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A gene trap vector system for identifying transcriptionally responsive genes

Enzo Medico[1,2]*, Giovanna Gambarotta[1], Alessandra Gentile[1], Paolo M. Comoglio[1], and Philippe Soriano[2]

We present a method for fast and efficient trapping of genes whose transcription is regulated by exogenous stimuli. We constructed a promoterless retroviral vector transducing a green fluorescent protein[1]–nitroreductase[2] (GFNR) fusion protein downstream from a splice acceptor site. Flow cytometric analysis of the infected population allows identification and sorting of cells in which the trap is integrated downstream from an active promoter. Conversely, the nitroreductase (NTR) moiety allows pharmacological selection against constitutive GFNR expression. Using hepatocyte growth factor (HGF) stimulation of liver cells[3] combined with either positive or negative selection, we recovered cell populations carrying traps in induced or suppressed genes, respectively. Several distinct responsive clones were isolated, and regulated expression of the trapped gene was confirmed at the RNA level. Positive and negative selection can be calibrated to recover traps in genes showing different levels of basal expression or transcriptional regulation. The flexibility and efficiency of the GFNR-based trap screening procedure make it suitable for wide surveys of transcriptionally regulated genes.

Gene traps are plasmid- or retrovirus-based vectors containing a reporter gene that is only expressed upon integration in a functional gene[4]. They were developed originally for studies of insertional mutagenesis in the mouse, on the basis of the disruption by trap integration of the endogenous transcript. Later, the gene trap approach was used to identify and characterize genes regulated by exogenous stimuli[5–9] or during development[10,11]. However, the trapping procedures developed in these cases were still too labor intensive for a genome-wide survey. Yet, gene traps have some intrinsic properties that make them complementary to RNA-based approaches such as DNA microarrays and serial analysis of gene expression (SAGE)[12,13]. First, cost-effective full-genome exploration takes place by random integration. Second, trapping generates a single-cell reporter of transcriptional activity, rather than assessing messenger RNA (mRNA) abundance in a cell population. Third, further functional studies may easily be accomplished directly on the trapped cells or on organisms derived from them. We therefore designed a new reporter gene and exploited the gene trap approach to achieve high efficiency in sequential positive and negative selection, as well as straightforward screening of numerous trapped clones.

We constructed a fusion between enhanced green fluorescent protein (EGFP, ref. 1; Clontech, Palo Alto, CA) and *Escherichia coli* NTR. We called this hybrid green fluorescent nitroreductase (GFNR). EGFP was chosen because it can be easily detected by flow cytometry and, owing to its long half-life (>24 h; ref. 1), allows detection of transient mRNA-level changes without prior knowledge of the response time course. NTR is capable of activating different prodrugs into alkylating agents that kill cells quickly and independently of their proliferation status[2]. We chose metronidazole (MN) among the possible prodrugs because it has no bystander effect[14]. GFNR functionality was evaluated by transient and stable transfection using a cytomegalovirus promoter–driven expression vector (pGFNR; Fig. 1A). Fusion of EGFP and NTR resulted in a slight loss of fluorescence and killing efficiency (10–15%; Fig. 1B). Green fluorescence correlated well with sensitivity to MN in a dose-dependent manner (Fig. 1C). We also observed that counterselection of low-GFNR expressing cells was progressively more effective with increased treatment duration (data not shown). After this preliminary validation, we incorporated GFNR in a retroviral trap (ROSA–GFNR; Fig. 1A). In the trap, the GFNR sequence is preceded by a splice acceptor (SA) that allows for expression of the reporter gene when integrated into an intron and that, together with the polyadenylation site, disrupts the tagged gene. The transcriptional orientation of GFNR is the reverse of that of the virus (i.e., RO, for reverse orientation), to avoid interference of the SA and polyA signals with the correct processing of retroviral RNA. Stable transfection of GP+E86 mouse ecotropic packaging cells[15] yielded a ROSA–GFNR producer line (GPE–ROSAGFNR) with a viral titer of 5×10^5 colony-forming units (CFU)/ml.

