Histol Histopathol (2014) 29: 635-639
DOI: 10.14670/HH-29.10.635

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Immunohistochemical and Western blot analysis of two protein tyrosine phosphatase receptors, R and Z1, in colorectal carcinoma, colon adenoma and normal colon tissues

Marta Woźniak[1], Elżbieta Gamian[1], Izabela Łaczmańska[2], Maria M. Sąsiadek[2], Kamila Duś-Szachniewicz[1] and Piotr Ziółkowski[1]

[1]Department of Pathology and [2]Department of Genetics, Wrocław Medical University, Wrocław, Poland

Summary. Two classes of proteins, namely tyrosine kinases (PTK) and phosphatases (PTP), play an important role in cell proliferation and differentiation, thus leading to an acceleration or inhibition of tumour growth. The role of the above proteins in colorectal carcinoma (CRC) growth is a well-known event. In this study we carried out immunohistochemical and Western blot analysis of colorectal carcinoma, adenoma and normal colon tissue in relation to two protein tyrosine phosphatase receptors, R and Z1. Twenty-five cases of CRC were analyzed and the results were compared with similar data obtained in non-malignant tissues. High expression of both PTP receptors was observed in all examined cases of CRC, adenoma and normal colon tissue in this study. These results are not in line with recently published data, showing that genetic coding for PTPRR and PTPRZ1 were hypermethylated in CRC's. We presume that the protein tyrosine phosphatase overexpression in colorectal carcinoma is not enough to protect from the progression of disease.

Key words: Colorectal carcinoma, Protein tyrosine phosphatase receptor R and Z1, Immunohistochemistry, Western blotting analysis

Offprint requests to: Piotr Ziółkowski, MD, PhD, Department of Pathology, Wrocław Medical University Ul. Marcinkowskiego 1, 50-368 Wrocław, Poland. e-mail: ziolkows@interia.pl

Introduction

Reversible tyrosine-specific phosphorylation of cellular proteins is a key signalling mechanism, evoking essential cellular processes, such as proliferation and differentiation. The regulation of these processes depends on the balanced activities of protein tyrosine phosphatases (PTP's) and protein tyrosine kinases (PTK's) (Hunter, 1987). Perturbed PTK signalling may be caused by alterations in the genetic coding of PTK's, such as mutations, deletions and amplifications and also by epigenetic mechanisms influencing gene expression. Deregulated kinase activity adds to mechanisms of malignant transformation (Blume-Jensen and Hunter, 2001). PTPs are classified as tumour suppressors on the basis that they counteract PTK activity (Julien et al., 2011). This view has been strengthened by data showing that members of the *PTP* gene superfamily are epigenetically silenced in several types of cancer, including hepatocellular and colorectal carcinoma (Motiwala et al., 2003; Menigattiet et al., 2009). Recently published data has shown a statistically significant difference between promoter methylation of those genes in cancerous and healthy tissue. These results support the hypothesis that the PTPR (protein tyrosine phosphates receptor) family plays an important role in the etiology of colorectal carcinoma, CRC (Łaczmańska et al., 2013). Moreover, an expression of PTPR's has been observed in gastrointestinal cancers with a strong association between PTPRA/PTPRZ expression and progression of cancer (Wu et al., 2006).

The *PTPRR* gene encodes the classical

transmembrane protein-tyrosine phosphatase (PTP) known as PTP receptor type, (PTPRR) (Alonso et al., 2004). The assessment of *PTPRR* expression levels in different human tissues reveals its preferential expression in the brain and in the lower gastrointestinal tract, therefore its downregulation might reasonably be expected to lead to the development of disease in these tissues (Menigatti et al., 2009). *PTPRR* silencing may thus represent one of the mechanisms by which neoplastic colorectal cells evade tumour suppression.

