; TA, transactivation domain.

Table 1
Macromolecule-production information.

	Runx1-related transcription factor 1†	Core-binding factor subunit β	Ets-1
Source organism	<i>Mus musculus</i>	<i>Mus musculus</i>	<i>Homo sapiens</i>
Forward primer	EcoRI–NdeI–GGCGAGCTAGTGCACCGAC	EcoRI–NdeI–CGCGCGTGTGTCGCGG	SalI–NdeI–AGCGTCAGCGTGTTCCTCCTC
Reverse primer	BamHI–stop–GGATCCAGGTACTGGTAGGACTGGTC	BamHI–stop–CTGTTGTGCTAATGCATCTTCC	BamHI–TCACTCGTCGGCATCTGGCTTGAC
Cloning vector	pBluescript KS	pBluescript KS	pBluescript KS
Expression vector	pET-24b	pAR2156	pET-24b
Expression host	<i>E. coli</i> BL21 (DE3)	<i>E. coli</i> BL21 (DE3)	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MGELVVRTDSPNLCVLPVTHWRCNKLPIAFKVVAKGD– GDVDPGLTVTMAGNDENYSAELRNATAAMKNQVAR– FNDLRFVGRSGRGSFTLTITVFTNPPQVATYHRAI– KITVDGPREPRHRKLDQTKPGSLFSERLSELE– QLRRITAMRVSPHAPPTPNPRASLNHSTAFNPQPQS– QMQDARQIQSPSPWSYDQSYQYLGS	MPRVDPQSRKFENEFFRKLRSRECEIKYTGFRDRPHE– ERQTRFQACNRDGRSEIAFVATGNTLSLQFFPASWQ– GEQRQTPSREYVDLEREAGKVYLKAPMILNGVCVW– KGWIDLHRLDGMGCLFDEERAQQEDALAQQQA	SLQRVPSYDSFDSYPAALPNHKKPGTKFDYVRDRAD– LNKDKVPVIAAALAGYTSGSPIQLWQFLELLTDKS– CQSFISWTGDGWEFKLSDPDEVARRWGKRKNPKMN– YEKLSRGLRYYDKNI IHKTAGKRVYRVCDDLQSL– LGYPTEELHMLDVKPDADAE

† Leu94 was mutated to Lys to facilitate crystallization.

a HiTrap Phenyl Sepharose HP column (GE Healthcare) pre-equilibrated with buffer *D* (50 mM Tris–HCl pH 7.4, 0.5 mM EDTA, 1.0 M ammonium sulfate) and the Runx1 fragment was eluted with a linear gradient of 1.0–0 M ammonium sulfate. Immediately after collecting the fractions containing the Runx1 fragment, α₂-macroglobulin was added to the solution to a final concentration of 0.05 mg ml^{−1} to protect the sample from degradation by proteases. The Runx1 fragment was then concentrated using a 5 kDa cutoff Vivaspin 20 (Sartorius) and loaded onto a HiLoad Superdex 75 column (GE Healthcare) pre-equilibrated with buffer *E* (50 mM Tris–HCl pH 8.0, 0.5 mM EDTA, 100 mM NaCl). The fractions containing the Runx1 fragment were collected, pooled and then concentrated with buffer exchange to distilled water containing 10 mM DTT using a 5 kDa cutoff Vivaspin 20 (Sartorius).

A human Ets1 fragment (amino acids 276–441), which contained the Ets domain and N-terminal extension, was overexpressed using the procedure described above for expressing the Runx1 fragment, except that the cells were cultivated for 3 h at 310 K after induction. The Ets1 fragment-expressing cells were harvested, lysed and centrifuged, and the supernatant was applied onto a P11 column and eluted in the same manner as for the case of the Runx1 fragment.

