Gamma Tropomyosin Gene Products Are Required for Embryonic Development

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Received 26 August 2003/Accepted 12 November 2003

The actin filament system is essential for many cellular functions, including shape, motility, cytokinesis, intracellular trafficking, and tissue organization. Tropomyosins (Tms) are rod-like components of most actin filaments that differentially affect their stability and flexibility. The Tm gene family consists of four genes, α Tm, β Tm, γ Tm (Tm5 NM, where "NM" indicates "nonmuscle"), and δ Tm (Tm4). Multiple isoforms of the Tm family are generated by alternative splicing of three of these genes, and their expression is highly regulated. Extensive spatial and temporal sorting of Tm isoforms into different cellular compartments has been shown to occur in several cell types. We have addressed the function of the low-molecular-weight Tms encoded by the γ Tm gene by eliminating the corresponding amino-terminal coding sequences from this gene. Heterozygous mice were generated, and subsequent intercrossing of the F₁ pups did not result in any viable homozygous knockouts. Genotype analysis of day 2.5 morulae also failed to detect any homozygous knockouts. We have failed in our attempts to delete the second allele and generate in vitro double-knockout cells, although 51 clones displayed homologous recombination back into the originally targeted locus. We therefore conclude that low-molecular-weight products from the γ Tm gene are essential for both embryonic development and cell survival.

The actin filament system is a fundamental structural network essential for many cellular functions, including the regulation of cell shape, motility, cytokinesis, intracellular trafficking, and tissue organization (1). Tropomyosins (Tms) are a highly conserved family of actin binding proteins, and these rod-shaped dimers have been shown to differentially affect the stability and flexibility of actin filaments (9, 14, 25). The Tm molecule consists of two parallel chains arranged in an α -helical coiled coil (3), and polymers of Tm insert into the major groove of the actin filament (34).

The mammalian Tm gene family consists of four genes, α Tm, β Tm, γ Tm (Tm5 NM, where NM indicates nonmuscle), and δ Tm (Tm4). Multiple isoforms (more than 40) of the Tm family are generated by alternative splicing (21, 25), and the expression of these isoforms appears to be highly regulated. Many nonmuscle isoforms are generated from the γ Tm gene (Fig. 1), and to date, 11 nonmuscle (NM1 to NM11) isoforms have been identified (12). The exact role for all of the 11 nonmuscle isoforms is yet to be determined. Extensive spatial sorting of some Tm isoforms into different cellular compartments has been shown for several cell types (33, 43). Studies of Tm isoform sorting suggest that individual isoforms may confer specific functional properties to actin filaments. It has been reported that some higher-molecular-weight Tms (Tm1, Tm2, and Tm3) contribute to the stability of actin filaments and to the regulation of cell morphology and division (5, 13, 14, 22, 29). In contrast, lower-molecular-weight Tm isoforms such as γ Tm nonmuscle (Tm5 NM) products have been reported at the leading edge of fibroblasts, suggesting that they may have

a specific role in membrane organization, motility, and growth (7, 15, 28, 29, 39). γ Tm gene products have been shown to be associated with Golgi apparatus-derived vesicles, which also suggests a role for low-molecular-weight Tms in vesicle trafficking (20). However, the overexpression of the γ Tm NM1 isoform in B35 cells shows a distinct increase in stress fibers, suggesting a broad role for isoforms from this gene (7).

Neuronal development and maturation are accompanied by dynamic spatial sorting of Tm isoforms into different cellular compartments (15, 16). Several isoforms from all four of the mammalian Tm gene families have been identified in brain tissue, and the lower-molecular-weight Tms are the most abundant of the various Tm gene products (17, 37, 42). The segregation of γ Tm nonmuscle gene products has been reported in the establishment of neuronal polarity during embryonic development, axon outgrowth (18, 36, 43), and maturation in the brain (41).

Tm isoforms have been reported to occur in mouse embryonic stem (ES) cells and in the four to eight cell stages of early embryo development (8, 31), with products from all four Tm genes being present in ES cells at various levels of expression. However, only low-molecular-weight Tm gene products are expressed in the early developing embryo, with high-molecular-weight Tm gene products being notably absent. Previous studies of the α Tm gene (4, 35) have shown that products from this gene are required for the normal growth and development of cardiac tissue.