Infection and trapping experiments were performed on the mouse embryo liver cell line MLP-29, which displays a specific change in morphology in response to HGF (ref. 3). In each experiment, 10^7 cells were infected with 10^6 CFU of ROSA–GFNR to avoid multiple integrations in the same cell, and selected with G418 for trap integration. We observed that 0.1% of the G418-resistant cells expressed detectable GFNR (data not shown). Variation in trap expression levels provided evidence for integration at distinct loci. We subsequently optimized the combination among G418 selection, FACS analysis, MN counterselection, and HGF stimulation. In all cases, we found that counterselection by multiple treatments with mild doses of MN (5 mM) was less toxic and more efficient than a single, high-dose (10–20 mM) treatment. Treatment combinations and sequences for selection of traps in HGF-induced or -suppressed genes are summarized by the flowcharts in Figure 2A and 2B, respectively.

Flow cytometric analysis was performed on populations of MLP-29 cells selected as above and kept in the absence or presence of HGF. After one round of selection for induced genes (Fig. 2C), three subpopulations could be distinguished in the unstimulated sample: negative (peak around 4), low fluorescence (peak around 8), and medium fluorescence (peak around 30). In response to HGF (24 h treatment), a significant proportion of the low-fluorescence population reached medium fluorescence. No changes were observed in the negative fraction, which is mostly composed of sorting contaminants that have a selective advantage during MN counterselection. We also considered that the population displaying medium fluorescence in the absence of HGF probably was composed of traps that were constitutively active but not completely counterselected. To verify this hypothesis, we repeated the selection for HGF-induced traps on this already selected population. This second round of positive/negative selection yielded a population of almost totally responsive cells, displaying a basal level of fluorescence that was consistently increased on HGF stimulation (Fig. 2D). The overall higher fluorescence in the reselected population is probably due to the higher threshold set for the second sorting (50 instead of 30). This indicates that, by combining different thresholds of fluorescence sorting with variable stringency in the MN treatment, it is possible to retrieve traps in regulated genes covering vir-

[1]*Institute for Cancer Research and Treatment, University of Torino School of Medicine, 10060 Candiolo, Italy.* [2]*Program in Developmental Biology, Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98109.* **Corresponding author (emedico@ircc.unito.it).*

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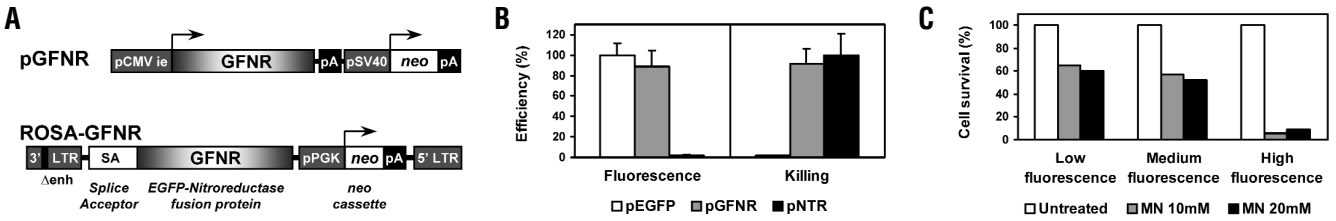


Figure 1. Construction and validation of GFNR. (A) GFNR expression vector (pGFNR) and retroviral trap (ROSA–GFNR). (B) Fluorescence and drug sensitivity of 293T cells transiently transfected with pEGFP, pGFNR, and pNTR. Relative pGFNR efficiency was estimated either by flow cytometric analysis (left) or by cell counting after 48 h of MN treatment (right). A fluorescence efficiency of 100% corresponds to the mean fluorescence intensity of the green fluorescent subpopulation in the pEGFP-transfected cells. A killing efficiency of 100% corresponds to the difference in cell number between mock-transfected and pNTR-transfected MN-treated cells. Error bars represent standard deviation of triplicates. (C) Correlation between fluorescence and MN sensitivity in stable transfectants. MLP-29 cells were transfected with pGFNR, selected with G418, and FACS-analyzed for green fluorescence before and after 48 h of MN treatment. For the analysis, three classes of fluorescence were identified and defined as low, medium, and high. In the untreated population, the number of cells in each class was assigned the 100% value. After treatment, the abundance of each fluorescent subpopulation was compared with the respective control sample, and percentage of survival was estimated. Error bars are not present because data were obtained by a FACS-based population analysis.

tually all ranges of basal expression and transcriptional response. Other gene trap approaches tend to completely eliminate traps with background expression[6–9], an event that may lead to loss of traps in regulated genes that have a basal activity. Also, in the case of suppressed genes, we could efficiently enrich for HGF-responsive traps (Fig. 2E), with the best fluorescence differential generally observed after 48 h of treatment, because of the long GFNR half-life (data not shown).