The P11-fractionated Ets1-containing solution with 50 mM NaCl was loaded onto a HiTrap SP FF column (GE Healthcare) pre-equilibrated with buffer *A*, after which the column was washed with buffer *A* and the Ets1 fragment was eluted with a linear gradient of 100 mM to 2.0 M NaCl in buffer *A*. Buffer *C* was then added to the pooled fractions containing the Ets1 fragment until an ammonium sulfate concentration of 1.0 M was reached. The resultant solution was loaded onto a HiTrap Phenyl Sepharose HP column (GE Healthcare) pre-equilibrated with buffer *D* and the Ets1 fragment was eluted with a linear gradient of 1.0–0 M ammonium sulfate. Fractions containing the Ets1 fragment were pooled, concentrated using a 5 kDa cutoff Vivaspin 20 (Sartorius) and loaded onto a HiLoad Superdex 75 column pre-equilibrated with buffer solution consisting of 1.0 M KPB pH 6.5, 0.5 mM EDTA. The fractions containing the Ets1 fragment were collected, pooled and then concentrated with buffer exchange to distilled water containing 200 mM NaCl and 10 mM DTT using a 5 kDa cutoff Vivaspin 20 (Sartorius).

Once purified, some of the Ets1 fragment in a 0.5 mM protein solution was phosphorylated by incubation with 200 nM recombinant CaMKII for 3 h at 303 K in a buffer solution consisting of 50 mM HEPES pH 7.5, 10 mM magnesium acetate, 0.5 mM CaCl₂, 2 mM DTT, 1 mM ATP, 0.01 mM calmodulin. Expression and purification of CaMKII were accomplished as described previously (Brickey *et al.*, 1990). The phosphorylated Ets1 fragment was purified using a Mono S column (GE Healthcare). The fractions containing the phosphorylated Ets1 fragment were collected, pooled and concentrated in the same manner as for the case of unmodified Ets1. The level of phosphorylation of Ets1 was monitored by SDS–PAGE.

Expression and purification of a mouse CBFβ fragment (amino acids 1–141) were accomplished as described previously (Tahirov *et al.*, 2001).

The single-stranded DNA fragments 5′-d(AGAGGATGTGGC–TTC)-3′ and 5′-d(GAAGCCACATCCTCT)-3′ derived from the TCRα enhancer region were purchased (BEX Co.) and purified using reversed-phase HPLC with a Wakopac Navi C18 column (Wako Pure Chemical Industries Ltd). The DNA-containing fractions were pooled, lyophilized and dissolved in 5 mM KPB pH 6.5. Equimolar amounts of the complementary DNA fragments were mixed, heated to 386 K and then gradually cooled to 298 K for annealing. To separate the double-stranded DNA from single-stranded material, the annealed DNA was loaded onto a hydroxyapatite column (Bio-Rad Laboratories) and eluted with a linear gradient of 5–250 mM KPB pH 6.5. Fractions containing the double-stranded DNA were collected, pooled and concentrated with buffer exchange to distilled water using a Centricon Centrifugal Filter Device with Ultracel YM-3 (Millipore). The concentrated sample was lyophilized and stored at

Figure 2
Preparation of protein–DNA complexes. (a, b) 8% nondenaturing PAGE images of complexes composed of unmodified Ets1 (lane 1) or phosphorylated Ets1 (lane 2), Runx1(L94K), CBFβ and the 15 bp TCRα enhancer DNA stained with ethidium bromide (a) or Coomassie Brilliant Blue (b). The single asterisk indicates the free DNA band and the double asterisks indicate the DNA (a) and protein (b) bands in the quaternary complexes.

277 K. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

A solution containing the complex composed of unmodified or phosphorylated Ets1 (amino acids 276–441), Runx1(L94K) (amino acids 60–263), CBF β (amino acids 1–141) and the 15 bp DNA, hereafter referred to as the Ets1–Runx1(L94K)–CBF β –DNA or the pEts1–Runx1(L94K)–CBF β –DNA complex, respectively, was prepared by mixing equimolar amounts of each component in the presence of 10–30 mM DTT. Such a high DTT concentration was used to avoid oxidation of the cysteine residues of Runx1 (Kurokawa *et al.*, 1996). The status of the complex formation was monitored using electrophoretic mobility shift assays (EMSAs; Fig. 2). After adjusting the concentrations of the complexes to 5.0 mg ml^{−1} using distilled water containing 10 mM DTT, they were frozen in liquid nitrogen and stored at 193 K.

