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Biochemical analysis of the human ENA/VASP-family proteins, MENA, VASP and EVL, in homologous recombination

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MENA, VASP and EVL are members of the ENA/VASP family of proteins and are involved in cytoplasmic actin remodeling. Previously, we found that EVL directly interacts with RAD51, an essential protein in the homologous recombinational repair of double-strand breaks (DSBs) and stimulates the RAD51-mediated recombination reactions *in vitro*. The EVL-knockdown MCF7 cells exhibited a clear reduction in RAD51-foci formation, suggesting that EVL may function in the DSB repair pathway through RAD51-mediated homologous recombination. However, the DSB repair defects were less significant in the EVL-knockdown cells, implying that two EVL paralogues, MENA and VASP, may complement the EVL function in human cells. Therefore, in the present study, we purified human MENA, VASP and EVL as recombinant proteins, and compared their biochemical activities *in vitro*. We found that all three proteins commonly exhibited the RAD51 binding, DNA binding and DNA-annealing activities. Stimulation of the RAD51-mediated homologous pairing was also observed with all three proteins. In addition, surface plasmon resonance analyses revealed that MENA, VASP and EVL mutually interacted. These results support the ideas that the ENA/VASP-family proteins are functionally redundant in homologous recombination, and that all three may be involved in the DSB repair pathway in humans.

Keywords: DNA repair/ENA/VASP family/homologous recombination/RAD51.

Abbreviations: AMPPNP, beta,gamma-imido-adenosine-5′-triphosphate; ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EVH, Ena/VASP homology; EVL, Ena/VASP-like; MENA, mammalian Enabled; PAGE, polyacrylamide gel electrophoresis; SD, standard deviation; SDS, sodium dodecyl sulfate; VASP, vasodilator-stimulted phosphoprotein.

RAD51 is an essential enzyme for the homologous recombinational repair (HRR) pathway in mitotic cell division and meiotic homologous recombination (*1*–*5*). In mice, the *Rad51*-gene knockout results in early embryonic lethality due to a defect in HRR (*6*, *7*), and disrupting the *Rad51*-gene in chicken DT40 cells causes cell death, accompanying the accumulation of spontaneous chromosome breaks (*8*). These facts indicate that RAD51 is essential for the maintenance of chromosome integrity through the HRR pathway in higher eukaryotes.

RAD51 was first identified as a eukaryotic homologue of bacterial RecA (*9*–*14*). During the homologous recombination processes, RAD51 binds to the ssDNA tail produced at the DNA double-strand break (DSB) sites, and forms a helical nucleoprotein filament. The RAD51-ssDNA filament then binds to the intact double-stranded DNA (dsDNA) to form a ternary complex, containing ssDNA, dsDNA and RAD51. In this ternary complex, RAD51 aligns homologous sequences between ssDNA and dsDNA, and promotes recombination reactions, such as homologous pairing and strand exchange (*15*–*19*).

To efficiently promote homologous pairing and strand exchange, human RAD51 requires auxiliary factors, such as RAD51 paralogues (*20*), BRCA2 (*21*–*24*), RAD52 (*25*), RAD54 (*26*, *27*), RAD54B (*28*), RAD51AP1 (*29*, *30*), PSF (*31*) and GEMIN2 (*32*). In addition, we previously identified human EVL as a RAD51 binding protein (*33*, *34*). Human EVL is a member of the ENA/VASP family, which is involved in actin-remodelling processes (*35*).

The ENA/VASP-family proteins bind actin and regulate the assembly and geometry of F-actin networks (*36*), and have been suggested to play a key role in neuronal migration and axonal outgrowth and guidance. However, the functional analysis of each ENA/VASP-family protein has been precluded, because the three proteins, MENA, VASP and EVL, exhibit similar functions (*37*) and overlapping expression patterns in the neocortex (*38*, *39*). The loss of all ENA/VASP-family proteins reportedly blocks axon fiber tract formation in the cortex, resulting from the failure of cortical neurons to produce neurites (*40*). These facts suggest that the ENA/VASP-family proteins may be required for neuritogenesis in the developing cortex.

We previously reported that EVL directly interacts with RAD51, and stimulates RAD51-mediated recombination reactions *in vitro* (*33*, *34*). The EVL-knockdown MCF7 cells exhibited a clear reduction in RAD51-foci formation (*33*). These results

suggested that EVL may function in DSB repair pathway defects through RAD51-mediated HRR, although the relationship between HRR and neuritogenesis is not well understood. However, the EVL reduction in the knockdown cells did not significantly increase the sensitivity to a DSB-inducing agent, mitomycin C, probably due to presence of two other ENA/VASP-family proteins, MENA and VASP, in humans. The ENA/VASP-family proteins contain three distinct domains, EVH1, Pro-rich and EVH2 (*35*). Our previous study revealed that the EVH2 domain, but not the EVH1 and Pro-rich domains, is responsible for the recombination-related activity of EVL (*34*, *41*). Two other ENA/VASP-family proteins, MENA and VASP, also contain these three domains; however, the significance of MENA and VASP in homologous recombination has not been elucidated.

In the present study, we purified human MENA, VASP and EVL as recombinant proteins, and tested their biochemical activities related to homologous recombination.