More than 200 individual clones were derived from five independent, progressively optimized trapping procedures, which involved sorting from a total of approximately 5×10^6 G418-resistant cells. After the first setup trappings, clones were derived in conjunction with fluorescence-activated cell sorting (FACS) to minimize trap redundancy. Screening clones for HGF responsiveness involved a simple and straightforward procedure: each clone was split in three wells, one to maintain the line and two for treatment with or without HGF for 24 h, followed by FACS analysis. Twenty to 40% of the clones turned out to be HGF responsive. The recovery of a high percentage of responsive clones was strictly dependent on the double-selection procedure, because randomly picked clones that had undergone only the positive selection showed no response to HGF (data not shown). Figure 3A illustrates the distribution of HGF responsiveness for 53 isolated clones (39 induced and 14 suppressed), as estimated by flow cytometric analysis before and after HGF stimulation. Note that many of the traps respond within a twofold range, which indicates that selection does not require extreme expression differences. Efficient exploration of a high number of genes showing minor transcriptional responses is a distinctive feature of this system; sampling and technical variability impair reliable detection of such changes by DNA arrays[16], and existing trap approaches have technical biases against handling large clone numbers or selecting minor responses[6–9].

If GFNR is to be an effective indicator of the promoter transcriptional activity, the fluorescence readout should reflect the abundance of GFNR-encoding cellular mRNAs. Indeed, GFP has been described as a reliable reporter[17]. This was confirmed by comparing flow cytometry data with GFNR northern blot analysis for two trapped clones, one induced and one suppressed by HGF (data not shown). To further validate the system, we obtained flanking sequences by 5′ rapid amplification of complementary DNA ends (RACE) on a total of 10 traps. In six cases, RACE

