

- berg, W. Heath, F. Köntgen, F. R. Carbone, K. Shortman, *J. Exp. Med.* **181**, 1643 (1995); B. Lucas and R. N. Germain, *Immunity* **5**, 461 (1996).
15. D. L. Wiest *et al.*, *Immunity* **6**, 663 (1997).
 16. Total $HD^{-/-}$ or WT thymocytes were stimulated with anti-TCR β and -CD4, lysed, and subjected to immunoprecipitation with anti-CD3 ζ . This leads to the isolation also of CD4-associated p56^{lck}, due to the presence of cross-linking anti-CD4 antibody in the lysate. To measure in vitro kinase activity (Fig. 2A), immune complexes were incubated with adenosine [γ -³²P]triphosphate and the exogenous kinase substrate enolase, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), blotted, and exposed to x-ray film. To detect CD3 ζ phospho-isoforms (Fig. 2B), immune complexes were separated by SDS-PAGE, blotted, and probed with phosphotyrosine antibody 4G10 (Upstate Biotech).
 17. V. P. Dave and D. J. Kappes, unpublished data.
 18. We coated 96-well U-bottom tissue culture plates overnight with anti-CD3 ϵ (2C11) or anti-TCR β (H57) (Pharmingen) (10 mg/ml) or with phosphate-buffered saline for unstimulated controls. Total $HD^{-/-}$, $I-A\beta^{-/-}$, or C57BL/6 splenocytes depleted of red blood cells by density gradient centrifugation over Lympholyte M (Cedarlane, ON) were plated (5×10^6 cells per well) in 200 ml of RPMI-1640 medium supplemented with 10% fetal calf serum. Proliferation was assayed after 3 days by addition of 1 mCi of [3 H]thymidine per well followed by incubation for an additional 16 hours. All assays were carried out in triplicate. Data are shown with background values from unstimulated controls already subtracted.
 19. R. Keefe and D. J. Kappes, unpublished data.
 20. J. Kaye *et al.*, *Nature* **341**, 746 (1989).
 21. We obtained AND TCR transgenic and $\beta_2M^{-/-}$ mice from Jackson Laboratories (Bar Harbour, ME) and backcrossed them for two generations to $HD^{-/-}$ mates to obtain $AND^+ HD^{-/-}$ and $\beta_2M^{-/-} HD^{-/-}$ mice. These were identified by flow cytometric analysis of peripheral blood lymphocytes with antibodies specific for CD4, CD8, H-2K^b, and V β 3. Typings of $\beta_2M^{-/-}$ mice were confirmed by polymerase chain reaction analysis of tail DNAs.
 22. J. Kaye, N. J. Vasquez, S. M. Hedrick, *J. Immunol.* **148**, 3342 (1992).
 23. A. Bendelac, P. Matzinger, R. A. Seder, W. E. Paul, R. H. Schwartz, *J. Exp. Med.* **175**, 731 (1992); W. Swat, M. Dessing, H. von Boehmer, P. Kisielow, *Eur. J. Immunol.* **23**, 739 (1993); I. Yamashita, T. Nagata, T. Tada, T. Nakayama, *Int. Immunol.* **5**, 1139 (1993); J. A. Punt, H. Suzuki, L. G. Granger, S. O. Sharrow, A. Singer, *J. Exp. Med.* **184**, 2091 (1996).
 24. M. J. Gabor, D. I. Godfrey, R. Scollay, *Eur. J. Immunol.* **27**, 2010 (1997).
 25. J. Kirberg *et al.*, *J. Exp. Med.* **180**, 25 (1994).
 26. B. H. Koller, P. Marrack, J. W. Kappler, O. Smithies, *Science* **248**, 1227 (1990).
 27. D. J. Kappes, unpublished data.
 28. R. Scollay, S. Jacobs, L. Jerabek, E. Butcher, I. Weissman, *J. Immunol.* **124**, 2845 (1980); B. Lucas, F. Vasseur, C. Penit, *J. Immunol.* **153**, 53 (1994).
 29. D. Allman and D. J. Kappes, unpublished data.
 30. B. Scott, H. Bluethmann, H. S. Teh, H. von Boehmer, *Nature* **338**, 591 (1989).
 31. Supported by National Institutes of Health grants CA74620 and AI34472, by institutional grant CA06927 from the National Institutes of Health, and by an appropriation from the Commonwealth of Pennsylvania. We thank R. Hardy for critical reading of the manuscript and M. Zdancewicz for technical assistance.

