

ERK2* CD domain mutation from a human cancer cell line enhanced anchorage-independent cell growth and abnormality in *Drosophila

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Abstract. In a human cancer cell line, we previously found a mutation in codon 322 of the extracellular signal-regulated kinase (*ERK2*^{E322K}), the protein showed a faster migration when compared to wild-type in SDS-PAGE and constitutive phosphorylation. However, the reason for the faster migration, and the biochemical and biological properties of the mutation is unknown. In this study, we report that the amino acid charge-change mutation in the common docking (CD) domain is important for fast migration. *In vitro* binding of *ERK2*^{E322K} to MKP1 and RSK2 was lost, resulting in constitutive activation and possibly contributing to a more efficient colony formation in soft agar. We established transgenic flies by carrying the corresponding CD domain mutation, *DERK*^{E335K}, which developed smaller and rougher eyes compared with the wild-type. Taken together, these data are consistent with *ERK2*^{E322K} loss of contact with downstream effectors and its constitutive activation, presenting an oncogenic potential and weak abnormality in differentiation.

Introduction

The extracellular signal-regulated kinase (ERK) belongs to the mitogen-activated protein kinase (MAPK) family, which plays important roles in cell growth, differentiation, and

survival (1,2). ERK is activated by extracellular growth factors through RTKs (3). ERK is up-regulated with the upstream dual kinase, MEK (4), which phosphorylates threonine and tyrosine residues of the TEY sequence in ERK (5). In contrast, ERK is down-regulated with ERK-regulated phosphatases with dual-specificity phosphatase activity, keeping ERK activation under control (2). Activated ERK activates RSK in the cytoplasm, enters the nucleus and phosphorylates various transcriptional factors such as ELK-1 (6) through phosphorylation at serine/threonine residues of the target proteins (7).

It has been reported that various signal transductions are mediated through the acidic amino-acid rich common docking (CD) domain. A gain-of-function mutant within this region was first reported in *sevenmaker of Drosophila* (*DERK*^{D334N/sem}) (8), resulting in loss of binding to MKP1 (9,10) and RSK (11).

We previously reported a CD domain mutant, *ERK2*^{E322K}, from a human cancer cell line that was detected as a faster-migrating band in SDS-PAGE (12). Herein, we examined the biochemical and biological properties of the faster-migrating *ERK2*^{E322K} and found that other amino acid charge-change mutations at other sites in the CD domain, including human counterparts of *ERK*^{sem/D321N}, resulted in faster migration. *ERK*^{E322K} induced the anchorage-independent growth of Balb3T3 cells in soft agar and weak abnormality in *Drosophila* eye development.

Materials and methods

Cell lines and culture. HaCaT (13), HEK 293T, HSC6, Ca922, Saos-2 and Balb3T3 cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceuticals, Tokyo, Japan) and supplemented with 10% FBS (JRH Biosciences, Tokyo, Japan) under humidified 5% CO₂/95% air.

Construction of HA-tagged and LTR plasmids. *ERK2*^{wt} or *ERK2*^{E322K}, originally cloned in pGEM®-T easy vector (Promega, Madison, WI), were PCR-amplified using 5'-TTG ATATCTTATGGCGGCGGCG-3' (forward) and 5'-GGCTC GAGTTAAGATCTGTATCCTGGCTG-3' (reverse) primers,

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digested with *EcoRV* and *XhoI* and cloned in-frame in pcDNA3-2HA. Two tandem copies of hemeagglutinin epitope sequences were inserted at *HindIII* and *EcoRI* sites.

To express in mouse cells the *NotI* fragment of ERK2, originally cloned in pGEM-T easy vector, the cells were cloned in a pLTR-SA vector (14) where the *BamHI* site was changed to *NotI* site by blunting the *BamHI* site followed by *NotI* linker ligation. A neomycin derivative (G418) gene was inserted.

Plasmid purification was performed using an endofree DNA purification kit (Qiagen Japan, Tokyo).