The aim of this study was to investigate whether morphogenesis and the establishment of tissue structure are dependent on the use of specific Tm isoforms or whether the presence of multiple Tm genes may give a degree of functional redundancy. Since little is known about the contribution of the γ Tm gene in early development, we eliminated all nonmuscle isoforms generated by the mouse γ Tm gene. The results es-

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FIG. 1. γ Tm (Tm5 NM) gene and nonmuscle isoforms. The entire γ Tm gene and alternatively spliced nonmuscle variants (12) are shown. All nonmuscle products contain the exon 1b promoter, with a choice of either exon 6a or 6b, and variations of carboxy-terminal exon 9. Open boxes represent untranslated regions (UTRs), lines represent introns, and A represents the poly(A) tail. The CG3 antibody (CG3 Ab) corresponds to a monoclonal antibody recognizing an epitope within exon 1b.

establish that embryonic development requires nonmuscle products from this gene as early as day 2.5. In addition, we show that ES cells require nonmuscle γ Tm products for cell growth. We conclude that products from the γ Tm gene are essential for both embryonic development and cell survival.

MATERIALS AND METHODS

Construction of a targeting vector. Genomic clones of the mouse γ Tm gene were isolated by screening a 129-mouse genomic library (lambda DASH II vector; Stratagene, La Jolla, Calif.), with oligonucleotide probes directed towards γ Tm exons 1a to 2b (180 bp) and exon 1b (150 bp). Two genomic clones of approximately 18 kb were isolated and subjected to restriction endonuclease mapping and sequencing. A 7.9-kb *Bam*HI fragment containing exon 1b and a flanking intron sequence was isolated and subcloned into an Sp72 vector (Promega Corporation, Madison, Wis.). A cassette containing LoxP-pGK thymidine kinsase-pGK Neo^r-LoxP (obtained from Allan Bradley) was inserted between the *Nco*I sites, replacing the 132-bp coding sequence of exon 1b as well as 64 bp of the intronic sequence 3' to exon 1b.

Generation of ES cells heterozygous for γ Tm. The targeting vector (40 μ g) was linearized with *Nde*I and transfected into R1 (32) ES cells by electroporation (0.24 kV; 500 μ F; time constant of 11.0 ms). ES cells were subsequently cultured for 10 days in knockout Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, Calif.) containing 10% fetal bovine serum, 2 mM glutamine, 10 mM non-essential amino acids, 100 μ M β -mercaptoethanol, and 1,000 U of ESGRO (Chemicon International, Temecula, Calif.) per ml, with selection done using 300 μ g of G418 (Invitrogen) per ml. A total of 240 clones were picked and grown to confluence in 24-well tissue culture plates. Genomic DNA was isolated, and 10 μ g was digested with *Bam*HI. Digested genomic DNA was subjected to Southern blot analysis by using a 340-bp external probe 5' to the region of homologous recombination. Homologous recombinant clones were expanded and reelectroporated with 40 μ g of cytomegalovirus-Cre recombinase (Stratagene), followed by selection in 1 μ M ganciclovir (Roche Products, Dee Why, New South Wales,

Australia). Surviving ES cell clones were isolated, expanded, screened by PCR (as described below), and sequenced for the presence of a single LoxP and for the absence of the coding region of exon 1b.

Generation of heterozygous mice. ES cell clones that had undergone both homologous and subsequent Cre-mediated recombination were injected into cultured BALB/c blastocysts as described by Lemckert et al. (26), followed by transfer into pseudopregnant foster mice. Chimeric males were bred against both BALB/c and 129/SvJ females. All animal experimentation was performed in accordance with institutional guidelines and guidelines of the National Health and Medical Research Council, Canberra, Australia.

Genomic tail DNA was isolated from the tails of F₁ pups and genotyped by PCR for the presence of both wild-type and knockout alleles. An oligonucleotide primer set common to both alleles (For. 9341, 5'-GGCTACAACGCCGAGCG GAG-3', and Rev. 9342, 5'-CGGGGCTCGATTCTTTCCAG-3') was made to regions on either side of the deletion and used in the PCR, based on the prediction that the For. 9341 and Rev. 9342 primers would generate a 405-bp fragment for the wild-type allele and a 355-bp fragment for the mutated allele. A primer further upstream (For. 9399, 5'-GAGGCACCGGATAAGAGAGG-3') was used as an alternative in the PCR and gave identical results. The PCR products were analyzed on a 2% agarose gel in 1 $\times$ Tris-acetate-EDTA.