Hypoxia inducible factors (HIF's) are the principal means by which cells upregulate genes in response to hypoxia and certain other stresses. It is also known that processes such as increased glycolysis, angiogenesis, and erythropoesis are mediated through activation of specific genes by hypoxia-inducible factors (HIF's), including HIF-2 (Semenza, 1998). Certain genes are preferentially activated by HIF-2. Among them, protein tyrosine phosphatase receptor-type Z polypeptide 1 (PTPRZ1) is overexpressed in a number of tumours, including hepatocarcinoma, renal carcinoma and glioblastoma (Foehr et al., 2006). Interestingly, like most of the HIF-2-specific genes, *PTPRZ1* is only minimally upregulated by hypoxia (Wang et al., 2005). The HIF-2-specific upregulation of *PTPRZ1* may provide another mechanism by which HIF-2 promotes tumourigenesis in a variety of tumours through activation of the ß-catenin pathway (Liu et al., 2007). *PTPRZ* is also widely expressed in the mammalian central nervous system and has been suggested to regulate oligodendrocyte survival and differentiation. It may play a role in the etiology of multiple sclerosis and periventricular leukomalacia. (Huang et al., 2012). In the present study, we showed strong and diffuse immunohistochemical reaction of CRC, adenoma and normal colon mucosa to antibodies against two receptor types: PTPRR and PTPRZ1.

Material and methods

Ethics statement

This investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and has been approved by the authors' institutional review board.

Tissue specimens were derived from patients surgically treated for colon adenocarcinoma (CRC) or colon adenoma (tubular or tubulovillous). These included 25 cases of CRC, grade 1, 2 or 3, 5 benign tumours (3 tubular and 2 tubulovillous) and 5 unaltered mucosae in surgical margins obtained from patients treated because of inflammatory bowel disease (Crohn's disease or ulcerative colitis) (Table 1). All specimens were routinely processed as formalin fixed and paraffin embedded samples and stained using the hematoxylin-eosin method. After a thorough histopathological examination and establishing the diagnosis, the paraffin blocks were cut again and thus additional slices matching the HE stained slides were used for immunohistochemical staining.

The specimens were used upon approval by the Local Ethics Committee.

Antibodies. Two antibodies were used in immunohistochemical staining and in Western blot analysis: rabbit polyclonal anti-PTPRR (product no: HPA011851) and polyclonal rabbit anti-PTPRZ1 (product no: HPA011772), both from: Atlas Antibodies AB, Sweden.

Table 1. Results of immunohistochemical stainings of PTPRR and PTPRZ1 in colorectal carcioma, colon adenoma and normal mucosa cases according to the Allred scoring system.

Sample	Patient	Grade	PTPRR	PTPRZ1
Colorectal carcinoma				
	1	1	6	4
	2	1	6	4
	3	1	5	4
	4	1	6	6
	5	1	6	5
	6	1	5	4
	7	1	4	4
	8	1	3	6
	9	1	4	3
	10	1	3	3
	11	1	6	5
	12	1	6	6
	13	2	6	4
	14	2	4	4
	15	2	5	6
	16	2	6	6
	17	2	6	5
	18	2	4	3
	19	3	6	3
	20	3	3	3
	21	3	6	4
	22	3	4	6
	23	3	6	6
	24	3	6	6
	25	3	6	3
Tubular adenoma				
	1	x	5	5
	2	x	5	5
	3	x	6	5
Tubulo-villous adenoma				
	1	x	5	5
	2	x	6	4
Healthy				
	1	x	5	3
	2	x	6	4
	3	x	4	4
	4	x	4	4
	5	x	5	4

The numerical value for overall intensity [intensity score (IS)] was based on a 4-point system: 0, 1, 2 and 3 (for none, light, medium, or dark staining). The numerical value for percent of stained cells [proportion score (PS)]: no stain = 0; ≤1/100 cells stained = 1; ≤1/10 cells stained = 2; ≤1/3 cells stained = 3, ≤2/3 cells stained = 4; all cells stained = 5. Thus, the total Allred score varied from 0 to 8.

IHC score: For immunohistochemical evaluation of slides we used the Allred scoring system. The numerical value for overall intensity [intensity score (IS)] was based on a 4-point system: 0, 1, 2 and 3 (for none, light, medium, or dark staining). A numerical value was obtained for percent of stained cells [proportion score (PS)]: no stain=0; ≤1/100 cells stained=1; ≤1/10 cells stained=2; ≤1/3 cells stained=3, ≤2/3 cells stained=4; all cells stained=5.