Site-directed mutagenesis. *In vitro* mutagenesis of ERK2 was performed as described in the QuickChange® site-directed mutagenesis kit (Stratagene, La Jolla) using the primer sets: D321K/5'-ATTACGACCCGAGTAAAGAGCCCATC-3' (forward) and 5'-GATGGGCTCTTTACTCGGGTCGTAAT-3' (reverse), K321N/5'-CGACCCGAGTAACGAGCCCATCG CCG-3' (forward) and 5'-CGGCGATGGGCTCGTTACTC GGGTCG-3' (reverse), D318K/5'-CTGGAGCAGTATTA CAAACCGAGT-3' (forward) and 5'-ACTCGGTTTGTA TACTGCTCCAG-3' (reverse), E250K/5'-TGGATCCCCA TCACAAAAGACCTGAATTG-3' (forward) and 5'-CAAT TCAGGTCTTTTGTGATGGGGATCCA-3' (reverse), and M13L/5'-GCGGGCCCCGAGCTGGTCCGCGGGCAGG-3' (forward) and 3'-CCTGCCCGCGGACCAGCTCCGGGCC CGC-3' (reverse). The mutations were introduced in HA-ERK2^{wt}. D321N was constructed from D321K. The mutant constructs were confirmed by sequencing (ABI PRISM version 1, ABI Japan, Tokyo).

Treatment with EGF and mammalian cell lysate preparation. Ca922 cells were serum-starved in DMEM for two days, and stimulated with EGF (10 ng/ml, Sigma-Aldrich, MO) for 1 h. Cells were then washed with ice-cold PBS and lysed in NP-40 lysis-buffer as previously described (12). Nuclear extracts were prepared from Saos-2 cells as previously described (15).

Western blotting. Western blotting was performed as previously described (16). Anti-MKP1, anti-ERK2 (D-2), anti-ERK1 (C-16) and anti-RSK2 (C-19) (Santa Cruz, CA), as well as anti-HA (Boehringer Mannheim, Germany) and the HRP conjugates (New England Biolabs) were used.

GST-tagged vector, bacterial growth and lysate preparation, and pulldown assay. GST-ERK2^{wt} and GST-ERK2^{E322K} were constructed (17), and used to transform *E. coli* BL21 (DE3), which was induced and lysed as previously described (16). GST-ERK2^{wt}, GST-ERK2^{E322K} and GST-vector containing bacterial lysates were used for the pulldown assay (18) of MKP1 and RSK2 proteins in Ca922 total cell and Saos-2 nuclear lysates.

Colony formation assay in soft agar. Balb3T3 cells were lipofected (LF2000, Invitrogen) by using the LTR-ERK2^{wt} or -ERK2^{E322K} or -vector alone according to the manufacturer's directions, and grown with 400 µg/ml G418. For clones stably expressing the wild-type or mutant proteins, the cells were left with G418 for three weeks. The cell colonies were isolated by using cloning cylinders and grown separately with