21 June 1999; accepted 27 August 1999

A Biochemical Genomics Approach for Identifying Genes by the Activity of Their Products

Mark R. Martzen,¹ Stephen M. McCraith,² Sherry L. Spinelli,¹ Francy M. Torres,¹ Stanley Fields,^{2,3} Elizabeth J. Grayhack,¹ Eric M. Phizicky^{1*}

For the identification of yeast genes specifying biochemical activities, a genomic strategy that is rapid, sensitive, and widely applicable was developed with an array of 6144 individual yeast strains, each containing a different yeast open reading frame (ORF) fused to glutathione S-transferase (GST). For the identification of ORF-associated activities, strains were grown in defined pools, and GST-ORFs were purified. Then, pools were assayed for activities, and active pools were deconvoluted to identify the source strains. Three previously unknown ORF-associated activities were identified with this strategy: a cyclic phosphodiesterase that acts on adenosine diphosphate-ribose 1''-2'' cyclic phosphate (Appr>p), an Appr-1''-p-processing activity, and a cytochrome c methyltransferase.

A major task in the analysis of any biochemical activity is the purification and identification of the polypeptide or polypeptides responsible for that activity. Purification is often difficult, time consuming, and expensive, yet it is often a necessary prerequisite for cloning of the gene and subsequent detailed biochemical and genetic study. An alternative to purification is expression cloning: the introduction of cDNA pools into various host

cells, followed by screening for activity and identifying the responsible cDNA (1). This method is inherently limited to those proteins that are easily detectable in the background of host cell proteins. Yet, given the accumulation of complete genome sequences, such as that of the yeast *Saccharomyces cerevisiae*, the sequences of genes encoding every biochemical activity of these organisms are already available. The challenge is how to use this information to connect biochemical activity with a specific gene.

We developed a rapid and sensitive genomic method for identifying yeast genes encoding biochemical activities, which is applicable for almost any detectable activity. We first constructed an array of 6144 strains, each of which bears a plasmid expressing a

different GST-ORF fusion under control of the P_{CUP1} promoter (2). To identify genes encoding particular biochemical activities, we purified this genomic set of GST-ORFs in 64 pools of 96 fusions each (3). Then, we assayed the pools for a particular activity and deconvoluted active pools to identify the strain and ORF responsible for the activity. Assay of the GST-ORF pools demonstrates that each of two previously known tRNA splicing activities is detected only in the pools that contain their respective GST fusions: tRNA ligase (4) in pool 35 (Fig. 1A) and 2'-phosphotransferase (5) in pool 46 (Fig. 1B).

The GST-ORFs were used to identify three previously unknown genes by biochemical assay of their products. A highly specific cyclic phosphodiesterase (CPDase) (6) that could convert Appr>p, produced during tRNA splicing (7), to Appr-1''-p was localized to pool 4 (Fig. 2A), and an otherwise uncharacterized Appr-1''-p-processing activity was found in pool 6 (Fig. 2B). We further explored the usefulness of the pools by searching for a protein-modifying enzyme. Yeast cytochrome c is known to have a trimethyllysine (8), and pool 23 has a methyltransferase that is active with horse cytochrome c, but not with bovine serum albumin (Fig. 2C).

To determine the strain responsible for each activity, we prepared and assayed the GST-ORFs from each of the 8 rows and 12 columns of strains from the corresponding microtiter plates. In this way, CPDase activity was associated with strain MRM 319 (expressing YGR247w) in row C and column 7 (position C7) of plate 4 (Fig. 3A), Appr-1''-p processing was associated with MRM 546 (expressing YBR022w) at position F6 of pool 6, and cytochrome c methyltransferase activity was associated with MRM 2122 (expressing YHR109w) at position A10 of plate 23.

¹Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, NY 14642, USA. ²Departments of Genetics and Medicine, ³Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195, USA.