Immunohistochemistry

Immunohistochemical staining was performed using the LSAB+ method (LSAB+ System HRP from DAKO, Denmark). 5 mm sections were cut from formalin-fixed and paraffin-embedded blocks. First, slides were deparaffinized in 2 changes of xylene for 9 minutes, then rehydrated in a series of graded alcohols (96%, 80% and 70%) for 1 minute each. Next, specimens were washed twice for 4 minutes in distilled water and placed in a citric buffer bath (pH 6.0) for 9 minutes to heat-induced epitope retrieval. After washing in distilled water twice for 4 minutes, specimens were incubated for 10 minutes with Peroxidase Blocking Reagent and rinsed 2x5 minutes with PBS. Incubation with Protein Block Reagent was performed for 10 minutes, then specimens were incubated with primary antibody (dilution 1:100) and stored overnight at 4°C. Next day, the slides were incubated for 15 minutes with each of the following two reagents: Biotinylated link antibody and Streptavidin-HRP. The reaction was detected and visualized by 3,3'-diaminobenzidine in chromogen solution. Finally, the specimens were counterstained with hematoxylin and then dehydrated through the above described alcohols for 3 minutes each, cleared in 2 changes of xylene for 5 minutes and mounted with xylene based mounting medium.

Negative and positive controls: For negative controls, the primary antibodies were omitted. Known positive controls (normal gastric mucosa for PTPRR and normal cerebral cortex for PTPRZ1) were used to confirm the specificity of primary antibodies.

Western blot analysis

For the Western blot, formalin fixed and paraffin-embedded histological sections were used. We used 6 CRC samples (2 of each grade), 5 adenoma and 3 control colon tissues. Proper parts were cut from blocks and homogenized in a lysis buffer consisting of 0.1 M Tris-HCL, pH 8.0, 0.1M DTT and 4% SDS. Then, samples were heated in a heating block with agitation (600 rpm) for 1 hour. Next, the crude extracts were clarified by centrifugation at 16.000 x g at 18°C for 10 minutes. The protein concentration in supernatant was measured at 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Inc). For Western blot analysis NuPAGENovexBis-Tris gels (12%), equipment, standards and buffers recommended by Invitrogen (USA) were used. The nitrocellulose was from AmershamHybond. After SDS-PAGE electrophoresis the membrane was briefly washed in PBS, incubated with 2.5% glutaraldehyde (Sigma-Aldrich, Germany) in PBS for 10 minutes and washed in PBS-Tween 0.1%. Next, the membrane was blocked with 5% normal goat serum (Sigma-Aldrich) in PBS-Tween 0.1% for 30 minutes and then incubated overnight with rabbit polyclonal primary antibody against protein tyrosine phosphatase, receptor type R (PTPRR, Cat. No. HPA011851, Atlas Antibodies, Sweden) in dilution 1:250 and with rabbit polyclonal anti- beta-actin antibody (for internal control, dilution 1:1000, RbpAb to beta-actin, Cat. No. ab8227, Abcam, UK) at 4°C. The following day, after washing with PBS-Tween 0.1%, the membrane was subjected to the peroxidase-conjugated secondary antibody (goat polyclonal antibody to rabbit IgG - H&L Abcam, UK). The specific protein bands were visualized by reaction with DAB (3,3-diaminobenzidine tetrahydrochloride) using DAB Enhanced Liquid Substrate System for Immunohistochemistry (Sigma-Aldrich) and results were documented using Molecular Imager Gel Doc TMXR+ (BioRad, USA).

Results

Strong staining for both protein tyrosine phosphatases was observed in this study in all examined cases (Table 1). We found the staining for PTPRR and PTPRZ1 in the cytoplasm of examined cells. The intensity of staining for PTPRR was strong and the number of stained cells was high in normal glandular epithelium (Fig. 1A), benign adenoma (Fig. 1B), as it also was in carcinoma cells (Fig. 1C).

Similarly, the intensity of staining for PTPRZ1 turned out to be strong and the number of reactive cells was high in all cases of normal glandular epithelium (Fig. 2A), adenoma (Fig. 2B), and in colorectal carcinoma (Fig. 2C).

Fig. 3 shows the results from the Western blot analysis. It is clearly shown that the bands for normal colon (control), adenoma and cancer are of similar size. The same, strong bands of expression have been found in all examined cases of CRC, adenoma and normal colon mucosa.