Breeding and genotyping of embryos and morulae. Mouse γ Tm gene heterozygous (F₁) pups were intercrossed in order to generate null pups. F₂ progeny were genotyped by PCR. As no live births were determined as $-/-$, day 10 to 12 embryos were isolated, and genomic DNA was prepared for genotyping by PCR as described above. Day 2.5 morulae were also isolated and cultured in M16 embryo medium (Sigma, St. Louis, Mo.) for a further 1.5 days to increase cell numbers. Individually cultured morulae were collected in minimum-volume M16 embryo medium, and DNA was prepared by lysing morulae with 10 μ l of 50 mM KOH at 95°C for 10 min, followed by the addition of 10 μ l of 50 mM Tris-HCl, pH 7.5. Morula DNA was genotyped by PCR as described above.

Western blot analysis of mouse tissue and cell lines. Protein was prepared from the brain tissue of adult mice heterozygous for the γ Tm gene and control littermates. Protein was also prepared from wild-type ES cells and primary mouse embryonic fibroblasts (PMEF) that were cultured as described above. Brain tissue, ES cells, and PMEF pellets were collected and homogenized in a sodium dodecyl sulfate (SDS) solubilization buffer (10 mM Tris-HCl [pH 7.6], 2% SDS, 2 mM dithiothreitol), and the protein concentration was determined by using a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). Total protein (10 μ g) was denatured and reduced in sample buffer containing β -mercaptoethanol. Proteins were analyzed by SDS-15% polyacrylamide gel electrophoresis containing a low level (0.9%) of bisacrylamide. Gels were transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.) via electroblotting techniques. The membranes were blocked in 5% skim milk powder in Tris-buffered saline (TBS), pH 7.5, and washed in TBS with 0.1% Tween 20 three times for 5 min each.

The primary antibodies used for Western analysis were mouse monoclonal antibody CG3 (28), a kind gift provided by J. Lin, University of Iowa; rabbit antiserum WS α /9d (36); and rabbit antiserum WD4/9d (19). The CG3 antibody recognizes an epitope within the 1b exon of the γ Tm gene, and therefore all 11 known nonmuscle products were detected. The WS α /9d rabbit polyclonal antiserum recognizes products from both the α Tm and β Tm genes that contain the 9d exon (Tm1, -2, -3, -5a, -5b, and -6). The WD4/9d rabbit polyclonal antiserum recognizes a single exon, 9d, containing product from the δ Tm gene. Primary antibodies were diluted at a ratio of 1:1,000 in TBS and were incubated with the membranes at room temperature for 1 h. The membranes were then washed in TBS with 0.1% Tween 20 (as described above) prior to addition of a secondary antibody at a 1:5,000 dilution (goat anti-mouse immunoglobulin [Ig] or goat anti-rabbit Ig conjugated to horseradish peroxidase; Jackson ImmunoResearch, West Grove, Pa.). Western bands were detected by using a Western Lightning kit (Perkin-Elmer Life Sciences, Boston, Mass.) followed by exposure to Fuji (Tokyo, Japan) Super RX X-ray film.

Screening for double-knockout ES clones. Two ES cell clones that had undergone both homologous and Cre-mediated recombination were reelectroporated with the targeting construct, followed by growth and selection in G418 as described above. Clones were expanded and selected for analysis by either PCR or Southern techniques as described above.

RESULTS

Targeting of the γ Tm gene. In order to eliminate all non-muscle Tm isoforms encoded by the γ Tm gene (Fig. 1) and to study the developmental impact in mice, the first coding exon (1b) and donor splice junction were knocked out of the γ Tm

FIG. 2. γ Tm gene and targeting construct. (A) γ Tm gene showing the subcloned 7.9-kb *Bam*HI fragment that includes nonmuscle exon 1b. A 340-bp probe for Southern analysis is shown. (B) The targeting construct was made by deleting a 200-bp region between the *Nco*I sites and inserting the 4-kb *LoxP*-thymidine kinase (TK)-*Neo*^R-*LoxP* cassette. Flanking DNA of approximately 2 kb is shown on either side of the cassette. (C) Homologously recombined alleles can be detected by *Bam*HI digestion and Southern analysis with a 340-bp probe 5' to the targeted region. Open boxes represent UTRs. Arrows indicate the direction of transcription.