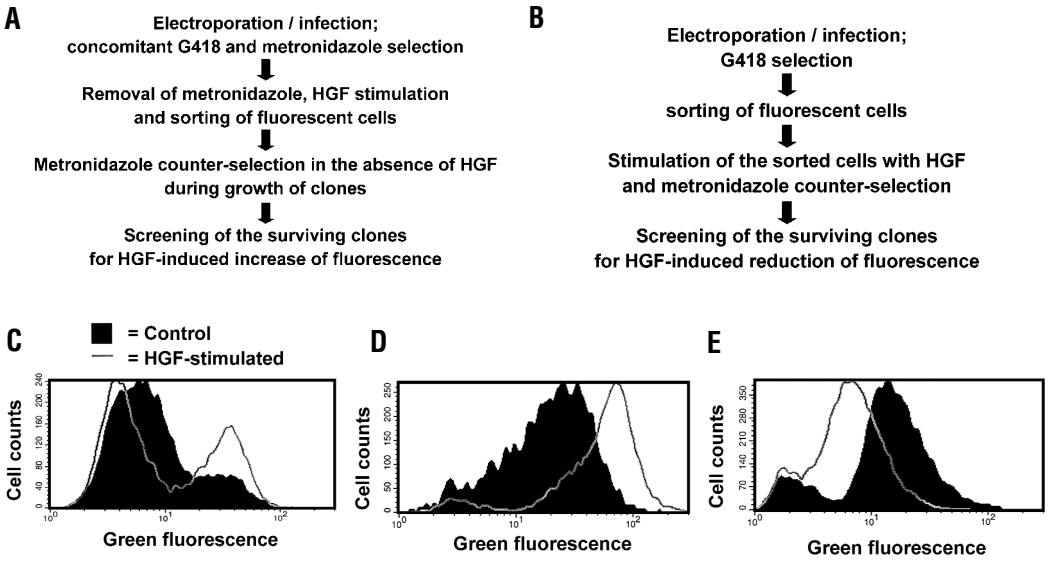


Figure 2. Selection of traps in HGF-regulated genes. (A) Schematic flowchart representation of the selection procedure for traps in HGF-induced genes. Infected cells first were treated with a combination of G418 (1.5 mg/ml) and MN (5 mM) to concomitantly achieve selection of integrants and counterselection of traps in constitutively expressed genes. Splitting cells daily reduces this treatment to three days, to avoid excessive cell duplication and consequent trap redundancy. MN then was removed for one day, and, after HGF stimulation, fluorescent cells were sorted by FACS and plated either as a population or as individual clones. A second MN treatment was performed on the sorted cells growing in the absence of HGF to increase the efficiency of selection against constitutively expressed traps. (B) Selection procedure for traps in HGF-suppressed genes. Infected cells were G418-selected and FACS-sorted to enrich for cells carrying traps in constitutively expressed genes. Selection of genes in which expression was downregulated by HGF was achieved by two or three rounds of HGF stimulation (24 h) and subsequent MN treatment (three to five days in the presence of HGF). (C) Fluorescence response of a population selected for HGF-induced traps analyzed by flow cytometry. (D) Response of the same population after a second round of selection for induced traps. Repeated selection yielded a population of almost totally responsive cells. (E) Response of a population selected for HGF-suppressed traps: upon HGF stimulation (48 h), most of the green fluorescent cells reduce their fluorescence.

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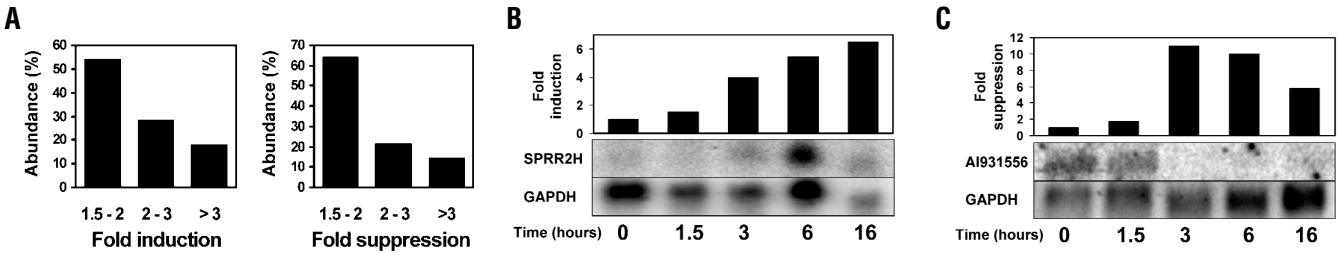


Figure 3. Responsiveness of trapped clones to HGF. (A) Individual clones were obtained from the trap selection procedures and subsequently analyzed by FACS for their response to HGF. Clones containing responsive traps were grouped into three classes according to the level of induction/suppression: between 1.5- and 2-fold, between 2- and 3-fold, and greater than 3-fold. Relative clone abundance was estimated for each class. Left panel, class distribution for HGF-induced traps (total clones = 39, maximum induction = 10 fold). Right panel, class distribution for HGF-suppressed traps (total clones = 14, maximum suppression = fivefold). (B, C) Time course northern blot analysis. Total RNA from untransfected MLP-29 cells stimulated with HGF for different times was blotted and hybridized with sequences from one induced and one suppressed trap, respectively: the SPRR2H gene (B; fivefold induction at 24 h by flow cytometry) and expressed sequence tag AI931556 (C; fivefold suppression at 24 h by flow cytometry).The housekeeper gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize samples for RNA abundance.