Experimental Procedures

Protein preparation

The human *MENA*, *VASP* and *EVL* genes were isolated from a human cDNA pool (Clontech, Mountain View, CA, USA) by polymerase chain reaction, and each was cloned separately into the pET-15b vector (Novagen, Darmstadt, Germany). In this construct, the hexa-histidine (His_6) tag sequence was fused at the N-terminal end of the gene. The MENA, VASP or EVL protein was expressed in the *Escherichia coli* BL21(DE3) strain, which also carried an expression vector for the minor tRNAs [Codon(+)RP, Stratagene, La Jolla, CA, USA]. The cells producing MENA, VASP or EVL were resuspended in 20 mM potassium phosphate buffer (pH 8.5 for EVL, pH 8.0 for VASP, pH 7.5 for MENA), containing 700 mM NaCl, 5 mM 2-mercaptoethanol, 10 mM imidazole (5 mM imidazole for MENA) and 10% glycerol, and were disrupted by sonication. The cell debris was removed by centrifugation for 20 min at 30,000 *g* and the lysate was mixed gently by the batch method with nickel–nitrilotriacetic acid (Ni–NTA) agarose beads (4 ml, Qiagen, Hilden, Germany) at 4°C for 1 h. The VASP or EVL-bound beads were washed with 40 ml of 20 mM potassium phosphate buffer (pH 8.5 for EVL, pH 8.0 for VASP), containing 700 mM NaCl, 5 mM 2-mercaptoethanol, 30 mM imidazole and 10% glycerol. The beads were then washed with 40 ml of 20 mM potassium phosphate buffer (pH 8.5 for EVL, pH 8.0 for VASP), containing 700 mM NaCl, 5 mM 2-mercaptoethanol, 60 mM imidazole and 10% glycerol, and then were washed again with 20 mM potassium phosphate buffer (pH 8.5 for EVL, pH 8.0 for VASP), containing 700 mM NaCl, 5 mM 2-mercaptoethanol, 30 mM imidazole and 10% glycerol. The MENA, VASP or EVL-bound beads were then packed into a column, and were washed with 150 ml of 20 mM potassium phosphate buffer (pH 8.5 for EVL, pH 8.0 for VASP, pH 7.5 for MENA), containing 100 mM NaCl (300 mM NaCl for MENA), 5 mM 2-mercaptoethanol, 30 mM imidazole (5 mM imidazole for MENA) and 10% glycerol. The His_6-tagged MENA, VASP or EVL protein was eluted with a 15-column volume linear gradient of 30–300 mM imidazole (5–300 mM imidazole for MENA), in 20 mM potassium phosphate buffer (pH 8.5 for EVL, pH 8.0 for VASP, pH 7.5 for MENA), containing 100 mM NaCl (300 mM NaCl for MENA), 5 mM 2-mercaptoethanol and 10% glycerol. The fractions containing the His_6-tagged MENA, VASP or EVL protein were diluted with the same volume of 10 mM potassium phosphate buffer (pH 8.5 for EVL, pH 8.0 for VASP, pH 7.5 for MENA), containing 100 mM NaCl, 5 mM 2-mercaptoethanol and 10% glycerol, and were mixed gently by the batch method with hydroxyapatite resin (8 ml, Bio-Rad Laboratories, Hercules, CA, USA) at 4°C for 1 h. The EVL-bound resin was then washed with 80 ml of 20 mM potassium phosphate buffer (pH 8.5 for EVL), 100 mM NaCl, 5 mM 2-mercaptoethanol and 10% glycerol. The MENA, VASP or EVL-bound resin was packed into an Econo-column (Bio-Rad Laboratories). The resin was further washed with 200 ml of 20 mM potassium phosphate buffer (pH 8.5 for EVL, pH 8.0 for VASP, pH 7.5 for MENA), containing 100 mM NaCl, 5 mM 2-mercaptoethanol and 10% glycerol, and then the His_6-tagged MENA, VASP or EVL protein was eluted with a 30-column volume linear gradient of 100–1000 mM NaCl and 10 to 300 mM potassium phosphate (pH 8.5 for EVL, pH 8.0 for VASP, pH 7.5 for MENA). The His_6 tag was uncoupled from the MENA, VASP or EVL protein portion by a digestion with 10 U (6 U for EVL) of thrombin protease (GE Healthcare Biosciences, Uppsala, Sweden) per mg of the His_6-tagged proteins, and then the proteins were immediately dialysed against 20 mM potassium phosphate buffer (pH 8.5 for EVL, pH 8.0 for VASP, pH 7.5 for MENA), containing 200 mM NaCl, 5 mM 2-mercaptoethanol and 10% glycerol, at 4°C. After uncoupling of the His_6 tag, MENA, VASP or EVL was further purified by Superdex 200 gel filtration column (HiLoad 26/60 prep grade, GE Healthcare Biosciences) chromatography, followed by Mono S column chromatography. The peak fractions were diluted with the same volume of 20 mM potassium phosphate buffer (pH 8.0 for EVL and VASP, pH 7.5 for MENA), containing 5 mM 2-mercaptoethanol and 10% glycerol, and were subjected to MonoS (GE Healthcare Biosciences) column chromatography. The column was washed with 20-column volumes of 20 mM potassium phosphate buffer (pH 8.0 for EVL and VASP, pH 7.5 for MENA), containing 100 mM NaCl, 5 mM 2--mercaptoethanol and 10% glycerol, and MENA, VASP or EVL was eluted with a 12-column volume linear gradient of 100–600 mM NaCl. The purified MENA, VASP and EVL proteins were dialysed against 20 mM HEPES–NaOH buffer (pH 7.5), containing 100 mM NaCl, 5 mM 2-mercaptoethanol and 30% glycerol, and were stored at −80°C. The concentrations of the purified MENA, VASP and EVL proteins were determined by the Bradford method (*42*), using bovine serum albumin (BSA) as the standard. The human RAD51 protein was purified by the methods described previously (*43–45*).

DNA substrates

Single-stranded ϕX174 viral (+) strand DNA and double-stranded ϕX174 replicative form I DNA were purchased from New England Biolabs (Ipswich, MA, USA). The linear dsDNA was prepared from the ϕX174 replicative form I DNA by PstI digestion. For the ssDNA annealing assay, the following high-performance liquid chromatography (HPLC)-purified oligonucleotides were purchased from Nihon Gene Research Laboratory: SAT1-50, 5′-ATT CCA ACG TCC ACC GAC CAA CTC TGA GTA ACG TCT TGC TGC TGT GTG TA-3′, and 5′-TAC ACA CAG CAG CAA GAC GTT ACT CAG AGT TGG TCG GTG GAC GTT GGA AT-3′. For the ssDNA substrate used in the D-loop assay, the following HPLC-purified oligonucleotide was purchased: 50-mer, 5′-GGA ATT CGG TAT TCC CAG GCG GTC TCC CAT CCA AGT ACT AAC CGA GCC CT-3′. The 5′-ends of the oligonucleotides were labelled with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-^{32}P]ATP, at 37°C for 30 min. The pB5Sarray DNA contained 11 repeats of a sea urchin 5 S rRNA gene (207-bp fragment) within the pBlueScript II SK(+) vector (*46*).

Surface plasmon resonance analysis

The surface plasmon resonance (SPR) signals were measured with a Biacore X100 instrument (GE Healthcare Biosciences). Flow cells were maintained at 25°C during the measurement, and the instrument was operated at the mid-flow rate (~30 µl/min). RAD51, MENA, VASP or EVL was conjugated to the activated surface of CM5 sensor chips (GE Healthcare Biosciences), using the standard amine coupling conditions recommended by the manufacturer. The reference SPR signals of the flow cell containing a sensor chip without the proteins were subtracted from those of the SPR signals of the flow cell containing the protein-conjugated sensor chips. The running buffer for the RAD51 binding assay contained 20 mM HEPES–NaOH (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 5% glycerol and 0.05% Tween-20. For the analysis of the mutual interaction between MENA, VASP and EVL, the running buffer contained 20 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, 5 mM 2-mercaptoethanol, 5% glycerol and 0.05% Triton X-100. In the RAD51 binding assay, the sensor chip was regenerated by 30 s washes with 1 mM citrate buffer (pH 2.65), followed by

0.5 mM NaOH, and then by a 25 min wash with the running buffer. In the analysis of the mutual interaction between MENA, VASP and EVL, the sensor chip was regenerated by 1 min washes with 3 M Guanidine–HCl, followed by 20 mM citrate buffer (pH 2.65), and then by a 25 min wash with the running buffer. For each binding assay, 0.5 μM analyte solution (*i.e.* MENA, VASP, EVL or BSA solution) was injected for 120 s.