Initial screening of crystallization conditions was carried out using Matrix, a crystallization reagent kit for nucleic acids (Hampton Research), and crystals were produced within 3–10 d under several conditions. Crystallization trials were then conducted using the sitting-drop vapour-diffusion method in 24-well plates at 298 K. In all experiments, a 0.5–2.0 μ l drop of a protein–DNA complex solution was mixed with 0.5–2.0 μ l reservoir solution and was equilibrated against 0.5 ml reservoir solution. The best crystals of the

(a)

(b)

Figure 3
Photomicrographs of crystals of complexes composed of Ets1, wild-type Runx1 (a) or Runx1 L94K mutant (b), CBF β and DNA.

Table 2

Data collection and processing.

Values in parentheses are for the outer shell.

	Ets1–Runx1–CBF β –DNA	Phosphorylated Ets1–Runx1–CBF β –DNA
Diffraction source	BL41XU, SPring-8	BL41XU, SPring-8
Wavelength (Å)	1.0	1.0
Temperature (K)	100	100
Detector	ADSC Quantum 315 CCD	ADSC Quantum 315 CCD
Crystal-to-detector distance (mm)	230	230
Rotation range per image (°)	1.0	1.0
Total rotation range (°)	320	180
Exposure time per image (s)	1.0	1.0
Space group	$P2_12_12_1$	$P2_12_12_1$
<i>a</i> , <i>b</i> , <i>c</i> (Å)	78.72, 102.06, 194.99	78.62, 101.72, 194.72
α , β , γ (°)	90, 90, 90	90, 90, 90
Mosaicity (°)	0.40	0.26
Resolution range (Å)	50.00–2.35 (2.43–2.35)	50.00–2.35 (2.43–2.35)
Total No. of reflections	802814	454641
No. of unique reflections	65368	64857
Completeness (%)	99.3 (96.7)	98.8 (94.7)
Multiplicity	12.30 (9.10)	7.00 (5.60)
$\langle I/\sigma(I) \rangle$	28.2 (2.34)	25.0 (2.33)
R_{int}	0.085 (0.585)	0.066 (0.573)
Overall <i>B</i> factor from Wilson plot (Å ²)	72.4	73.0

Ets1–Runx1(L94K)–CBF β –DNA and pEts1–Runx1(L94K)–CBF β –DNA complexes were obtained after 3–10 d in a 1.5 μ l drop consisting of 0.5 μ l of either complex solution and 1.0 μ l reservoir solution: 50 mM Tris–HCl pH 7.5, 100 mM ammonium acetate, 10%(w/v) PEG 4000 for the unmodified complex and 50 mM sodium acetate pH 5.5, 250 mM ammonium acetate and 10%(w/v) PEG 4000 for the phosphorylated complex.

2.3. Data collection and processing

The obtained crystals were first soaked in a reservoir solution to which cryoprotectant solutions were gradually added over a period of 5–10 min. They were then mounted in a nylon-fibre loop and flash-cooled in a stream of cold nitrogen gas (100 K). The cryoprotectant solutions consisted of 50 mM Tris–HCl pH 7.5, 100 mM ammonium acetate, 10%(w/v) PEG 4000, 30% glycerol for crystals of the Ets–Runx1(L94K)–CBF β –DNA complex and 50 mM sodium acetate pH 5.5, 250 mM ammonium acetate, 10%(w/v) PEG 4000, 30%(v/v) glycerol for crystals of the pEts1–Runx1–CBF β –DNA complex. Preliminary X-ray examinations of the crystals were carried out on beamlines NW12A and BL17A at the Photon Factory. The final diffraction data were collected at 100 K using an ADSC Quantum 315 CCD area detector and synchrotron radiation on beamline BL41–XU at SPring-8. The intensity data were indexed, integrated and scaled using the *HKL-2000* software (Otwinowski & Minor, 1997; Table 2).

3. Results and discussion

We obtained approximately 1.0, 20 and 19 mg of the highly purified fragments of Runx1 (amino acids 60–263), Ets1 (amino acids 276–441) and CBF β (amino acids 1–141), respectively, from 1 l of bacterial culture. We noted that the Ets1 fragment (amino acids 276–441) was less soluble in water at a salt concentration lower than about 200 mM NaCl and that the C-terminal extension (amino acids 183–263) of the Runx1 Runt domain was sensitive to proteolysis and tended to be degraded. To prevent degradation, we added α_2 -macroglobulin to the Runx1-containing fractions immediately after the hydrophobic

interaction (Phenyl Sepharose) column chromatography during the purification.