*To whom correspondence should be addressed. E-mail: eric_phizicky@urmc.rochester.edu

REPORTS

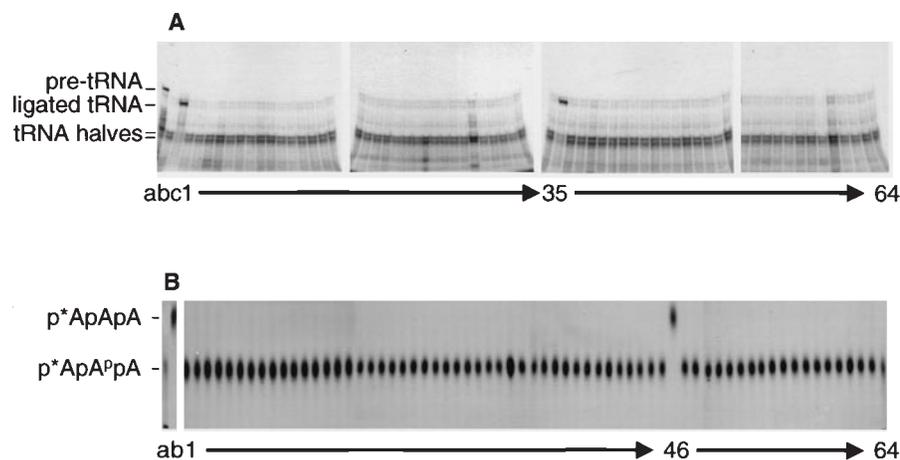


Fig. 1. Genomic assay of GST-ORF pools. **(A)** Transfer RNA ligase activity. [α - 32 P]adenosine triphosphate (ATP)-labeled precursor tRNA^{Phe} transcript was incubated in 10- μ l mixtures containing endonuclease (70), ATP, and 2 μ l of each GST-ORF pool, and products were resolved by PAGE (4). Lane a, precursor-tRNA (pre-tRNA); lane b, treated with endonuclease; lane c, treated with endonuclease and tRNA ligase. **(B)** 2'-phosphotransferase activity. [5'- 32 P]-ApApA was incubated in 10- μ l mixtures containing nicotinamide adenine dinucleotide and 0.1 μ l of GST-ORF pools, and products were resolved on polyethyleneimine-cellulose thin-layer plates (17). Asterisk indicates position of label. Lane a, substrate; lane b, with Tpt1 protein.

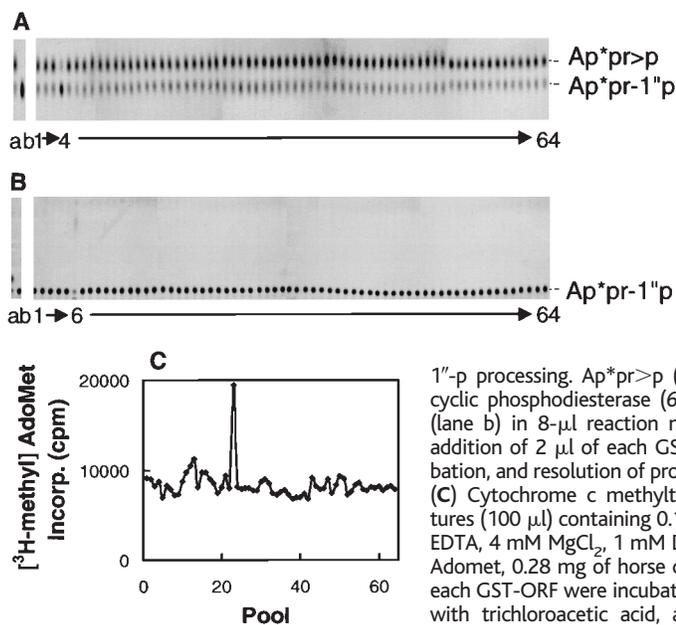
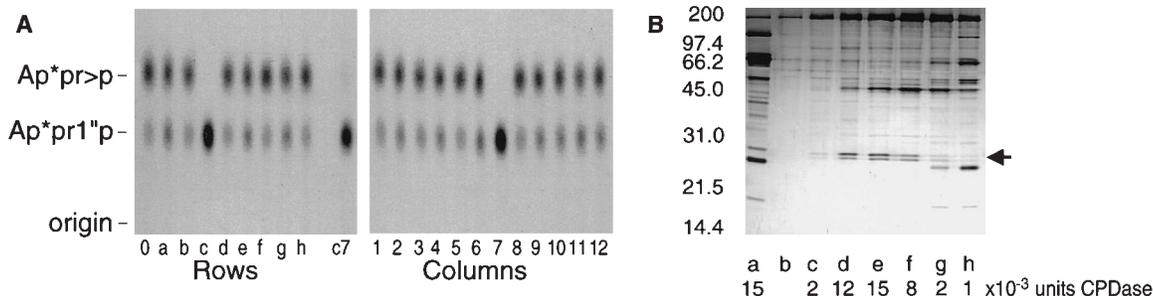