Assays for DNA binding

The ϕX174 circular ssDNA (20 μM) or the ϕX174 linear dsDNA (20 μM) was mixed with MENA, VASP or EVL in 10 μl of a standard reaction solution, containing 36 mM HEPES-NaOH (pH 7.5), 1 mM DTT, 4 mM 2-mercaptoethanol, 80 mM NaCl, 1 mM $MgCl_2$, 24% glycerol and 0.1 mg/ml BSA. The reaction mixtures were incubated at 37°C for 15 min, and were then analysed by 0.8% agarose gel electrophoresis in 1× TAE buffer (40 mM Tris–acetate and 1 mM EDTA) at 3 V/cm for 2 h. In the competitive DNA binding assay, the ϕX174 circular ssDNA (20 μM) and the linear ϕX174 dsDNA (20 μM) were mixed with MENA, VASP or EVL in 10 μl of a standard reaction solution, containing 34 mM HEPES–NaOH (pH 7.5), 70 mM NaCl, 1 mM DTT, 3.5 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 21% glycerol and 0.1 mg/ml BSA. The reaction mixtures were incubated at 37°C for 15 min, and were then analysed by 0.8% agarose gel electrophoresis in 1× TAE buffer at 3 V/cm for 2 h. The bands were visualized by ethidium bromide staining.

ssDNA-annealing assay

The ssDNA-annealing assay was performed as described previously (*47*). Briefly, the SAT1-50 ssDNA oligonucleotide 50-mer (1 μM) was incubated with the indicated amounts of MENA, VASP or EVL at 24°C for 5 min, in 9 μl of reaction buffer, containing 28 mM HEPES-NaOH (pH 7.5), 40 mM NaCl, 2 mM 2-mercaptoethanol, 12% glycerol, 1 mM DTT and 0.1 mg/ml BSA. The reactions were initiated by the addition of 1 μM antisense ^{32}P-labelled 50-mer oligonucleotide, and were continued at 24°C for 2.5 min. After this incubation, the reactions were quenched with an excess of the unlabelled 50-mer oligonucleotide. The DNA substrates and products were deproteinized by a treatment with 0.2% SDS and 1.5 mg/ml proteinase K at 24°C for 15 min. The products were fractionated by 15% PAGE in 0.5× TBE. The gels were dried, exposed to an imaging plate and visualized using an FLA-7000 imaging analyzer (Fujifilm, Tokyo, Japan).

The D-loop formation assay

For the D-loop formation assay, the superhelical dsDNA (pB5Sarray DNA) was prepared by a method to prevent the irreversible denaturation of the dsDNA substrate, by avoiding alkaline treatment of the cells harbouring the plasmid DNA. The cells were gently lysed using sarkosyl, as described previously (*48*). The indicated amount of MENA, VASP or EVL was incubated in the presence of RAD51 (0.1 μM) at 37°C for 5 min, in a reaction buffer containing 26 mM HEPES–NaOH (pH 7.5), 40 mM NaCl, 0.02 mM EDTA, 0.9 mM 2-mercaptoethanol, 5% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 2 mM AMPPNP and 0.1 mg/ml BSA. After this incubation, the ^{32}P-labelled 50-mer oligonucleotide (1 μM) was added, and the samples were further incubated at 37°C for 5 min. The reactions were then initiated by the addition of the pB5Sarray superhelical dsDNA (30 μM) along with 9 mM $MgCl_2$ and were continued at 37°C for 30 min. The reactions were stopped by the addition of 0.2% SDS and 1.5 mg/ml proteinase K and were further incubated at 37°C for 15 min. After adding 6-fold loading dye, the deproteinized reaction products were separated by 1% agarose gel electrophoresis in 1× TAE buffer at 3.6 V/cm for 2 h. The gels were dried, exposed to an imaging plate and visualized using an FLA-7000 imaging analyzer (Fujifilm).

Dynamic light scattering measurements

Dynamic light scattering analyses of MENA, VASP and EVL (0.4 mg/ml) were performed with Zetasizer μV light scattering system (Malvern Instruments), in 20 mM potassium phosphate buffer (pH 7.5), containing 0.1 M NaCl, and 5 mM 2-mercaptoethanol at 25°C.

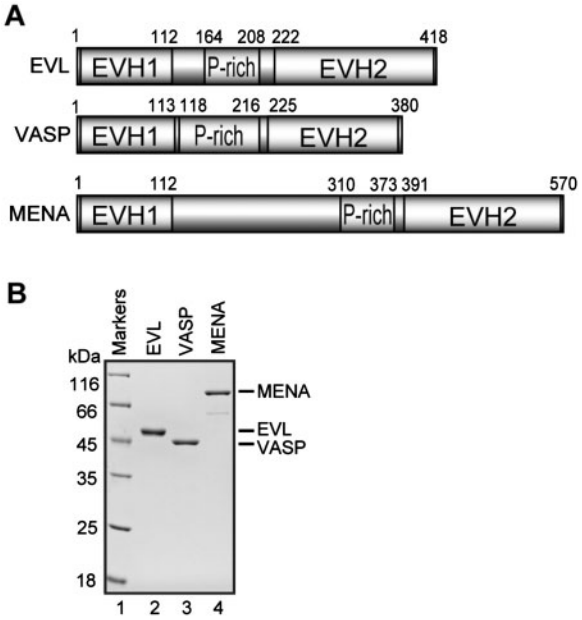

Fig. 1 The human MENA, VASP and EVL proteins. (A) Schematic representation of human MENA, VASP and EVL. Boxes denoted as EVH1, P-rich and EVH2 represent regions corresponding to the EVH1, Pro-rich and EVH2 domains, respectively. (B) Purification of MENA, VASP and EVL. The purified proteins were analysed by 12% SDS–PAGE with Coomassie brilliant blue staining. Lane 1 indicates the molecular mass markers. Lanes 2–4 represent EVL, VASP and MENA, respectively.

Results

RAD51 binding activities of human MENA, VASP and EVL

We purified human MENA, VASP and EVL (Fig. 1A), as bacterially expressed recombinant proteins. In this procedure, we expressed MENA, VASP and EVL as N-terminally hexahistidine (His_6)-tagged proteins, which were purified by Ni–NTA agarose column chromatography and hydroxyapatite column chromatography. The His_6 tags were then removed by thrombin protease treatment, and the proteins were further purified by gel filtration chromatography and Mono S chromatography (Fig. 1B).