To prepare the phosphorylated Ets1 fragment (276–441), we carried out *in vitro* phosphorylation using recombinant CaMKII. The degree of Ets1 phosphorylation could be monitored by SDS–PAGE, in which the phosphorylated Ets1 fragment was electrophoresed more slowly than the unphosphorylated fragment. We found that the Ets1 fragment was almost completely phosphorylated on SDS–PAGE.

Because the obtained Ets1 and Runx1 fragments bound to the TCR α enhancer DNA fragment with a high degree of cooperativity, we were able to prepare a solution containing predominantly the Ets1–Runx1–CBF β –DNA quaternary complex with almost no other protein–DNA binary complexes (*e.g.* Ets1–DNA or Runx1–CBF β –DNA complexes) (Fig. 2). The fact that we also obtained the quaternary complex containing the phosphorylated Ets1 fragment was unexpected (Fig. 2), because phosphorylation of Ets1 is known to markedly reduce Ets1–DNA binding (Hagman & Grosschedl, 1992; Jonsen *et al.*, 1996; Lim *et al.*, 1992; Skalicky *et al.*, 1996; Wasylyk *et al.*, 1992). This may indicate that the inhibitory effect of Ets1 phosphorylation on DNA binding was countered by Runx1 binding to the TCR α gene enhancer.

In the crystallization trials, we first attempted to crystallize the Ets1–Runx1–CBF β –DNA complex containing a wild-type Runx1 fragment (without the L94K mutation) and obtained stereo-microscopically fine crystals (Fig. 3a). However, these crystals diffracted poorly. To overcome this problem, we examined the structures of the previously described Runx1-containing crystals and mutated Leu94 to a charged amino-acid residue, lysine, because crystal-packing contacts commonly occur between Runx1 molecules at an exposed hydrophobic patch centred around Leu94 (Supplementary Fig. S1; Bravo *et al.*, 2001; Warren *et al.*, 2000). This mutation improved the size, shape and diffraction quality of the crystals. In the obtained crystal structures, we found that the mutated residue, Lys94, is not involved in any molecular interaction, including crystal packing.

In macromolecular X-ray crystallography, crystallization has consistently been a bottleneck in the process, despite the development of various crystallization techniques to facilitate it. Introducing amino-acid mutations onto the protein surface is known to be an effective approach to facilitating crystallization, particularly when the affected residues are located in a crystal-packing area (Mizutani *et al.*, 2008). Surface-entropy reduction (SER), in which surface residues with large flexible side chains (*e.g.* lysine or glutamate) are replaced by residues having smaller side chains (*e.g.* alanine) (Cooper *et al.*, 2007; Derewenda, 2004) has been especially effective in improving crystal quality. In the present study, we attempted to reduce the hydrophobic interactions involved in the crystal-packing contacts by introducing an L94K mutation. This would seem to be the opposite approach to SER. Nonetheless, we suggest that this approach is worth trying when hydrophobic interactions appear to interfere with ordered crystal packing and crystal growth.

We thank Shunsuke Ishii, Akinori Sarai and Akio Yamashita for helpful discussions. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, a Grant-in-Aid for Scientific Research on Innovative Areas ‘Transcription Cycle’ 24118005 and the Special Coordination Funds for Promoting Science and Technology ‘Creation of Innovation Centers for Advanced Interdisciplinary Research Areas’ from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan to KO.