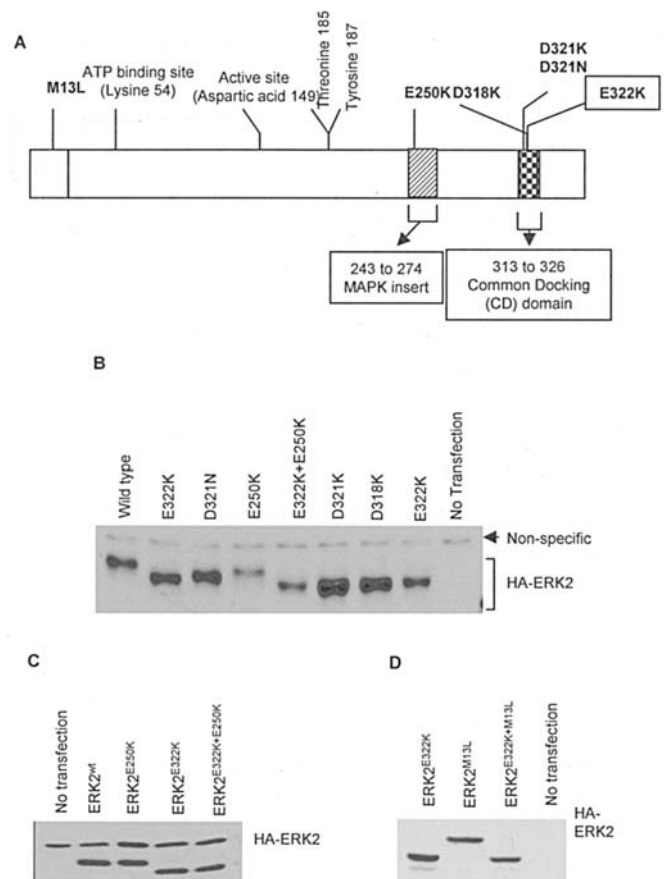


Figure 1. (A) Schematic illustration of the functional domains (sites) in human ERK2, in which the mutants constructed are shown in bold letters. (B) Various mutants in the CD domain of ERK2 proteins with amino acid charge-change migrated faster in SDS-PAGE. 293T cells were transiently transfected with one of the HA-tagged various mutant genes or with HA-ERK2^{wt}. Cell lysates (equal amount of proteins) were electrophoresed in 12% SDS-PAGE, and then Western-blotted using anti-HA antibody. (C) Migration of the ERK2^{E322K+E250K} double mutant in SDS-PAGE. 293T cells were transfected with HA-tagged ERK2^{wt}, ERK2^{E322K}, ERK2^{E250K} and the double mutant ERK2^{E322K+E250K} DNA and Western-blotted using anti-HA antibody. (D) Migration of ERK2^{M13L} in SDS-PAGE. 293T cells were transfected with HA-tagged ERK2^{M13L}, ERK2^{E322K} and ERK2^{M13L+E322K} DNA and Western-blotted using anti-HA antibody.

fresh complete medium. The clones positively expressing ERK2^{wt} were identified by using anti-ERK1 (C-16) antibody, which predominantly recognizes human ERK1 and to a lesser extent human ERK2. Mutant ERK2^{E322K} was identified by its faster-migrating property. For cells transiently expressing wild-type or mutant ERKs or just the vector, cells were grown for 2 days in the presence of G418 and used for the soft agar assay. A bottom agar in DMEM with 0.5% agarose and top agar with 3000 cells and 400 µg/ml G418 in DMEM and 0.33% agarose were made, incubated in 5% CO₂ and monitored for colony formation as previously described (17), with a few modifications.

Preparation of *Drosophila* cDNA. Total RNA was isolated using a TRIzol® reagent (Invitrogen) and cDNA was prepared with an AgPath-ID™ one-step RT-PCR kit (ABI) as described in the respective kits.

SPANDIDOS¹ERK^{E335K} plasmid construction. pUAST (19) is a *Drosophila* t vector (pCaSpeR3) containing the P3' and P5' ends of P element and the white gene which acts as a selection marker for successful integration into the *Drosophila* genome. To construct the pUAST-DERK^{wt} and pUAST-DERK^{E335K} plasmids, *Drosophila* ERK cDNA was amplified by using 5'-TAGCGGCCGCATGGAGGAATTTAATTTCG-3' (forward, with *NotI* site) and 5'-AACTCGAGTTAAGGCGCATTGTC TGG-3' (reverse, with *XhoI* site) primers, and amplicon was inserted into pGEM-T easy vector. The clones were PCR-amplified using the above-mentioned forward and 5'-AAGT CGACTTAAGGCGCATTGTCTGG-3' (reverse, with *SalI* site) primers and cloned into the pUAST vector. Then, *in vitro* mutagenesis was performed using 5'-GATCCTGGAGATAA GCCTGTCGCTGAAG-3' (forward) and 5'-CTTCAGCGA CAGGCTTATCTCCAGGATC-3' (reverse) primers and the mutagenized sequence was confirmed by sequencing.

Establishment of transgenic flies. The plasmids pUAST-DERK^{wt} or -DERK^{E335K} or the empty vector were injected in fertilized *Drosophila* eggs and the P-element-mediated germ line transformation was carried out as described earlier (20). F1 transformants were selected on the basis of white eye-color rescue (21). The established transgenic flies were crossed with the transgenic fly line (number 16) carrying GMR-GAL4 (22).

Scanning electron microscopy. Adult transgenic flies were anesthetized, mounted on stages and observed with a VE-7800 scanning electron microscope (Keyence Inc., Osaka, Japan) in a high vacuum mode.