We then tested the RAD51 binding activities of MENA, VASP and EVL. To do so, we performed SPR analyses, which were successfully used to detect the EVL-RAD51 interaction previously (*33*). In this study, RAD51 was covalently conjugated to the sensor chip, and the RAD51 binding activities of MENA, VASP and EVL were detected as SPR signals. As shown in Fig. 2A, the SPR signals were observed when MENA, VASP or EVL was injected as an analyte. These results indicated that MENA and VASP, like EVL, directly bind to RAD51. Interestingly, the SPR signal intensity of MENA was quite high, as compared with those of EVL and VASP (Fig. 2A). This suggested that MENA may have higher affinity to RAD51 than EVL and VASP. The higher SPR signal from MENA may also be explained by its larger multimer formation, because SPR signal intensities depend on the apparent molecular mass of the analyte. However, the gel filtration analysis revealed that all three proteins formed homomultimers with similar

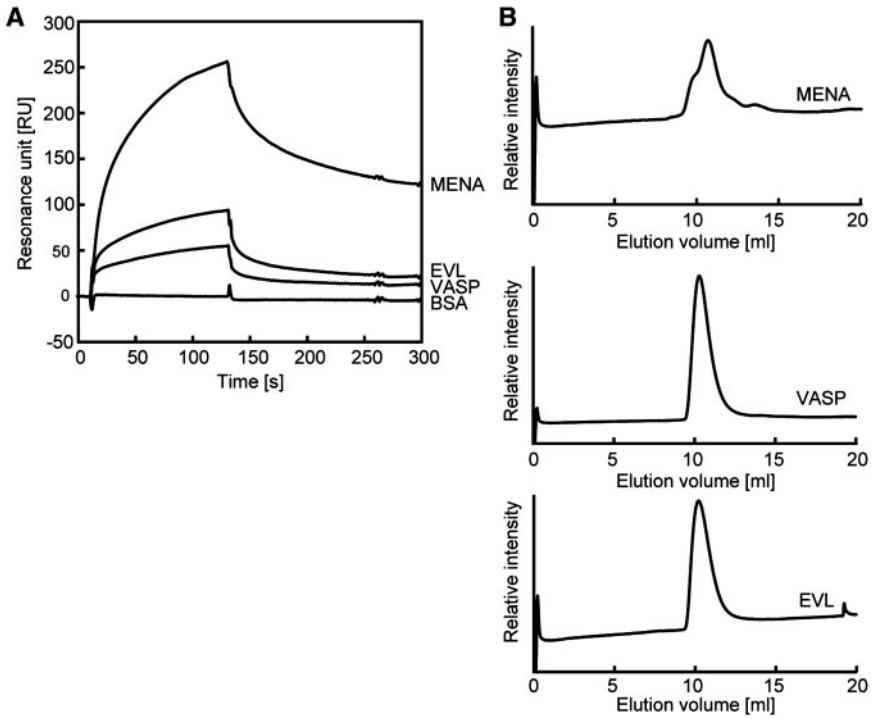

Fig. 2 RAD51 binding activities of MENA, VASP and EVL. (A) SPR analyses of the MENA-RAD51, VASP-RAD51 and EVL-RAD51 interactions. Sensorgrams for MENA, VASP and EVL binding to the immobilized RAD51 protein are presented. The protein concentrations were 0.5 μM. (B) Gel filtration analyses of MENA, VASP and EVL. The protein concentrations were described in the 'Experimental Procedures' section.

apparent molecular masses, of ~490 kDa (MENA), 580 kDa (VASP) and 600 kDa (EVL), which correspond to a 7–8-mer, a 14–15-mer and a 13–14-mer, respectively (Fig. 2B). Therefore, MENA may possess higher RAD51 binding affinity than EVL and VASP.

Dynamic light scattering analyses revealed that the Stokes radii of the MENA, VASP and EVL multimers were 8.83, 8.45 and 8.71 nm, respectively, suggesting that their hydrodynamic diameters are similar. It should be noted that ~20% of MENA formed larger aggregates with a Stokes radius of ~185 nm, as revealed by the dynamic light scattering analysis.

DNA binding activities of MENA, VASP and EVL

We next tested the ssDNA- and dsDNA binding activities of MENA, VASP and EVL. Previously, we found that EVL binds to ssDNA and dsDNA (*33*). As shown in Fig. 3A and B, VASP bound to ssDNA and dsDNA with almost the same affinity as EVL (lanes 1–9). MENA also bound to ssDNA and dsDNA, although its apparent DNA binding activity was weaker than those of EVL and VASP (Fig. 3A and B, lanes 10–13). The competitive DNA binding assay revealed that both VASP and MENA, like EVL, preferred to bind ssDNA over dsDNA (Fig. 3C–E). These results indicated that the ENA/VASP-family proteins possess the DNA binding activity in common, and preferentially bind to ssDNA.

DNA annealing activities of MENA, VASP and EVL

As previously reported, EVL promotes annealing between complementary ssDNA strands (*33*, *41*). Therefore, we performed the ssDNA annealing assay (Fig. 4A). In this assay, a ^{32}P-labelled ssDNA 50-mer and a complementary ssDNA 50-mer were used as substrates, and the dsDNA product was detected by native polyacrylamide gel electrophoresis (Fig. 4A). Consistent with the previous report, EVL exhibited robust annealing activity (Fig. 4B—lanes 2–4 and C). Intriguingly, VASP promoted the annealing reaction with almost the same efficiency as EVL (Fig. 4B—lanes 5–7 and C). On the other hand, MENA also promoted the annealing reaction, but with lower efficiency than those of EVL and VASP. These differences in the annealing activity may reflect their ssDNA binding activities (Fig. 3).

Homologous-pairing stimulation activities of MENA, VASP and EVL

Since EVL is known to stimulate homologous pairing by RAD51 (*33*, *34*), we then tested whether VASP and MENA also possess this activity. To do so, we employed the D-loop formation assay. In this assay, an ssDNA 50-mer and superhelical dsDNA were used as substrates, and D-loops, in which the ssDNA forms new Watson–Crick base pairs with a complementary strand of dsDNA, are formed as a product of homologous pairing by RAD51 (Fig. 5A). As reported previously, EVL stimulated the RAD51-mediated homologous pairing (Fig. 5B—lanes 5–8 and C). Similarly, VASP and MENA also enhanced the RAD51-mediated homologous pairing (Fig. 5B—lanes 9–14 and C). Therefore, VASP and MENA also possess the ability to stimulate RAD51-mediated homologous pairing.