Discussion

Carcinogenesis is a complicated process resulting from alterations in a variety of molecular pathways, thus leading to deregulation of the biological functions of transformed cells. Amongst others, protein tyrosine phosphatases (PTP's) and protein tyrosine kinases (PTK's) play key roles in regulation of a variety of cellular processes and are suspected of being involved in this process.

Recently, mutations in genetic coding for PTP's and PTK's have been observed in different tumors, including

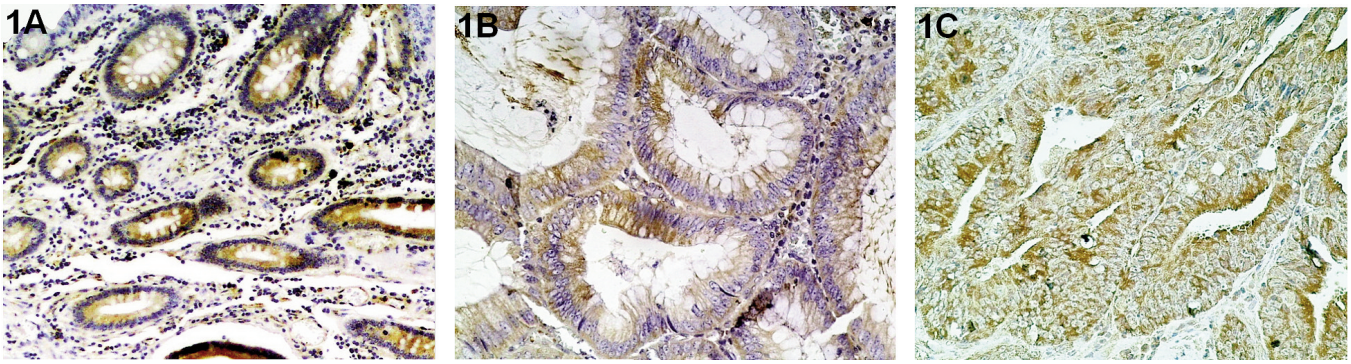

Fig. 1. Cytoplasmic pattern of immunohistochemical staining for PTPRR in normal colon glands **(A)**, in cells of tubular adenoma **(B)**, and in cells of colorectal carcinoma **(C)**. Rabbit polyclonal anti-PTPRR (product no: HPA011851), diluted 1:100, LSAB+ method, DAB, hematoxylin counterstained. x 200

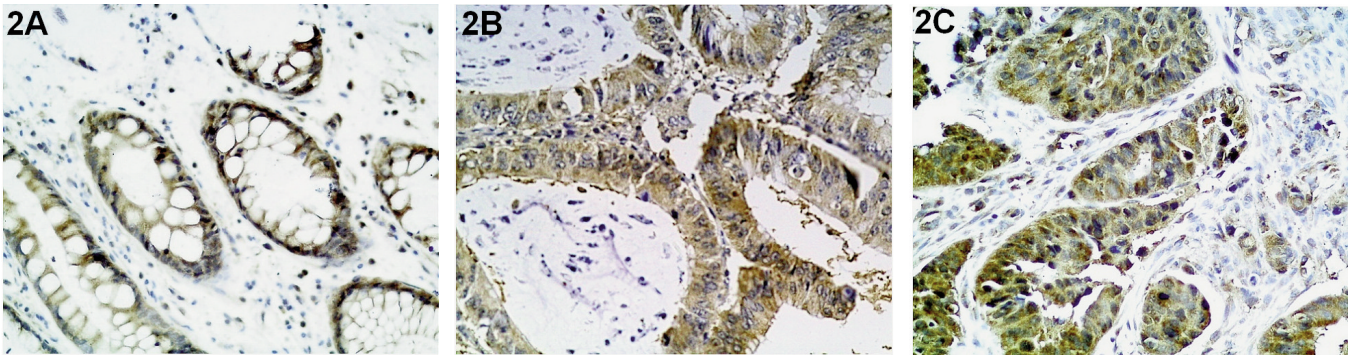

Fig. 2. Cytoplasmic pattern of immunohistochemical staining for PTPRZ1 in normal colon glands **(A)**, in cells of tubular adenoma **(B)**, and in cells of colorectal carcinoma **(C)**. Rabbit polyclonal anti-PTPRZ1 (product no: HPA011772), diluted 1:100, LSAB+ method, DAB, hematoxylin counterstained. x 200