Statistical analysis. A comparison between the numbers of colonies formed by wild-type or mutant ERKs in soft agar was made with unpaired Student's t-test for continuous outcomes. P-values were two-sided and $p < 0.05$ was considered statistically significant.

Results

ERK2 proteins with amino acid charge-change mutations in the CD domain migrate faster in SDS-PAGE. Our earlier study revealed that the faster migrating mutant ERK2^{E322K}, found in the HSC6 cancer cell line (12), was ~40 kDa. Since the protein was detected with a C-terminal anti-ERK2-specific antibody, we excluded the presence of premature termination as the reason for faster migration. A possibility is the presence of an internal start site at codon 13, resulting in a constructed HA-ERK2^{M13L}, which was examined for migration, while another possibility is the amino acid charge-change mutation in the CD domain or in the MAPK insert region. Thus, we constructed other HA-tagged mutants in the CD domain (23) and MAPK insert region (24) as shown in Fig. 1A.

293T cells transiently expressing HA-ERK2 mutants showed that charge-change mutation in the MAPK insert region (ERK2^{E250K}) did not markedly affect its migration in SDS-PAGE. However, mutants in the CD domain (ERK2^{E322K}, ERK2^{D321N} and ERK2^{D318K}) resulted in a faster migration (Fig. 1B). In addition, the double mutant (ERK2^{E322K+E250K}) migrated as ERK2^{E322K} but not as ERK2^{E250K} (Fig. 1C), confirming that the amino acid charge-change mutation in the

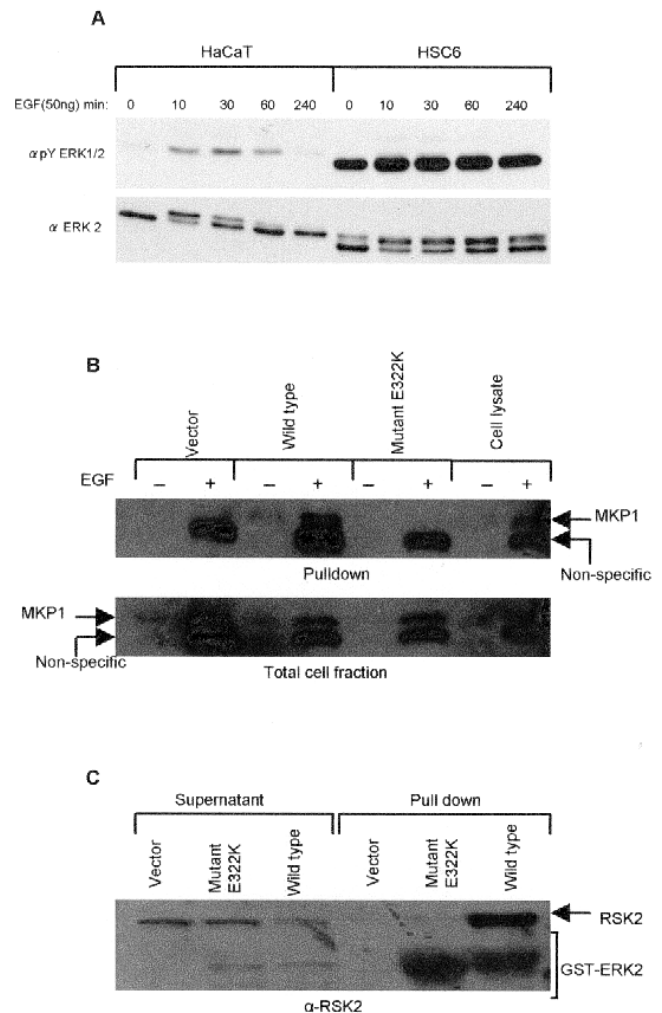


Figure 2. Biochemical and biological characteristics of ERK2^{E322K} mutation. (A) Constitutive phosphorylation of ERK2^{E322K} in HaCaT and HSC6 cells. Cell lysates were prepared from EGF-treated HaCaT and HSC6 cells for the indicated time period. The blotted lysates were then probed using anti-phospho-tyrosine and anti-ERK2 antibodies. (B) Loss of binding of ERK2^{E322K} to MKP1. Cell lysates were prepared from serum-starved or EGF-treated Ca922 to induce MKP1. After pull-down with GST-ERK2^{wt} or GST-ERK2^{E322K}, the amounts of MKP1 were examined by Western blotting using anti-MKP1 antibody. (C) Loss of binding to RSK2 by pull-down assay. The binding of RSK2 to GST-ERK2^{wt} or GST-ERK2^{E322K} were examined as described in Fig. 2A, using anti-RSK2 antibody.