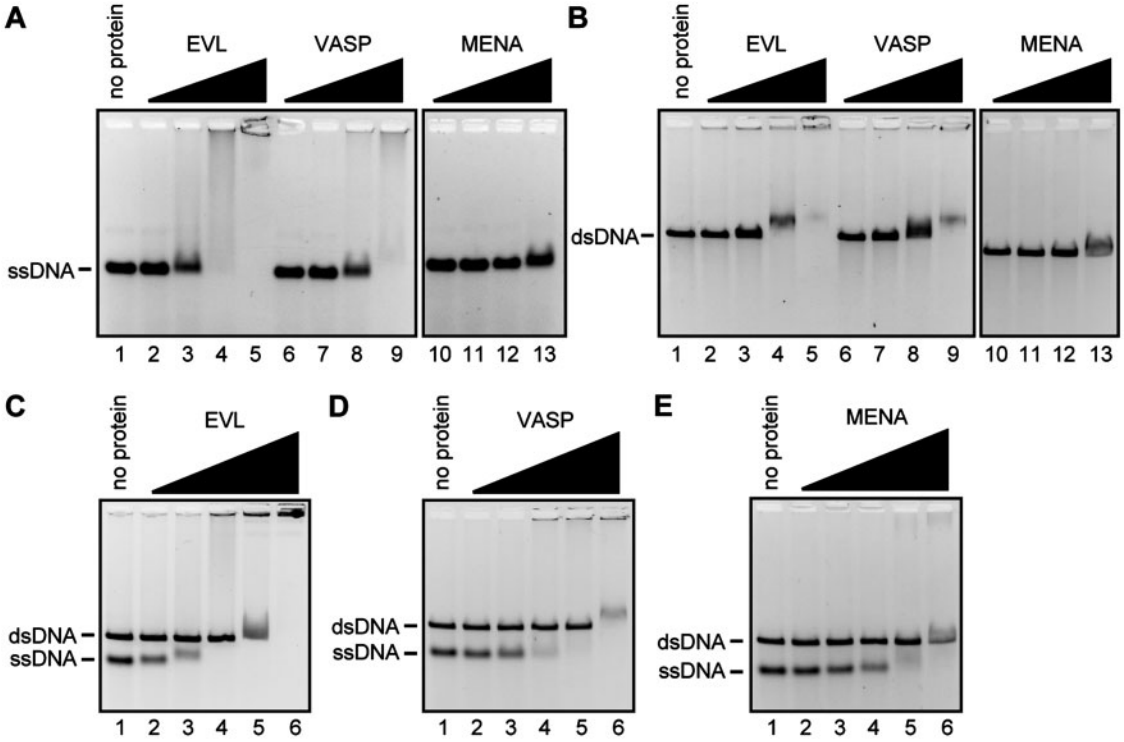

Fig. 3 The ssDNA- and dsDNA binding activities of MENA, VASP and EVL. ϕX174 ssDNA (20 μM) and ϕX174 linear dsDNA (20 μM) were each incubated with MENA, VASP or EVL at 37°C for 15 min. The samples were then separated by 0.8% agarose gel electrophoresis in TAE buffer, and were visualized by ethidium bromide staining. (A) The ssDNA binding assay. (B) The dsDNA binding assay. Lane 1 indicates the negative control experiments without the protein. Lanes 2–5, 6–9 and 10–13 indicate the experiments with EVL, VASP and MENA, respectively. The concentrations of the protein used in the DNA binding experiments were 0.1 μM (lanes 2, 6 and 10), 0.2 μM (lanes 3, 7 and 11), 0.4 μM (lanes 4, 8 and 12) and 0.8 μM (lanes 5, 9 and 13). (C–E) Competitive DNA binding assay. ϕX174 ssDNA (20 μM) and ϕX174 linear dsDNA (20 μM) were used as substrates in this assay. (C) EVL. (D) VASP. (E) MENA. Lane 1 indicates the negative control experiment without the protein. The protein concentrations were 0.1 μM (lane 2), 0.2 μM (lane 3), 0.4 μM (lane 4), 0.8 μM (lane 5) and 1.2 μM (lane 6).

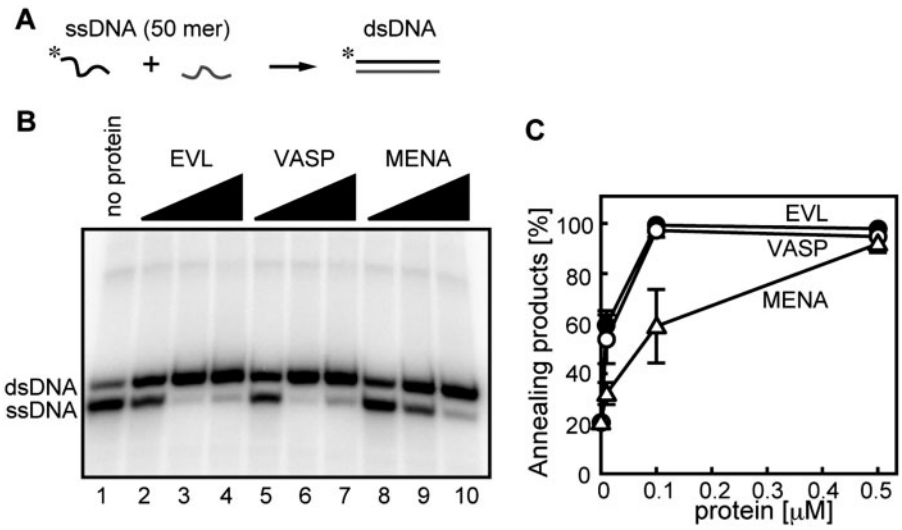

Fig. 4 ssDNA annealing activity of MENA, VASP and EVL. (A) Schematic representation of the ssDNA annealing assay. Asterisks indicate the ^{32}P-labelled end of the DNA. (B) Protein titration. MENA, VASP and EVL were first complexed with 1 μM ssDNA, followed by the addition of the ^{32}P-labelled ssDNA. The reactions were conducted at 24°C for 2.5 min. The samples were then deproteinized, and were separated by 15% PAGE. Lane 1 indicates a control experiment without protein. Lanes 2–4, 5–7 and 8–10 indicate the experiments with EVL, VASP and MENA, respectively. The protein concentrations were 0.01 μM (lanes 2, 5 and 8), 0.1 μM (lanes 3, 6 and 9) and 0.5 μM (lanes, 4, 7 and 10). (C) Graphical representation of the experiments shown in panel A. The band intensities of the annealing products were quantified, and the average values of three independent experiments are shown with the SD values. Closed circles indicate experiments with EVL. Open circles and open triangles indicate experiments with VASP and MENA, respectively.