colon cancer (Menigatti et al., 2009). Moreover, a genome-wide analysis of methylation pattern of colorectal carcinoma revealed about 400 differentially methylated CpG's, including four located in the promoters of the tyrosine phosphatase family receptors T, R, M and Z1. A methylation of these genes, either partial or complete has been observed in 81 to 99% of tumors. In 30-100% of corresponding normal colon tissue samples no methylation of these genes has been shown. Moreover, a complete methylation of *PTPRM, PTPRT, PTPRR* and *PTPRZ1* was extremely rare in normal colon tissues. Epigenetic silencing of *PTPRR, PTPRG, PTPRD* and *PTPRO* was also observed in other cancers (Motiwala et al., 2003; Menigatti et al., 2009; Veeriah et at., 2009; Van Roon et al., 2011). Hypermethylation of genes encoding tyrosine phosphatases may result in decreased expression of corresponding proteins (Menigatti et al., 2009).

To verify this hypothesis, our study focused on an immunohistochemical analysis of PTPRR and PTPRZ1 in 25 cases of colon cancer and corresponding normal

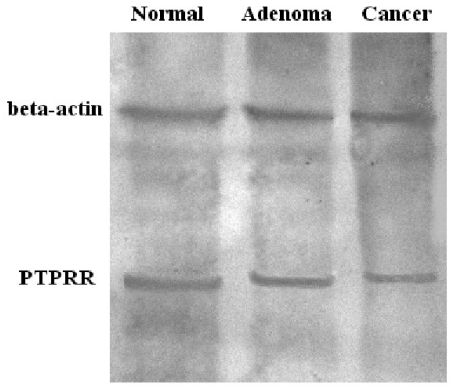

Fig. 3. Western blot results of protein expression of PTPRR (22 kDa) and beta-actin (42 kDa). A distinct expression of PTPRR was found in this study in all examined samples, normal colon, adenoma and colorectal carcinoma. Antibody for PTPRR used in this analysis was the same as used for the immunohistochemistry, diluted 1:250. Antibody for beta-actin for internal control was from Abcam, UK (product no. ab8227), diluted 1:1000.

tissues. Strong and diffuse staining for both proteins has been observed in all examined types of tissues. Moreover, the pattern of staining was cytoplasmic, with no nuclear reaction observed. Both malignant and benign tissues stained with similar intensity and showed a similar number of positively stained cells. This means that the synthesis of PTPRR and PTPRZ1 in normal mucosa, benign adenoma and in CRC remained steady. Western blot analysis confirmed that in all examined samples the PTPRR was strongly expressed. We did not, however, carry out a relevant Western blot analysis of PTPRZ1, because the available antibody is not recommended for such analysis. We presume that both proteins seen in immunohistochemical staining could lose their tumour suppressor function due to the biallelic form of inactivation. Further studies are, however, needed in order to elucidate this hypothesis. It has been thought that tyrosine phosphatases act as tumor suppressors, although this statement has been recently questioned because dephosphorylation events induced by some PTP's stimulate the growth of some tumours. PTP's could then have a dual role as tumour suppressors and oncogenes.

Our observation is not in line with recently published data, showing that genes coding for PTPRR and PTPRZ1 are hypermethylated in CRC's (Łaczmańska et al., 2013), which allowed the authors to speculate that PTPRR and PTPRZ1 would be downexpressed in CRC tissues. However, according to Knudson's two-hit hypothesis, an inactivation of only one allele of tumour suppressor genes is not enough to inactivate the gene.

A similar discrepancy between the results of immunohistochemical and genetic analyses has been observed in breast cancer, where a low level of Her-2 amplification did not correlate with protein overexpression (Rygiel et al., 2010).

We presume that protein tyrosine phosphatase overexpression in colorectal carcinoma is not enough to protect from the progression of disease. Regardless, the present results point toward the need for next extensive studies on the correlations between tumour suppressor genes and relevant protein expression in malignant tumours.

Acknowledgements. This paper has been supported by the State Committee for Scientific Research, Polish Ministry for Scientific Research and Information Technology (grant no. N N401 601438), 2010-2013.

References

Alonso A., Sasin J., Bottini N., Friedberg I., Osterman A., Godzik A., Hunter T., Dixon J. and Mustelin T. (2004). Protein tyrosine phosphatases in the human genome. Cell 117, 699-711.