CD domain resulted in faster migration, whereas the mutation in the MAPK insert region did not. Not surprisingly, the ERK2^{M13L} mutant did not migrate as ERK2^{E322K} but the ERK2^{M13L+E322K} double mutant did (Fig. 1D). It is likely that any other charge-change mutations in the CD domain of ERK2 may also result in faster migration.

ERK2^{E322K} is constitutively phosphorylated and loses its interaction (in vitro) with MKP1 and RSK2. The constitutive phosphorylation of ERK2^{E322K} expressed in HSC6 cells was reported earlier (12). A time course treatment of HaCaT (an immortalized human skin keratinocyte cell line) and HSC6 cells with EGF show that the ERK2^{E322K} phosphorylation is maintained even after 4 h of EGF treatment. This is in contrast with the control HaCaT cells in which the dephosphorylation started after 30 min of EGF treatment (Fig. 2A). The

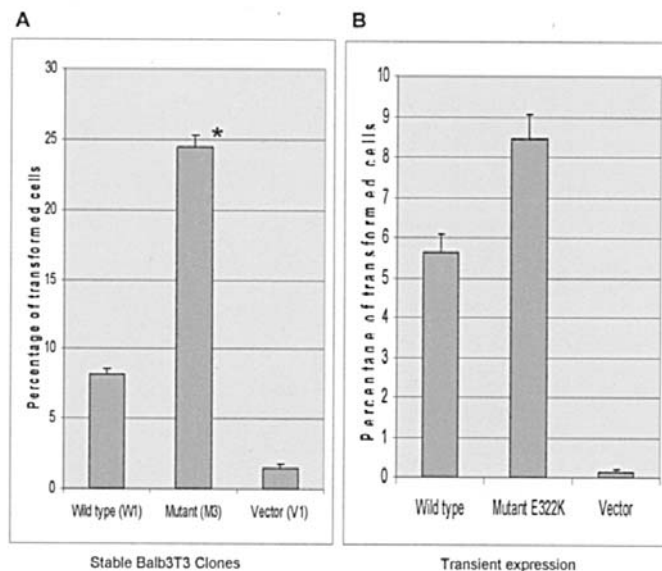


Figure 3. Colony forming efficiency of Balb3T3 cells in soft agar. (A) Colony formation by stable cell clones of Balb3T3 cells in soft agar. Balb3T3 cells transfected with LTR-ERK2^{wt}, LTR-ERK2^{E322K} or vector were G418-selected and grown as single colonies to establish stable cell clones. The clones were grown in duplicate with soft agar medium. After 4 weeks, colonies >0.1 mm in size were counted and analysed. (B) Colony formation by transiently transfected Balb3T3 cells in soft agar. Balb3T3 cells were transfected with LTR-ERK2^{wt}, LTR-ERK2^{E322K} or vector. Soft agar assay was performed as explained in Fig. 3A and statistically analysed (*p=0.0035, the difference is statistically significant). The results were an average of two independent experiments.

expression of MKP1 was at its peak at 1 h in HSC6 cells (data not shown).

The CD domain mutation of DERK^{sem} was reported to have reduced binding to a number of downstream effectors which included MKP1/2/3, and RSK *in vitro* and *in vivo* (9-11). As EGF induces MKP1/2, we used EGF-treated Ca922 cell lysates to examine the interaction of MKP1 with GST-ERK2^{wt} and -ERK2^{E322K}. The pulldown assay showed that ERK2^{wt} bound to MKP1 but ERK2^{E322K} did not (Fig. 2B). The result indicates that this CD domain mutation of ERK2^{E322K} lost its capacity to bind to MKP1.

In Fig. 2C, it is evident that ERK2^{wt} strongly binds to RSK2 but not to ERK2^{E322K}. Thus, as seen in Fig. 2B and C, together they suggest that the ERK2^{E322K} mutation resulted in loss of communication with the downstream target proteins, MKP1 and RSK2, both of which were reported to contribute to the constitutive activation of ERK2.