Fig. 2. Genomic assays of previously unassigned activities. **(A)** Appr>p cyclic phosphodiesterase. Reaction mixtures (10 μ l) contained buffer, Ap*pr>p, and 2 μ l of each GST-ORF pool, and products were resolved on thin-layer plates (6). Lane a, substrate; lane b, with cyclic phosphodiesterase. **(B)** Appr-1''-p processing. Ap*pr>p (lane a) was treated with cyclic phosphodiesterase (6) to generate Ap*pr-1''-p (lane b) in 8- μ l reaction mixtures, followed by the addition of 2 μ l of each GST-ORF pool, further incubation, and resolution of products on thin-layer plates. **(C)** Cytochrome c methyltransferase. Reaction mixtures (100 μ l) containing 0.1 M Hepes (pH 7.9), 2 mM EDTA, 4 mM MgCl₂, 1 mM DTT, 1 μ Ci of [3 H-methyl] Adomet, 0.28 mg of horse cytochrome c, and 2 μ l of each GST-ORF were incubated for 1 hour, precipitated with trichloroacetic acid, and counted to measure incorporation (12). cpm, counts per minute.

Fig. 3. Association of YGR247w with CPDase activity. **(A)** Deconvolution of CPDase from pool 4 implicates YGR247w. GST-ORFs prepared from rows and columns of strains on microtiter plate 4 and from MRM 319 (position C7) were assayed for CPDase. **(B)** CPDase is associated with YGR247w during conventional purification. Portions from the penultimate hydroxyapatite peak (lane a) and fractions 11 through 17 from the final Orange A Sepharose column (lanes b through h), containing the indicated number of units of activity, were resolved by SDS-PAGE and stained with silver. Arrow indicates the 25-kD band judged to copurify with activity.



In all three cases, we separately confirmed that the GST-ORF preparation from the individual strain had the expected activity (Fig. 3A) and that the plasmid DNA contained the expected ORF. No homologs of these ORFs were detected by routine BLAST searches.

Although these GST-ORFs copurify with the corresponding activities, they may instead interact with or be a subunit of their respective enzyme. For example, conventional purification of CPDase from crude extracts of wild-type cells confirms that ORF YGR247w copurifies with activity (9). SDS-polyacrylamide gel electrophoresis (PAGE) analysis of fractions from the final column indicated that the upper band of the 25-kD doublet copurified with CPDase activity (Fig. 3B, arrow), and mass spectrometry indicated that this band is YGR247w. However, we infer that YGR247w requires some other limiting factor for CPDase activity because overexpression of the ORF (or the GST-ORF) in yeast or the His6 ORF in *Escherichia coli* does not result in increased activity in extracts. It is also conceivable that any of these three enzymes might use other substrates in vivo; more biochemical and in vivo analyses are required to fully assess their cellular roles.

In principle, this biochemical genomics approach can be used to identify the gene associated with any biochemical activity, provided that the GST-ORF is functional, is solubilized during extraction, and retains other required components when purified. A large number of NH₂-terminal fusions are functional (for example, in two-hybrid screens), and simple modifications of our procedure could be used for the study of membrane proteins, protein complexes, and proteins that might be toxic when overproduced.

This biochemical genomics strategy has distinct advantages over conventional purification or expression cloning. First, it is rapid. The identification of an ORF-associated activity presently takes ~2 weeks, starting with the 64 purified GST-ORF pools, and with appropriate pooling strategies, identification could take 1 day. In contrast, months or years are required for purification or expression

cloning. Second, it is sensitive. Activities can be detected in the purified GST-ORF pools that simply cannot be detected in extracts or cells, the starting point of both conventional purification and expression cloning. Because the GST-ORFs are individually expressed at high levels and are largely free of extract proteins after purification, activities can be measured for hours without competing activities that destroy the substrate, the product, or the enzymes.