Blume-Jensen P. and Hunter T. (2001). Oncogenic kinase signalling. Nature 411, 355-365.

Foehr E.D., Lorente G., Kuo J., Ram R., Nikolich K. and Urfer R. (2006). Targeting of the receptor protein tyrosine phosphatase beta with a monoclonal antibody delays tumor growth in a glioblastoma model. Cancer Res. 66, 2271-2278.

Huang J.K., Ferrari C.C. and Monteiro de Castro G. (2012). Accelerated axonal loss following acute CNS demyelination in mice lacking protein tyrosine phosphatase receptor type Z. Am. J. Pathol. 181, 1518-1523.

Hunter T. (1987). A thousand and one protein kinases. Cell 50, 823-829.

Julien S.G., Dube N., Hardy S. and Tremblay M.L. (2011). Inside the human cancer tyrosine phosphatome. Nat. Rev. Cancer 11, 35-49.

Liu Y.T., Shang D., Akatsuka S., Ohara H., Dutta K.K., Mizushima K., Naito Y., Yoshikawa T., Izumiya M., Abe K., Nakagama H., Noguchi N. and Toyokuni S. (2007). Chronic oxidative stress causes amplification and overexpression of ptprz1 protein tyrosine phosphatase to activate beta-catenin pathway. Am. J. Pathol. 17, 1978-1988.

Łaczmańska I., Karpiński P., Bębenek M., Sedziak T., Ramsey D., Szmida E. and Sąsiadek M.M. (2013). Protein tyrosine phosphatase receptor-like genes are frequently hypermethylated in sporadic colorectal cancer. J. Human Genet. 58, 11-15.

Menigatti M., Cattaneo E., Sabates-Bellver J., Ilinsky V.V., Went P., Buffoli F., Marquez V.E., Jiricny J. and Marra G. (2009). The protein tyrosine phosphatase receptor type R gene is an early and frequent target of silencing in human colorectal tumorigenesis. Mol. Cancer 8, 124.

Motiwala T., Ghoshal K., Das A., Majumder S., Weichenhan D., Wu Y.Z., Holman K., James S.J., Jacob S.T. and Plass C. (2003). Suppression of the protein tyrosine phosphatase receptor type O gene (PTPRO) by methylation was found in hepatocellular carcinomas. Oncogene 22, 6319-6331.

Rygiel A.M., Milano F., Ten Kate F.J., Bergman J.J. and Krishnadath K.K. (2010). Low level of Her-2 locus amplification by fluorescent in situ hybridization does not correlate with Her-2 protein overexpression by immunohistochemistry in Barrett's esophagus. J. Oncol. 2010, 382582.

Semenza G.L. (1998). Hypoxia-inducible factor 1: master regulator of O_2 homeostasis. Curr. Opin. Genet. Dev. 8, 588-594.

Wang V., Davis D.A., Haque M., Huang L.E. and Yarchoan R. (2005). Differential gene up-regulation by hypoxia-inducible factor-1alpha and hypoxia-inducible factor-2alpha in HEK293T cells. Cancer Res. 65, 3299-3306.

Wu C.W., Kao H.L., Li A.F., Chi C.W. and Lin W.C. (2006). Protein tyrosine-phosphatase expression profiling in gastric cancer tissues. Cancer Lett. 242, 95-103.

Van Roon E.H., De Miranda N.F. and Van Puijenbroek M. (2011). Tumour-specific methylation of PTPRG intron 1 locus in sporadic and Lynch syndrome colorectal cancer. Eur. J. Hum. Genet. 19, 307-312.

Veeriah S., Brennan C., Meng S., Singh B., Fagin J.A., Solit D.B., Paty P.B., Rohle D., Vivanco I., Chmielecki J., Pao W., Gerald W.L., Liau L., Cloughesy T.C., Mischel P.S., Sander C., Taylor B., Schultz N., Major J., Heguy A., Fang F., Mellinghoff I.K. and Chan T.A. (2009). The tyrosine phosphatase PTPRD is a tumor suppressor that is frequently inactivated and mutated in glioblastoma and other human cancers. Proc. Natl. Acad. Sci. USA 106, 9435-9440.

Accepted November 15, 2013