Anchorage-independent growth of ERK2^{E322K}-expressing Balb3T3 cells in soft agar. As we initially found this mutation in a human cancer cell line and it was constitutively activated, we explored whether this mutant ERK2^{E322K} was able to enhance anchorage-independent growth. Balb3T3 cells stably (Fig. 3A) and transiently (Fig. 3B) expressing ERK2^{E322K} formed more numbers of colonies than the cells expressing wild-type ERK2 (Fig. 3A, *p=0.0035 and B, p=0.065).

Effect of CD domain mutation DERK^{E335K} in *Drosophila* eye development. To delineate the physiological changes induced

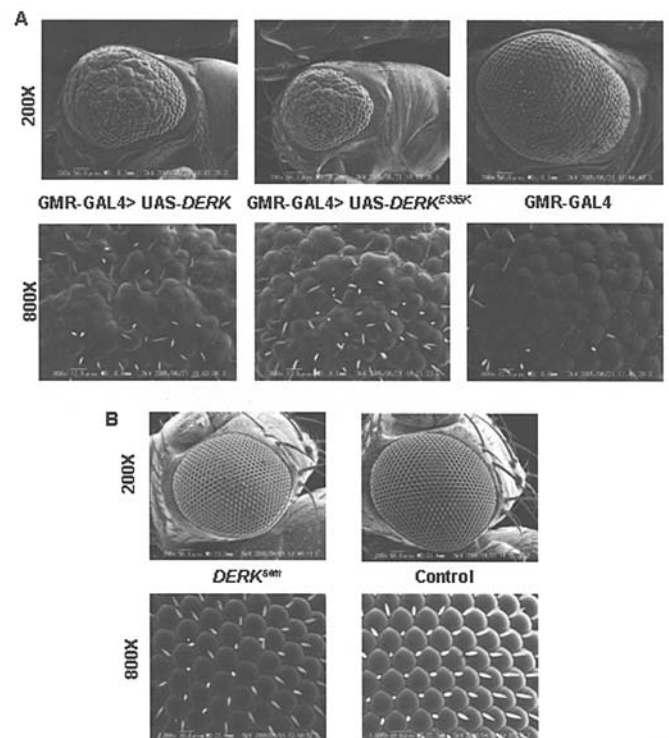


Figure 4. *Drosophila* transgenic for DERK^{E335K} induced weakly abnormal eye development. (A) Scanning electron microscopy of the development of eyes in transgenic flies for DERK^{wt} or DERK^{E335K} or GAL4 vector only. The upper and lower panels show the x200 and x800 resolutions of the transgenic *Drosophila* eyes, respectively. (B) Scanning electron microscopy of the development of eyes in DERK^{sem} expressing fly and the control fly eye. Resolutions of x200 and x800 are shown as in A.

by the CD domain mutation in *Drosophila* eye development, we established transgenic *Drosophila* by carrying the DERK^{wt} or the CD domain mutation DERK^{E335K}, corresponding to the human ERK2^{E322K}. The transgenic fly was crossed with GMR-GAL4 driver strain to examine the effect of DERK^{E335K} on eye development, and compared with the *sevenmaker* mutant DERK^{sem}. Scanning electron microscopy of the transgenic fly for DERK^{E335K} showed a more severe abnormality in adult *Drosophila* eye morphology than the transgenic flies for DERK^{wt}, by exhibiting a perturbed eye development with a smaller size and rougher eyes (Fig. 4A). In Fig. 4B, a comparison of the DERK^{sem} mutant expressing fly and the control fly eye is shown.