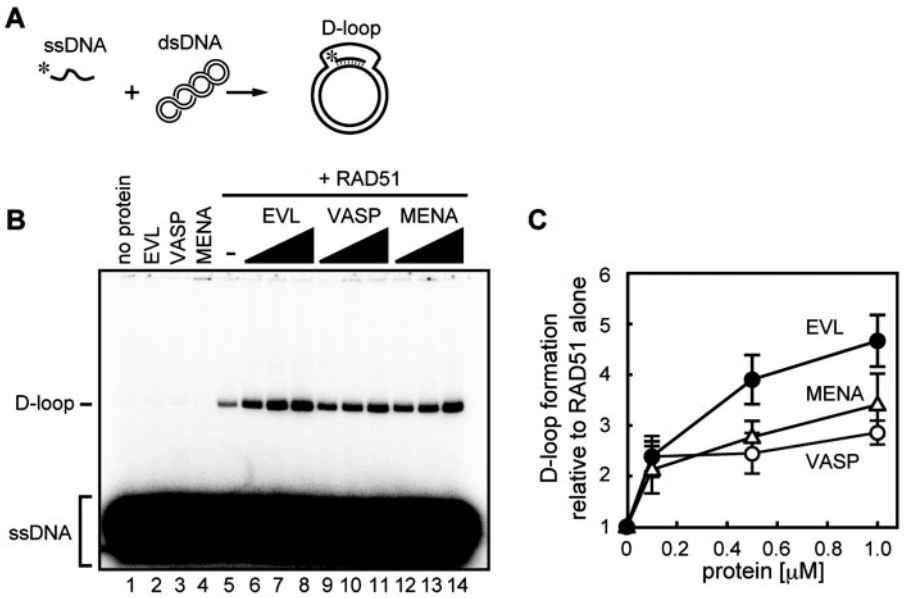

Fig. 5 RAD51-mediated homologous pairing stimulation by MENA, VASP and EVL. (A) A schematic representation of the homologous-pairing (D-loop formation) assay. Asterisks indicate the ^{32}P-labelled end of the 50-mer ssDNA. (B) The D-loop formation assay. Lane 1 indicates a negative control experiment without protein. Lanes 2–4 indicate control experiments without RAD51 in the presence of EVL (1 μM), VASP (1 μM) and MENA (1 μM). Lanes 5–14 indicate experiments with RAD51 (0.1 μM) in the presence of increasing amounts of EVL (lanes 6–8), VASP (lanes 9–11) and MENA (lanes 12–14). The EVL, VASP and MENA concentrations were 0.1 μM (lanes 6, 9 and 12), 0.5 μM (lanes 7, 10 and 13) and 1 μM (lanes 8, 11 and 14). (C) Graphic representation of the experiments shown in panel B. Closed circles indicate experiments with EVL. Open circles and open triangles indicate experiments with VASP and MENA, respectively.

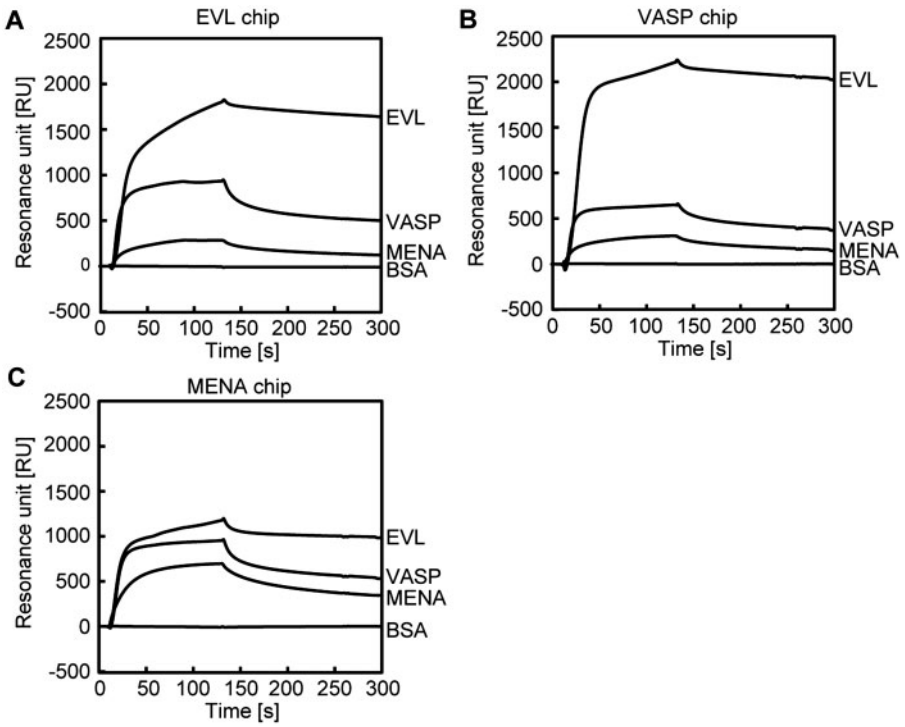

Fig. 6 SPR analyses of the mutual interactions among MENA, VASP and EVL. Sensorgrams for MENA, VASP and EVL binding to the immobilized EVL protein (A), VASP protein (B) and MENA protein (C) are presented. The protein concentrations were 0.5 μM.

Mutual interactions among MENA, VASP and EVL

We tested the mutual interactions among MENA, VASP and EVL by SPR spectroscopy. To do so, we prepared sensor chips conjugated with MENA, VASP or EVL. As shown in Fig. 6, mutual interactions among all three proteins, MENA, VASP and EVL, were detected with the three sensor chips conjugated with MENA, VASP or EVL. In all cases, EVL generated the highest SPR signals followed by VASP and

MENA (Fig. 6). These results suggested that EVL possesses the highest binding affinity to MENA, VASP and EVL itself.

Discussion

The ENA/VASP-family proteins are involved in actin-remodelling processes in the cytoplasm (*35*). We previously reported that an ENA/VASP-family protein, EVL, is a RAD51 binding protein (*33*, *34*), suggesting that the ENA/VASP-family proteins may also have a nuclear function, because RAD51 is an essential protein in the HRR pathway for DSB repair. Actually, purified EVL stimulates RAD51-mediated recombination reactions *in vitro*, and the EVL knockdown reduces the RAD51 accumulation at the DSB sites in MCF7 cells (*33*). These results support the idea that EVL functions in the nucleus as a novel RAD51 co-factor.

The DSB repair defect was clearly observed when the amount of a *bona fide* HRR protein, such as RAD51B, was reduced in human cells (*33*, *49*). RAD51B, one of the RAD51 paralogues in humans, also directly interacts with EVL, as well as RAD51 (*33*). Therefore, the reduction in the amount of EVL may cause a significant HRR defect in human cells, because it may affect both the RAD51 and RAD51B functions. Although the DSB repair defect in the EVL-knockdown MCF7 cells was certainly observed, it was less significant as compared to that of the RAD51B-knockdown cells (*33*). These results imply that two EVL paralogues, MENA and VASP, may complement the EVL function in human cells. Since the EVL-knockdown did not show a clear deficiency in DSB repair *in vivo* (*33*), a MENA or VASP knockdown experiment may not be appropriate to analyse the functional redundancy of the ENA/VASP-family proteins.