gene (Fig. 2A). The coding sequences and splice donor site of exon 1b were replaced by a 4-kb cassette containing *Neo*^R and thymidine kinase genes flanked by *LoxP* sites (Fig. 2B). Homologous recombination events in multiple ES cell clones were identified by Southern analysis (Fig. 2C), where both the parental allele at 7.9 kb and the mutated allele at 5.0 kb were observed. The two ES cell clones shown in Fig. 2C were expanded, and the selection cassette was removed by transient transfection with a cytomegalovirus-Cre recombinase plasmid to yield the mutated gene structure shown in Fig. 3A.

Generation of heterozygous animals. One of the heterozygous cell lines was used to generate a male chimera that was backcrossed to a 129/SvJ female. Germ line transmission was assessed by the PCR screening strategy shown in Fig. 3, and the identities of the two products obtained at 405 and 355 bp were verified by DNA sequence analysis to be the wild-type and mutant genes, respectively (data not shown). Genotyping results obtained from F₁ pups showed that the knockout allele was transmitted to 5 out of the 11 F₁ animals. Subsequent intercrossing of the heterozygous F₁ mice has generated over

FIG. 3. PCR screening of ES lines and mouse F₁ and F₂ progeny. (A) Oligonucleotide primers (For. 9341 and Rev. 9342) were made to the 5' UTR of exon 1b and to an intron region 3' of exon 1b. The expected sizes of PCR products from both the wild-type (WT or wt) and disrupted alleles following Cre-mediated (*Cre*^d) recombination are shown. (B) PCR screening of both F₁ and F₂ progeny yielded products with the expected sizes of 405 and 355 bp, respectively. -ve, negative; +ve, positive.

70 F₂ pups. PCR genotype analysis of these mice has failed to identify any homozygous knockout animals (Table 1). We therefore conclude that one or more products from the γ Tm gene are essential for full embryonic development.

Genotyping of embryos. Developing mouse embryos at days 10 to 12 were collected and genotyped by PCR screening. This approach failed to identify any -/- genotype (data not shown). In order to determine how early during development lethality occurs, day 2.5 morulae were collected and individually cultured to increase cell numbers before PCR analysis. Genotype analysis of the cultured morulae also failed to show any γ Tm homozygous knockouts (PCR genotyping of a total of 31 morulae on day 2.5 showed 5 +/+ morulae, 26 +/- morulae, and 0 -/- morulae). These results suggest that the activity of the γ Tm gene is required very early in development and certainly before implantation.

Screening for double-knockout ES cells. We attempted to mutate the wild-type allele in our single-knockout ES cells to generate double-knockout cells. This seemed to be a realistic

TABLE 1. PCR genotype analysis of F₁ and F₂ mouse pups

Generation	No. of mice that are:			Total
	+/+	+/-	-/- ^a	
F ₁	6	5	N/A	11
F ₂	17	55	0	72

^a N/A, not applicable.

TABLE 2. Screening for double-knockout ES cells

ES line and screen (no. of clones)	No. of clones targeting:		
	Original locus	Random recombinants	Double-knockout ES cells
Line 1			
Southern screen (144)	24	120	0
PCR screen (109)	20	89	0
Line 2: PCR screen (144)	7	137	0

approach since we had observed that the targeting of this locus in the original electroporation experiments had occurred with frequencies between 10 and 15%. We used both the PCR method and Southern analysis for genotyping the heterozygous pups. More than 300 ES clones were assayed, and we were not able to detect any homozygous-knockout ES cells in either of the two ES lines (Table 2). From the originally observed recombination rate, we expected to detect multiple clones targeted in the remaining wild-type allele. Indeed, a total of 51 clones showed targeting of the originally targeted allele, indicating that both ES clones tested were competent for homologous recombination into this locus. We therefore conclude that the activity of the γ Tm gene is required for ES cell viability.