In addition to the conventional use demonstrated here, this array could be used in two other ways: (i) to determine the range of potential substrate proteins for any protein-modifying enzyme (such as a protein kinase) before genetic or biochemical tests to establish authentic substrates and (ii) to identify genes encoding proteins that bind any particular macromolecule, ligand, or drug. Thus, one could rapidly ascribe function to many presently unclassified yeast proteins, complementing other genomic approaches to deduce gene function from expression patterns, mutant phenotypes, localization of gene products, and identification of interacting partners.

References and Notes

1. H. Simonsen and H. F. Lodish, *Trends Pharmacol. Sci.* **15**, 437 (1994).
2. Plasmid pYEX 4T-1 (Clontech, Palo Alto, CA) was modified by the addition of a 140-nucleotide recombination domain, 3' of its Eco RI site, linearized within the recombination domain by restriction digestion, and cotransformed with a genomic set of reamplified ORFs that had the same ends as the linearized plasmid [J. R. Hudson Jr. et al., *Genome Res.* **7**, 1169 (1997)] into strain EJ 758 [*MATa his3-Δ200, leu2-3,112, ura3-52, pep4::URA3*], a derivative of JHRY-20-2Ca (5). Transformants obtained on synthetic minimal (SD) - Ura drop-out plates [F. Sherman, *Methods Enzymol.* **194**, 3 (1991)] (>100 in all cases, and more than five times the cut vector in 97% of the cases) were eluted in batch and saved in 96-well microtiter plates. The library contains 6080 ORF-containing strains and 64 strains with vector only.
3. Cell patches were inoculated in SD - Ura liquid medium, grown overnight, reinoculated, and grown overnight in SD - Ura - Leu medium, and then inoculated into 250 ml of SD - Ura - Leu medium, grown to absorbance at 600 nm of 0.8, and induced with 0.5 mM copper sulfate for 2 hours before harvest [I. G. Macreadie, O. Horaitis, A. J. Verkuylen, K. W. Savin, *Gene* **104**, 107 (1991)]. Cells were resuspended in 1 ml of buffer [50 mM tris-HCl (pH 7.5), 1 mM EDTA, 4 mM MgCl₂, 5 mM dithiothreitol (DTT), 10% glycerol, and 1 M NaCl] containing leupeptin (2 μg/ml) and pepstatin (1 μg/ml), and extracts were made with glass beads [S. M. McCraith and E. M. Phizicky, *Mol. Cell. Biol.* **10**, 1049 (1990)], followed by supplementation with 1 mM phenylmethylsulfonyl fluoride and centrifugation. GST-ORF fusion proteins were purified by glutathione agarose chromatography in buffer containing 0.5 M NaCl, essentially as described [J. R. Nelson, C. W. Lawrence, D. C. Hinkle, *Science* **272**, 1646 (1996)], followed by dialysis in 20 mM tris-HCl, 2 mM EDTA, 4 mM MgCl₂, 1 mM DTT, 55 mM NaCl, and 50% (v/v) glycerol and storage at -20°C.
4. C. L. Greer, C. L. Peebles, P. Gegenheimer, J. Abelson, *Cell* **32**, 537 (1983); E. M. Phizicky, R. C. Schwartz, J. Abelson, *J. Biol. Chem.* **261**, 2978 (1986); E. M. Phizicky, S. A. Consaul, K. W. Nehrke, J. Abelson, *J. Biol. Chem.* **267**, 4577 (1992).
5. S. M. McCraith and E. M. Phizicky, *J. Biol. Chem.* **266**, 11986 (1991); G. M. Culver, S. M. McCraith, S. A.

- Consaul, D. R. Stanford, E. M. Phizicky, *J. Biol. Chem.* **272**, 13203 (1997); S. L. Spinelli, S. A. Consaul, E. M. Phizicky, *RNA* **3**, 1388 (1997).
6. G. M. Culver, S. A. Consaul, K. T. Tycowski, W. Filipowicz, E. M. Phizicky, *J. Biol. Chem.* **269**, 24928 (1994).
7. G. M. Culver et al., *Science* **261**, 206 (1993).
8. R. J. DeLange, A. N. Glazer, E. L. Smith, *J. Biol. Chem.* **245**, 3325 (1970).
9. Crude extract from 430 g of strain JHRY-20-2Ca (5) was chromatographed successively on Blue Sepharose, heparin agarose, hydroxyapatite, and Orange A Sepharose to purify cyclic phosphodiesterase activity 4000-fold in relation to crude extract.
10. C. L. Peebles, P. Gegenheimer, J. Abelson, *Cell* **32**, 525 (1983); C. R. Trotta et al., *Cell* **89**, 849 (1996).