Discussion

Our earlier report on the CD domain mutation in the human cancer cell line, HSC6, demonstrated its constitutive phosphorylation by MEK and a faster migration in SDS-PAGE (12). Constitutively phosphorylated (25) and over-expressed (26) ERK had been reported in many cancers such as hepatocellular carcinomas, renal cell carcinomas, and gastric adenocarcinomas (27-29). In this study, we found that the other CD domain mutations with amino acid charge-change migrated faster and thus, may be constitutively active. Such a CD domain mutation was first reported as a *sevenmaker* mutation in *Drosophila* (8).



activity depends on the interaction with upstream effectors. The CD domain is necessary for interaction with several downstream effectors, and the MAPK insert region is important for interaction with the upstream effector, PEA-15 for nuclear entry (30). MKP1 is a phosphothreonine/tyrosine dual-specificity phosphatase, which dephosphorylates ERK1/2 *in vitro* and the expression of MKP1 in the cell block activation of ERK1/2 (31). In addition, ERK1/2 phosphorylates and stabilizes the MKP1 protein (32) and in turn, MKP binds to the CD domain and inactivates ERK2 (23). Our preliminary results with control HaCaT cells showed that the time course of MKP1 stimulation resulted in the decrease of ERK1/2 activation. However, this activation was not affected in HSC6 cells (Fig. 2A). Based on these results, further experiments conducted by us show that ERK2^{E322K} mutation lost its binding to MKP1, thus avoiding inactivation. Since it is known that MKP3 interacts with ERK2 through the CD domain, it is presumed that the interaction between them is also lost. It is known that RSK physically interacts with ERK2 and negatively regulates the nuclear entry of ERK2 (11). It was recently reported that mutant ERK2^{E320K} (rat ERK2 homolog to human ERK2^{E322K}) is actively translocated into the nucleus as efficiently as ERK2^{wt} (30). Unlike the MAPK insert mutant ERK2, the CD domain double mutant D316A and D319A failed to interact to MKP3 and RSK1 in a yeast two-hybrid system showing the importance of the CD domain in the interaction of ERK2 with its cellular targets (33). It is thus suggested that loss of binding to MKP1 and RSK2 conferred to the constitutive activation on ERK2^{E322K}, though more experiments should confirm whether this facilitated proliferation and oncogenic transformation. Our preliminary data showed three important results: i) HSC6 cells maintained phospho-threonine/tyrosine and phosphotyrosine ERK2 proteins even at 4 h after EGF stimulation, ii) MKP1 levels were maintained at high levels at 1 h after EGF stimulation and iii) serum-starved HSC6 cells (expressing ERK2^{E322K}, i.e. 75% of the total ERK2 in the cell) had two times more kinase activities than the control HaCaT cells (Arvind *et al*, unpublished data) showing its constitutive activation by the mutant ERK2.

The constitutively active mutant ERK2^{E322K} is likely to present an oncogenic potential, due to loss of control with negative signaling. It should be noted that this mutation was originally identified in a human cancer cell line. These results are consistent with enhanced anchorage-independent growth by this mutation. We showed that the mutant ERK2^{E322K} overexpressing Balb3T3 cells induced more numbers of colonies and thus, anchorage-independent cell growth. Though the cells transiently expressing ERK2^{E322K} formed more numbers of colonies than those expressing wild-type (Fig. 3B), the values were not statistically significant ($p=0.065$). However, a higher number of colonies formed by the stable cells expressing ERK2^{E322K} were statistically very significant ($p=0.0035$) (Fig. 3A).

The DERK^{E335K} CD domain mutation that we constructed, which corresponded to the ERK2^{E322K} mutation, revealed that this mutation resulted in a perturbed, smaller and rougher eye development in *Drosophila*. In Fig. 4B, though the external eye development of DERK^{D334N/sem} did not appear to be as abnormal as DERK^{E335K}, DERK^{D334N/sem} induced an R7

differentiation and abnormal wing formation, which were not observed with DERK^{E335K} in our study (data not shown). This may be due to UAS mediating overexpression of ERK2, and inducing marked morphological changes such as small and rough eyes. Since DERK^{D334N/sem} is not overexpressed, it did not show such morphological abnormality. The difference in position of the mutation and the difference in the amino acid charge-change between DERK^{E335K} and DERK^{D334N/sem} may influence the differences in inducing abnormality. It has been reported that the *Drosophila* ERK^{D334N/sem} mutant failed to bind RSK2 (11). The importance of RSK in PC12 cell differentiation was reported earlier (34).

In summary, our results suggest that the ERK2^{E322K} CD domain mutation is involved in enhanced cell growth and abnormal differentiation. Further studies of the CD domain are needed to elucidate the more novel functions of ERK2.

Acknowledgements

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