Heterozygous deletion of exon 1b does not impact protein levels derived from the γ Tm gene. Western analysis on whole adult brain tissue of wild-type and heterozygous mice was performed. The Western blot was exposed to an antibody, CG3, that recognizes products from the coding region of exon 1b and therefore will detect all 248-amino-acid γ Tm nonmuscle isoforms (Fig. 1). The results in Fig. 4A show no difference in the amounts of γ Tm gene nonmuscle products expressed between wild-type and heterozygous mouse brains. We therefore conclude that haploinsufficiency of this gene does not result in a reduced accumulation of protein.

Western blot analysis of ES clones. The failure to generate any double-knockout ES cells was surprising since Tms are encoded by four different genes. The results therefore suggest that none of the remaining Tm genes are capable of rescuing the deletion of the exon 1b-containing products of the γ Tm gene although two of the other genes make similar 248-amino-acid Tms. In order to confirm that these genes are active in ES cells, we performed Western blot analysis on ES cell protein. The expression of products from all four Tm genes was observed by comparing wild-type ES cells to PMEF by using Tm antibodies. Figure 4B shows that there is substantial expression of the other Tm gene products in ES cells, and it is therefore apparent that these Tm isoforms were not able to rescue the phenotype of the γ Tm homozygous knockouts.

DISCUSSION

The γ Tm gene is not functionally redundant. We have shown in this paper that one or more products from the mouse γ Tm gene are absolutely required for ES cell survival and early embryogenesis. This result occurs despite the finding that three other Tm genes are active in both ES cells and early mouse embryogenesis (8, 31) (Fig. 4). The essential function provided by the γ Tm gene, therefore, cannot be rescued by the products expressed by the other Tm genes in ES cells and early embryos.

However, it does remain a formal possibility that there is a Tm isoform not expressed in ES cells or in early embryos, which could rescue the γ Tm gene knockout if it were expressed in these cells.

The essential function provided by the γ Tm gene most likely involves the γ Tm NM1 and/or NM2 isoforms because the exon 9d carboxy terminus accounts for most if not all products from this gene in ES cells (B. Vrhovski and P. Gunning, unpublished observation), and these two isoforms are the only isoforms which use this carboxy terminus (Fig. 1). Neither the 9a nor the 9c alternative carboxy terminus can be detected in ES cells (J. Hook, B. Vrhovski, and P. Gunning, unpublished observation). The isoforms most similar to γ Tm NM1 and NM2 are Tm4 from the δ Tm gene and Tm5a and Tm5b from the α Tm gene. Since all three isoforms are expressed in ES cells (Fig. 4B), it seems unlikely that the failure to rescue the γ Tm gene knock-

FIG. 4. Western blot analysis of mouse tissue, ES cells, and PMEF. (A) Total adult brain tissue protein (10 μ g) from both wild-type and heterozygous mice was analyzed with the CG3 antibody to detect all products from the 1b exon of the γ Tm gene. (B) Total cell protein (10 μ g) from ES cells (wild type) and PMEF were analyzed with antibodies for the presence of other high- and low-molecular-weight Tm gene products. The antibodies CG3, Tm4/9d, and WS α /9d recognize all nonmuscle γ Tm products; Tm4; and Tm6, -1, -2, -3, -5a, and -5b, respectively.

out is due to a lack of expression of the most closely related isoforms. Rather, it appears more likely that γ Tm NM1 and/or NM2 is not functionally redundant in terms of its role in ES cell survival.

The essential role(s) of γ Tm isoforms may be any one of a number of possibilities. It has previously been shown that γ Tm isoforms are sorted to specific intracellular sites in neurons (18, 19, 36, 42), the brain (41), epithelial cells, and fibroblasts (33) and that their location is subject to developmental (43) and cell cycle (33) regulation. In particular, a number of γ Tm isoforms have been shown to be associated with the cell cortex (28), stress fibers (7), areas of polarized growth (18, 36), and the Golgi apparatus (20). A number of isoforms from the other Tm genes are also found in the cell cortex (28, 29, 39), stress fibers (7, 33), and areas of polarized growth (18, 36), but γ Tm products are the only ones thus far detected in the Golgi apparatus (20). It is therefore possible that the elimination of γ Tm NM1 and/or NM2 from the Golgi apparatus cannot be rescued by another isoform because no other isoform may be sorted to that structure. Alternatively, Clayton and Johnson (8) have observed products from the γ Tm gene, but not high-molecular-weight products from the α Tm and β Tm genes, associated with the cleavage furrow of dividing cells in early embryos. Failure to produce γ Tm gene products may therefore compromise cell division, which would explain embryonic lethality and the lack of ES cell viability, again based on the cell's inability to sort another isoform to the cleavage furrow.