identified unique sequences, as illustrated in Table 1. Two sequences matched known genes – TMP (ref. 18) and a member of the small proline-rich gene family, SPRR2H (ref. 19) – that were never previously linked to the biology of HGF. TMP constitutes a good candidate because it is a tumorigenic, proinvasive gene whose expression is upregulated by the nuclear transcription factor Myc[18], and Myc is transcriptionally induced by HGF[20]. Moreover, its transcriptional regulation by HGF has been confirmed independently using DNA microarrays. Interestingly, microarray analysis also confirmed expressed sequence tag AI931556 as an HGF-suppressed gene expressed just above the threshold of significant detection (E. Medico, unpublished data). Two of four clones derived at the end of the same selection procedure (3E1) were found to carry the same trap in the TMP gene, which confirmed that cloning should be done as early as possible to minimize redundancy. In four cases, we found that a cryptic splice donor from the long terminal repeat was used instead of an upstream endogenous splice donor. This occurs when the retroviral gene trap vector is inserted in the 5′-terminal exon of the gene (P. Soriano, unpublished data). To assess an alternative gene identification approach, we successfully sequenced the genomic region flanking one of these traps by inverse PCR (ref. 21). Indeed, progress in the mouse genome sequencing probably will render inverse PCR the method of choice to concomitantly identify the trapped gene and define the precise integration site.

To verify that the transcriptional behavior of the wild-type genes is correctly mirrored by the trapped counterparts, we generated probes from two RACE fragments, derived from an induced and a suppressed trap, and used them in northern blot analysis on untransfected cells (Fig. 3B,C). For both genes, transcriptional regulation was found to correlate well with data obtained by GFNR fluorescence. Endogenous mRNA levels were found to be just above the detection limit of the northern blot technique, which indicates that GFNR is a sensitive reporter when related to RNA levels.

These data show that ROSA–GFNR trapping allows efficient generation and selection of reporter cells in which the transcriptional control of the trapped genes can be studied easily by FACS analysis. Virtually any cell line can be trapped to identify genes regulated by any exogenous stimulus. Preliminary setup can be conveniently performed at the cell population level, because optimal selection leads to >30% responsive traps, clearly detectable by FACS. More finely tuned analysis, such as isolation of specifically responsive genes, can be efficiently pursued either during the selection procedure or after obtaining individual clones. If embryonic stem cells are used in the screening procedure, responsive clones also might be used to generate mice in which the target genes are replaced by the reporter trap to monitor gene expression during development and search for loss-of-function phenotypes. Finally, cell clones bearing a trapped gene of particular biological or clinical interest can be used in high-throughput screens to identify genes, small molecules, or peptides that interfere with its function by modifying its expression.

Table 1. Identification of HGF-responsive trapped genes

Clone identification	HGF response (fold induction)[a]	Method of identification	Gene identity
3E1-6	+2.98	5′-RACE	Unknown
3E1-7	+2.12	5′-RACE	TMP
3E1-10	+2.62	5′-RACE	EST AA274109
3E2-6	+1.94	5′-RACE	Repeat
E3-2	+2.52	Inverse PCR	Unknown
H5-9	+4.61	5′-RACE	SPRR2H
H5-16	+1.57	5′-RACE	Repeat
HF-57	–5.26	5′-RACE	EST AI931556
Supp12	–1.58	5′-RACE	Unknown

[a]Estimated by FACS analysis of the trapped clone before and after HGF stimulation.

Experimental protocol

Construction of plasmid and retroviral vector. pGFNR was constructed by inserting the NTR coding sequences (gift of R. Palmiter) into pEGFP-C1 (Clontech) downstream from EGFP and in the same translational frame. pNTR subsequently was constructed by removing the EGFP coding sequence from pGFNR. To generate the gene trap cassette, we excised GFNR from pGFNR and inserted it by blunt-end ligation into pSA-βGal-PGKneobpA (ref. 22), from which the βGal moiety had been removed. The trap cassette SA-GFNR-PGKneobpA was inserted into the self-inactivating, MoMuLV-derived retroviral vector pGen⁻ (ref. 23) to produce the retroviral vector pGen⁻-ROSAGFNRPGKneobpA (pROSA–GFNR).

Cell culture and viruses. MLP-29 cells were cultured as described[3]. 293T cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FBS and transiently transfected using the Lipofectin Reagent (Gibco BRL, Grand Island, NY), according to the manufacturer's protocol. MN (Sigma, St. Louis, MO) was directly diluted in serum-free medium to obtain a 30 mM stock solution. Recombinant HGF was obtained as described[24]. Derivation of retrovirus-producing cells in the GP+E86 packaging line, infection, and estimation of the virus titer were performed as described[23].