11. S. L. Spinelli, R. Kierzek, D. H. Turner, E. M. Phizicky, *J. Biol. Chem.* **274**, 2637 (1999).
12. H. H. Liao and F. Sherman, *J. Bacteriol.* **138**, 853 (1979); H. Morino, T. Kawamoto, M. Miyake, Y. Kaki-moto, *J. Neurochem.* **48**, 1201 (1987).
13. We thank M. Dumont, M. Gorovsky, and L. Tabak for comments and B. Pinsky for assistance. Supported by the University of Rochester Medical School and the Merck Genome Research Institute (grant 196 to E.M.P. and E.J.G.), NIH (grant GM52347 to E.M.P.), American Cancer Society (grant RPG-95-049-05-MBC to E.J.G.), the National Center for Research Resources (grant P41 RR11823 to S.F.), and the Merck Genome Research Institute (to S.F.). S.F. is an investigator of the Howard Hughes Medical Institute.

26 July 1999; accepted 23 September 1999

Nongenomic Transmission Across Generations of Maternal Behavior and Stress Responses in the Rat

Darlene Francis, Josie Diorio, Dong Liu, Michael J. Meaney*

In the rat, variations in maternal care appear to influence the development of behavioral and endocrine responses to stress in the offspring. The results of cross-fostering studies reported here provide evidence for (i) a causal relationship between maternal behavior and stress reactivity in the offspring and (ii) the transmission of such individual differences in maternal behavior from one generation of females to the next. Moreover, an environmental manipulation imposed during early development that alters maternal behavior can then affect the pattern of transmission in subsequent generations. Taken together, these findings indicate that variations in maternal care can serve as the basis for a nongenomic behavioral transmission of individual differences in stress reactivity across generations.

Individual differences in personality traits appear to be transmitted from parents to offspring. A critical question, however, concerns the mode of inheritance. Concordance studies with mono- and dizygotic twins have provided evidence for a genetic mechanism of transmission even of complex traits (1). In addition, parental behavior influences the development of the offspring (2) and could therefore serve as a mechanism for a nongenomic behavioral mode of inheritance. In the Norway rat (*Rattus norvegicus*), variations in maternal care are associated with the development of individual differences in behavioral and endocrine responses to stress in the offspring (3, 4). In the studies reported here we have examined the possibility that such variations in maternal care might be the mechanism for a behavioral transmission of individual differences across multiple generations.

Mother-pup contact in the rat occurs primarily within the context of a nest bout that begins

when the mother approaches the litter, gathers the pups under her, licks/grooms her pups, and nurses her offspring while continuing to occasionally lick/groom the pups, and terminates when the mother leaves the nest (5). Naturally occurring variations in maternal licking/grooming and arched-back nursing (LG-ABN) have been associated with the development of individual differences in hypothalamic-pituitary-adrenal (HPA) and behavioral responses to stress in the offspring (3, 4). As adults, the offspring of high LG-ABN mothers are behaviorally less fearful and show more modest HPA responses to stress than do the offspring of low LG-ABN mothers. The variation in maternal behavior may thus constitute a mechanism for the nongenomic behavioral transmission of fearfulness from parent to offspring. Alternatively, of course, the differences in fearfulness and those in maternal behavior may both be associated with a common genotype so that the observed continuity of individual differences from mother to offspring is mediated by a genomically based pattern of inheritance.

We found that the female offspring of high LG-ABN mothers showed significantly increased licking/grooming of pups in comparison with those of low LG-ABN mothers (12.9 ± 1.0 versus 6.9 ± 1.1; *P* < 0.001) (6),

Developmental Neuroendocrinology Laboratory, Douglas Hospital Research Centre, Departments of Psychiatry and of Neurology and Neurosurgery, McGill University, Montreal, H4H 1R3, Canada.

*To whom correspondence should be addressed. E-mail: mdmm@musica.mcgill.ca