Intrinsic to the idea of functional redundancy is the ability of isoforms to perform the same or similar functions in the same spatial and temporal context. The differential sorting of Tm isoforms may therefore preclude, at least in some situations, functional rescue of one isoform by another. Whereas the mechanism of isoform sorting is unclear, it is unlikely that Tm isoforms are all competing for inclusion into all the same structures. The more highly regulated the specific targeting of Tm isoforms, the less likely that isoforms can shift from one compartment to another based on the absence of an isoform in a particular compartment.

A similar lack of functional redundancy has been observed in the actin gene family. The elimination of either cardiac or skeletal actin cannot be rescued by other actin genes (10, 24). This finding suggests that neither of the core components of the microfilament easily accommodate the loss of specific isoforms.

Tms perform essential functions in many organisms. The identification of essential roles played by γ Tm isoforms in cell survival and early mouse development sits well with studies of a number of organisms. In *Saccharomyces cerevisiae*, the elimination of both TPM1 and TPM2 genes is lethal, indicating that Tm performs an essential function (11). It is also notable that the elevated expression of the yeast TPM2 gene cannot compensate for the loss of the TPM1 gene, indicating a lack of functional redundancy (11). Recent studies have shown that Tm is required to stabilize actin filaments in yeast, which are required for vesicle transport from the Golgi apparatus to sites of polarized growth (6). Similarly, if γ Tm NM1 and/or NM2 is involved in short-range transport of vesicles along actin filaments, its elimination is expected to have very serious consequences for cell survival (20). In *Caenorhabditis elegans*, inactivation of the third and fourth isoforms of the *tmy-1* gene have

revealed a requirement for low-molecular-weight Tm isoforms during development (2).

The deletion of the only known cytoskeletal Tm genes in *Drosophila melanogaster* (*TmI* and *TmII*) leads to altered head morphogenesis, shorter sarcomeres, and disruptions to thick and thin filament packing (23, 38, 40). However, a rescue experiment with a nonflight muscle Tm isoform showed that the phenotype of the mutant indirect flight muscle Tm could be restored (30). More recently, mutations of the *TmII* gene were shown to impact the regulation of dendritic growth (27). In mice, targeted elimination of only the striated muscle isoform (4) or all isoforms (35) from the α Tm gene leads to embryonic lethality at embryonic day 9.5 (E9.5) to E13.5 or E8 to E11.5, respectively. It is therefore quite unequivocal that Tms perform essential cellular functions, and where multiple isoforms are generated, there is little evidence for functional equivalence of isoforms.

The α Tm gene findings together with the γ Tm gene findings presented in this paper show evidence for three different stages of development requiring three different isoforms or groups of isoforms. The difference between the α Tm striated muscle isoform knockout (4) and the knockout of all α Tm isoforms (35) suggests that one or more nonstriated isoforms are required at approximately E8 to E9.5 for normal development and that the striated isoform is required at E9.5 to E13.5. It is notable, however, that the elimination of all α Tm isoforms is not as severe as that of just the low-molecular-weight γ Tm isoforms.

The impact of Tm elimination on different organisms also indicates that Tms are required for a number of different cellular functions. The yeast Tm knockout and mouse γ Tm knockout results suggest a role for Tm in cell survival possibly related to vesicle transport and/or cell division. Results from the *C. elegans*, *Drosophila*, and α Tm studies of mice indicate a role in development. Finally, the α Tm knockout also indicates a role in cardiac function, as might be expected because of its pivotal role in regulating myosin head engagement with the actin filament. The data are therefore consistent with the proposal that Tm isoforms are responsible for a diverse set of biological functions and that this is underpinned by the temporal and spatial regulation of isoform expression.

ACKNOWLEDGMENTS

This work was supported by grants to P.G. and G.S. by the National Health and Medical Research Council (NH&MRC). P.G. is a Principal Research Fellow of the NH&MRC.

We thank past and present members of the ORU, particularly B. Vhrovski, for helpful discussions.

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