Therefore, in the present study, we purified all three ENA/VASP-family proteins, MENA, VASP and EVL, and compared their biochemical activities for homologous recombination *in vitro*. We found that MENA, VASP and EVL share common biochemical activities, such as RAD51 binding, DNA binding, DNA annealing and stimulation of the RAD51-mediated homologous pairing. In addition, we found that MENA, VASP and EVL interacted with each other, suggesting that the ENA/VASP-family proteins may function as complexes. These findings provide important new evidence that the ENA/VASP-family proteins may redundantly function in the homologous recombination process, in addition to actin remodelling, in cells.

Our biochemical analyses suggested that, among the ENA/VASP-family proteins, EVL possesses the highest stimulation activity for RAD51-mediated homologous pairing. Given that the ENA/VASP-family proteins function in the HRR pathway, EVL may be a central player for the stimulation of the RAD51-mediated homologous pairing. Further studies, including genetic and cell biological analyses, will clarify the HRR functions of the ENA/VASP-family proteins.

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Conflict of interest

None declared.

References

1. Symington, L.S. (2002) Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol. Mol. Biol. Rev.* **66**, 630–670
2. West, S.C. (2003) Molecular views of recombination proteins and their control. *Nat. Rev. Mol. Cell. Biol.* **4**, 435–445
3. Sung, P., Krejci, L., Van Komen, S., and Sehorn, M.G. (2003) Rad51 recombinase and recombination mediators. *J. Biol. Chem.* **278**, 42729–42732
4. Neale, M.J. and Keeney, S. (2006) Clarifying the mechanics of DNA strand exchange in meiotic recombination. *Nature* **442**, 153–158
5. San Filippo, J., Sung, P., and Klein, H. (2008) Mechanism of eukaryotic homologous recombination. *Annu. Rev. Biochem.* **77**, 229–257
6. Lim, D.-S. and Hasty, P. (1996) A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53. *Mol. Cell. Biol.* **16**, 7133–7143
7. Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaga, Y., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y., and Morita, T. (1996) Targeted disruption of the Rad51 gene leads to lethality in embryonic mice. *Proc. Natl Acad. Sci. USA* **93**, 6236–6240
8. Sonoda, E., Sasaki, M.S., Buerstedde, J.-M., Bezzubova, O., Shinohara, A., Ogawa, H., Takata, M., Yamaguchi-Iwai, Y., and Takeda, S. (1998) Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. *EMBO J.* **17**, 598–608
9. Aboussekhra, A., Chanet, R., Adjiri, A., and Fabre, F. (1992) Semidominant suppressors of Srs2 helicase mutations of Saccharomyces cerevisiae map in the RAD51 gene, whose sequence predicts a protein with similarities to procaryotic RecA proteins. *Mol. Cell. Biol.* **12**, 3224–3234
10. Basile, G., Aker, M., and Mortimer, R.K. (1992) Nucleotide sequence and transcriptional regulation of the yeast recombinational repair gene RAD51. *Mol. Cell. Biol.* **12**, 3235–3246
11. Shinohara, A., Ogawa, H., and Ogawa, T. (1992) Rad51 protein involved in repair and recombination in S. cerevisiae is a RecA-like protein. *Cell* **69**, 457–470
12. Shinohara, A., Ogawa, H., Matsuda, Y., Ushio, N., Ikeo, K., and Ogawa, T. (1993) Cloning of human, mouse and fission yeast recombination genes homologous to RAD51 and recA. *Nat. Genet.* **4**, 239–243
13. Morita, T., Yoshimura, Y., Yamamoto, A., Murata, K., Mori, M., Yamamoto, H., and Matsushiro, A. (1993) A mouse homolog of the Escherichia coli recA and Saccharomyces cerevisiae RAD51 genes. *Proc. Natl. Acad. Sci. USA* **90**, 6577–6580
14. Yoshimura, Y., Morita, T., Yamamoto, A., and Matsushiro, A. (1993) Cloning and sequence of the