References

- Bravo, J., Li, Z., Speck, N. A. & Warren, A. J. (2001). *Nature Struct. Biol.* **8**, 371–378.
- Brickey, D. A., Colbran, R. J., Fong, Y.-L. & Soderling, T. R. (1990). *Biochem. Biophys. Res. Commun.* **173**, 578–584.
- Cooper, D. R., Boczek, T., Grelewska, K., Pinkowska, M., Sikorska, M., Zawadzki, M. & Derewenda, Z. (2007). *Acta Cryst.* **D63**, 636–645.
- Cowley, D. O. & Graves, B. J. (2000). *Genes Dev.* **14**, 366–376.
- Derewenda, Z. S. (2004). *Structure*, **12**, 529–535.
- Dittmer, J. (2003). *Mol. Cancer*, **2**, 29.
- Dyson, H. J. & Wright, P. E. (2005). *Nature Rev. Mol. Cell Biol.* **6**, 197–208.
- Giese, K., Kingsley, C., Kirshner, J. R. & Grosschedl, R. (1995). *Genes Dev.* **9**, 995–1008.
- Goetz, T. L., Gu, T.-L., Speck, N. A. & Graves, B. J. (2000). *Mol. Cell. Biol.* **20**, 81–90.
- Gu, T.-L., Goetz, T. L., Graves, B. J. & Speck, N. A. (2000). *Mol. Cell. Biol.* **20**, 91–103.
- Hagman, J. & Grosschedl, R. (1992). *Proc. Natl Acad. Sci. USA*, **89**, 8889–8893.
- Jonsen, M. D., Petersen, J. M., Xu, Q.-P. & Graves, B. J. (1996). *Mol. Cell. Biol.* **16**, 2065–2073.
- Kim, W.-Y., Sieweke, M., Ogawa, E., Wee, H.-J., Englmeier, U., Graf, T. & Ito, Y. (1999). *EMBO J.* **18**, 1609–1620.
- Kurokawa, M., Tanaka, T., Tanaka, K., Hirano, N., Ogawa, S., Mitani, K., Yazaki, Y. & Hirai, H. (1996). *J. Biol. Chem.* **271**, 16870–16876.
- Lee, G. M., Pufall, M. A., Meeker, C. A., Kang, H.-S., Graves, B. J. & McIntosh, L. P. (2008). *J. Mol. Biol.* **382**, 1014–1030.
- Lim, F., Kraut, N., Frampton, J. & Graf, T. (1992). *EMBO J.* **11**, 643–652.
- Mittag, T., Kay, L. E. & Forman-Kay, J. D. (2010). *J. Mol. Recognit.* **23**, 105–116.
- Mizutani, H., Saraboji, K., Malathy Sony, S. M., Ponnuswamy, M. N., Kumarevel, T., Krishna Swamy, B. S., Simanshu, D. K., Murthy, M. R. N. & Kunishima, N. (2008). *Acta Cryst.* **D64**, 1020–1033.
- Ogawa, E., Inuzuka, M., Maruyama, M., Satake, M., Naito-Fujimoto, M., Ito, Y. & Shigesada, K. (1993). *Virology*, **194**, 314–331.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Pufall, M. A., Lee, G. M., Nelson, M. L., Kang, H.-S., Velyvis, A., Kay, L. E., McIntosh, L. P. & Graves, B. J. (2005). *Science*, **309**, 142–145.
- Rabault, B. & Ghysdael, J. (1994). *J. Biol. Chem.* **269**, 28143–28151.
- Shrivastava, T., Mino, K., Babayeva, N. D., Baranovskaya, O. I., Rizzino, A. & Tahirou, T. H. (2014). *Leukemia*, doi:10.1038/leu.2014.111.
- Skalicky, J. J., Donaldson, L. W., Petersen, J. M., Graves, B. J. & McIntosh, L. P. (1996). *Protein Sci.* **5**, 296–309.
- Tahirou, T. H., Inoue-Bungo, T., Morii, H., Fujikawa, A., Sasaki, M., Kimura, K., Shiina, M., Sato, K., Kumasaka, T., Yamamoto, M., Ishii, S. & Ogata, K. (2001). *Cell*, **104**, 755–767.
- Warren, A. J., Bravo, J., Williams, R. L. & Rabbitts, T. H. (2000). *EMBO J.* **19**, 3004–3015.
- Wasylyk, C., Kerckaert, J. P. & Wasylyk, B. (1992). *Genes Dev.* **6**, 965–974.
- Wotton, D., Ghysdael, J., Wang, S., Speck, N. A. & Owen, M. J. (1994). *Mol. Cell. Biol.* **14**, 840–850.