Flow cytometry. MLP-29 cells were detached by trypsin–ethylenediamine tetraacetic acid treatment, diluted in cold DMEM–10% FBS, carefully mixed to disrupt cell aggregates and allowed to sediment for 1 min to eliminate residual clumps. Flow cytometry and cell sorting were conducted on Becton Dickinson (San Jose, CA) FACS Calibur and FACS Vantage cytometers, respectively. To improve sensitivity in GFNR detection, we carried out analyses by comparing for each cell the fluorescence in the green channel (FL1) with fluorescence in the red channel (FL3), which indicated individual autofluorescence. For quantita-

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tive analysis of GFNR expression, average red fluorescence was subtracted from average green fluorescence to obtain an estimate of specific GFNR fluorescence.

Identification of trapped genes and northern blot. Total RNA was prepared from cell cultures using the Trizol reagent (Gibco BRL), according to the manufacturer's protocol. To recover and identify trapped genes, we did a 5′ RACE on total RNA by using the Smart RACE cDNA amplification kit (Clontech), according to the manufacturer's protocol, using two EGFP primers (5′-CTTGTGGC-CGTTTACGTCGCCG-3′, 5′-CGGTGAACAGCTCCTCGCC-3′) in the first round and nested PCR, respectively. Inverse PCR was performed as described[21], using a SupF primer (5′-GGAGCAGGCCAGTAAAAGCATTACCCGTG-3′) and a NTR primer (5′-AGTAGCGTTTTGATCTGCTCGGCCTGTTCC-3′), followed by PCR with two nested primers (5′-CTTCCCCCACCACCATCACTTT-3′, 5′-TAGTGGAATGACGCTTTAAGGC-3′). PCR products were cloned using the Invitrogen (Carlsbad, CA) TOPO TA cloning kit and sequenced using fluorescent dye terminators on a Perkin-Elmer (Foster City, CA) 310 sequence analyzer, according to the manufacturer's protocols. For quantitative northern blot analysis, bound radioactivity was detected and quantified using a STORM 840 phosphorimager apparatus (Molecular Dynamics, Sunnyvale, CA).

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Efficient FLPe recombinase enables scalable production of helper-dependent adenoviral vectors with negligible helper-virus contamination

Pablo Umaña[1,4], Christian A. Gerdes[1], Daniel Stone[1], Julian R.E. Davis[2], Daniel Ward[3], Maria G. Castro[1,5], and Pedro R. Lowenstein[1,5]*

Helper-dependent (HD), high-capacity adenoviruses are one of the most efficient and safe gene therapy vectors, capable of mediating long-term expression[1–12]. Currently, the most widely used system for HD vector production avoids significant contamination with helper virus by using producer cells stably expressing a nuclear-targeted Cre recombinase and an engineered first-generation helper virus with parallel *loxP* sites flanking its packaging signal[1,3–12]. The system requires a final, density-based separation of HD and residual helper viruses by ultracentrifugation to reduce contaminating helper virus to low levels. This separation step hinders large-scale production of clinical-grade HD virus[13]. By using a very efficient recombinase, *in vitro*–evolved FLPe (ref. 14), to excise the helper virus packaging signal in the producer cells, we have developed a scalable HD vector production method. FLP has previously been shown to mediate maximum levels of excision close to 100% compared to 80% for Cre (ref. 15). Utilizing a common HD plasmid backbone[1,7,8,10–12], the FLPe-based system reproducibly yielded HD virus with the same low levels of helper virus contamination before any density-based separation by ultracentrifugation. This should allow large-scale production of HD vectors using column chromatography–based virus purification[13].

*[1]Molecular Medicine and Gene Therapy Unit, Room 1.302 Stopford Building, School of Medicine, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom. [2]Endocrine Sciences Research Group and [3]ARC Epidemiology Unit, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, United Kingdom. [4]Current address: GlycArt Biotechnology AG, Einsteinstrasse, 8093 Zurich, Switzerland (pablo.umana@glycart.com). [5]Address from 1 July 1 2001: Board of Governors Gene Therapeutics Research Institute, 5th Floor Room R-5089, Research Pavilion, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, CA 90048-1860, USA. *Corresponding author (lowenstein @man.ac.uk or PRLowenstein@cs.com).*

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