human RecA-like gene cDNA. *Nucleic Acids Res.* **21**, 1665
15. Sung, P. (1994) Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. *Science* **265**, 1241–1243
16. Baumann, P., Benson, F.E., and West, S.C. (1996) Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions in vitro. *Cell* **87**, 757–766
17. Maeshima, K., Morimatsu, K., and Horii, T. (1996) Purification and characterization of XRad51.1 protein, *Xenopus* RAD51 homologue: recombinant XRad51.1 promotes strand exchange reaction. *Genes Cells* **1**, 1057–1068
18. Gupta, R.C., Bazemore, L.R., Golub, E.I., and Radding, C.M. (1997) Activities of human recombination protein Rad51. *Proc. Natl. Acad. Sci. USA* **94**, 463–468
19. Tracy, R.B., Baumohl, J.K., and Kowalczykowski, S.C. (1997) The preference for GT-rich DNA by the yeast Rad51 protein defines a set of universal pairing sequences. *Genes Dev.* **11**, 3423–3431
20. Sigurdsson, S., Van Komen, S., Bussen, W., Schild, D., Albala, J.S., and Sung, P. (2001) Mediator function of the human Rad51B-Rad51C complex in Rad51/RPA-catalyzed DNA strand exchange. *Genes Dev.* **15**, 3308–3318
21. Shivji, M.K., Davies, O.R., Savill, J.M., Bates, D.L., Pellegrini, L., and Venkitaraman, A.R. (2006) A region of human BRCA2 containing multiple BRC repeats promotes RAD51-mediated strand exchange. *Nucleic Acids Res.* **34**, 4000–4011
22. Jensen, R.B., Carreira, A., and Kowalczykowski, S.C. (2010) Purified human BRCA2 stimulates RAD51-mediated recombination. *Nature* **467**, 678–683
23. Liu, J., Doty, T., Gibson, B., and Heyer, W.D. (2010) Human BRCA2 protein promotes RAD51 filament formation on RPA-covered single-stranded DNA. *Nat. Struct. Mol. Biol.* **17**, 1260–1262
24. Thorslund, T., McIlwraith, M.J., Compton, S., Lekomtsev, S., Petronczki, M., Griffith, J.D., and West, S.C. (2010) The breast cancer tumor suppressor BRCA2 promotes the specific targeting of RAD51 to single-stranded DNA. *Nat. Struct. Mol. Biol.* **17**, 1263–1265
25. Benson, F.E., Baumann, P., and West, S.C. (1998) Synergistic actions of Rad51 and Rad52 in recombination and DNA repair. *Nature* **391**, 401–404
26. Sigurdsson, S., Van Komen, S., Petukhova, G., and Sung, P. Homologous DNA pairing by human recombination factors Rad51 and Rad54. *J. Biol. Chem.* **277**, 42790–42794
27. Mazina, O.M. and Mazin, A.V. (2004) Human Rad54 protein stimulates DNA strand exchange activity of hRad51 protein in the presence of Ca^{2+}. *J. Biol. Chem.* **279**, 52042–52051
28. Wesoly, J., Agarwal, S., Sigurdsson, S., Bussen, W., Van Komen, S., Qin, J., Van Steeg, H., Van Benthem, J., Wassenaar, E., Baarends, W.M., Ghazvini, M., Tafel, A.A., Heath, H., Galjart, N., Essers, J., Grootegoed, J.A., Arnheim, N., Bezzubova, O., Buerstedde, J.M., Sung, P., and Kanaar, R. (2006) Differential contributions of mammalian Rad54 paralogs to recombination, DNA damage repair, and meiosis. *Mol. Cell. Biol.* **26**, 976–989
29. Modesti, M., Budzowska, M., Baldeyron, C., Demmers, J.A., Ghirlando, R., and Kanaar, R. (2007) RAD51AP1 is a structure-specific DNA binding protein that stimulates joint molecule formation during RAD51-mediated homologous recombination. *Mol. Cell* **28**, 468–481
30. Wiese, C., Dray, E., Groesser, T., San Filippo, J., Shi, I., Collins, D.W., Tsai, M.S., Williams, G.J., Rydberg, B., Sung, P., and Schild, D. (2007) Promotion of homologous recombination and genomic stability by RAD51AP1 via RAD51 recombinase enhancement. *Mol. Cell* **28**, 482–490
31. Morozumi, Y., Takizawa, Y., Takaku, M., and Kurumizaka, H. (2009) Human PSF binds to RAD51 and modulates its homologous-pairing and strand-exchange activities, *Nucleic Acids Res. 37* 4296–4307
32. Takizawa, Y., Qing, Y., Takaku, M., Ishida, T., Morozumi, Y., Tsujita, T., Kogame, T., Hirota, K., Takahashi, M., Shibata, T., Kurumizaka, H., and Takeda, S. (2010) GEMIN2 promotes accumulation of RAD51 at double-strand breaks in homologous recombination. *Nucleic Acids Res.* **38**, 5059–5074
33. Takaku, M., Machida, S., Hosoya, N., Nakayama, S., Takizawa, Y., Sakane, I., Shibata, T., Miyagawa, K., and Kurumizaka, H. (2009) Recombination activator function of the novel RAD51- and RAD51B-binding protein, human EVL. *J. Biol. Chem.* **284**, 14326–14336
34. Takaku, M., Machida, S., Nakayama, S., Takahashi, D., and Kurumizaka, H. (2009) Biochemical analysis of the human EVL domains in homologous recombination. *FEBS J.* **276**, 5841–5848
35. Kwiatkowski, A.V., Gertler, F.B., and Loureiro, J.J. (2003) Function and regulation of Ena/VASP proteins. *Trends Cell Biol.* **13**, 386–392
36. Krause, M., Dent, E.W., Bear, J.E., Loureiro, J.J., and Gertler, F.B. (2003) Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. *Annu. Rev. Cell Dev. Biol.* **19**, 541–564
37. Loureiro, J.J., Rubinson, D.A., Bear, J.E., Baltus, G.A., Kwiatkowski, A.V., and Gertler, F.B. (2002) Critical roles of phosphorylation and actin binding motifs, but not the central proline-rich region, for Ena/vasodilator-stimulated phosphoprotein (VASP) function during cell migration. *Mol. Biol. Cell* **13**, 2533–2546
38. Lanier, L.M., Gates, M.A., Witke, W., Menzies, A.S., Wehman, A.M., Macklis, J.D., Kwiatkowski, D., Soriano, P., and Gertler, F.B. (1999) Mena is required for neurulation and commissure formation. *Neuron* **22**, 313–325
39. Goh, K.L., Cai, L., Cepko, C.L., and Gertler, F.B. (2002) Ena/VASP proteins regulate cortical neuronal positioning. *Curr. Biol.* **12**, 565–569
40. Kwiatkowski, A.V., Rubinson, D.A., Dent, E.W., van Veen, E., Leslie, J.D., Zhang, J., Mebane, L.M., Philippar, U., Pinheiro, E.M., Burds, A.A., Bronson, R.T., Mori, S., Fässler, R., and Gertler, F.B. (2007) Ena/VASP Is Required for Neuritogenesis in the Developing Cortex. *Neuron* **56**, 441–455
41. Takaku, M., Takahashi, D., Machida, S., Ueno, H., Hosoya, N., Ikawa, S., Miyagawa, K., Shibata, T., and Kurumizaka, H. (2010) Single-stranded DNA catenation mediated by human EVL and a type I topoisomerase. *Nucleic Acids Res.* **38**, 7579–7586
42. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
43. Kurumizaka, H., Aihara, H., Kagawa, W., Shibata, T., and Yokoyama, S. (1999) Human Rad51 amino acid residues required for Rad52 binding. *J. Mol. Biol.* **291**, 537–548
44. Matsuo, Y., Sakane, I., Takizawa, Y., Takahashi, M., and Kurumizaka, H. (2006) Roles of the human Rad51 L1 and L2 loops in DNA binding. *FEBS J.* **273**, 3148–3159

45. Ishida, T., Takizawa, Y., Sakane, I., and Kurumizaka, H. (2008) The Lys313 residue of the human Rad51 protein negatively regulates the strand-exchange activity. *Genes Cells* **13**, 91–103
46. Logie, C. and Peterson, C.L. (1997) Catalytic activity of the yeast SWI/SNF complex on reconstituted nucleosome arrays. *EMBO J.* **16**, 6772–6782
47. Kagawa, W., Kagawa, A., Saito, K., Ikawa, S., Shibata, T., Kurumizaka, H., and Yokoyama, S. (2008) Identification of a second DNA binding site in the human Rad52 protein. *J. Biol. Chem.* **283**, 24264–24273
48. Cunningham, R.P., DasGupta, C., Shibata, T., and Radding, C.M. (1980) Homologous pairing in genetic recombination: recA protein makes joint molecules of gapped circular DNA and closed circular DNA. *Cell* **20**, 223–235
49. Date, O., Katsura, M., Ishida, M., Yoshihara, T., Kinomura, A., Sueda, T., and Miyagawa, K. (2006) Haploinsufficiency of RAD51B causes centrosome fragmentation and aneuploidy in human cells. *Cancer Res.* **66**, 6018–6024