

Tuberous sclerosis complex: A *Drosophila* connection

Recent findings based on experiments with *Drosophila melanogaster* significantly advance our understanding of a human disease known as tuberous sclerosis complex (TSC). The present note begins with background information and goes on to explain what these findings are.

Tuberous sclerosis complex is a neuro-cutaneous genetic disorder which affects several organs in the human body including the brain, heart, kidneys, eyes, skin, spleen, liver and lungs. TSC is characterized by hamartomas (benign outgrowths, predominantly of a cell or tissue type that occurs normally in the organ) which rarely change to malignancy in the affected organs. Clinical symptoms of TSC include cortical tubers in the brain, seizures, mental retardation, autism, unguis and periungual fibromas, shagreen patches and hypopigmented macules on the skin, angiofibromas on the face, micro or macrocysts and angiomyolipomas in kidneys, cardiac rhabdomyomas, retinal hamartomas, hyper-inflated lungs and dental pits. In a majority of cases clinical symptoms are not evident or overlooked. Seizures are the most frequent clinical symptoms and are present in 86% of TSC patients. Patients who had frequent seizures in infancy develop mental retardation to some extent during the later part of their lives; approximately 55% of TSC patients are mentally retarded. A definite diagnosis of TSC is based on the presence of any one of the following symptoms: cortical tubers or subependymal nodules in the brain as diagnosed by computerized tomography (CT) and/or magnetic resonance imaging (MRI), retinal hamartomas, facial angiofibromas, unguis fibromas, and angiomyolipomas with or without cysts in kidneys as diagnosed by ultrasound, CT scan or MRI (NTSA 1988). A patient may have only one, some or all of the above symptoms. The estimated prevalence of TSC is 1 in 5,000–6,000 live births in western populations, and it occurs in all racial groups. The actual incidence of TSC is not known in India, but it seems to be as common as in western countries. It is estimated that approximately two-thirds of TSC cases are either sporadic or new mutations (i.e. with no previous family history).

TSC is apparently an autosomal dominant disorder which exhibits both incomplete penetrance and variable expression. It shows genetic heterogeneity with two known loci: TSC1 on chromosome 9q34 mapped in 1987 and TSC2 on chromosome 16p13-3 mapped in 1992. Multi-generation families are evenly divided between both loci. Patients with mutations in TSC1 and TSC2 genes cannot be separated clinically, suggesting that both genes work probably in the same pathway. The TSC1 gene, which was isolated in 1997, has 23 known exons (exons 1 and 2 being non-coding) with an approximately 8.6 kb long transcript including an unusually long 4.5 kb 3' untranslated region (3' UTR) with five non-overlapping polyA signals (AATAAA or ATTAAA). The significance of a long 3' UTR and five polyA signals is not yet obvious. TSC1 encodes a protein, hamartin, of 1164 amino acids (130 kDa protein) with a single potential transmembrane domain at amino acids 127–144 coded by exon 6 and a predicted 266 amino acids long coiled-coil domain (beginning at amino acid residue 730) coded by exons 17–23. The GenBank search shows a possible yeast homologue of TSC1 encoding a protein of 103 kDa with unknown function.

The TSC2 gene, which was isolated in 1993, has at least 42 known exons (41 coding and one non-coding leader exon 1a) and codes for a 5.5 kb long transcript with two overlapping polyA signals. The TSC2 gene encodes a protein, tuberin of, 1784 amino acids (198 kDa protein) which shows stretches of homology of approximately 160 amino acid residues (encoded by exons 34–38) to a GTPase activating protein (GAP) GAP3 (or rap1GAP), a protein involved in signalling pathways. In fact, the eukaryotically expressed tuberin protein has been shown to have a rap1GAP activity *in vitro*. Further, tuberin and hamartin associate physically *in vitro*, suggesting that both proteins play a closely related role. Tuberin has two small coiled-coil domains at amino acids 346–371 and 1008–1021 coded by exons 10

and 26, respectively. Rap1GAP is known to stimulate the hydrolysis of active GTP-bound rap1a and rap1b to their inactive GDP-bound forms. Both rap1a and rap1b are members of the ras superfamily of small GTP-binding proteins whose functions include the transduction of mitogenic signals from plasma membrane receptors to the nucleus (Cheadle *et al* 2000). By converting GTP-binding proteins to their inactive forms, GAPs can function as negative regulators of cellular processes including cell proliferation (Cheadle *et al* 2000). Other important domains in tuberlin include four potential tyrosine kinase phosphorylation sites that could be involved in signalling and a leucine zipper consensus domain from amino acids 81–102 coded by exon 3 (Cheadle *et al* 2000). Tuberlin does not seem to have any transmembrane domain. Alternate splicing of exon 25, the first 3 bp of exon 26 and exon 31 have been shown in human, mouse, rat and *Fugu* (Cheadle *et al* 2000). Both *TSC* genes are expressed widely in different tissues. The loss of heterozygosity (LOH) at *TSC1* and *TSC2* loci in *TSC*-associated hamartomas (tumours), and patients with germline mutations showing somatic mutations in hamartomas, suggest that both genes function as tumour suppressors. In other words, although the disease seems to display an autosomal dominant mode of inheritance in pedigrees, the hamartomas develop due to the loss of gene function of both alleles in a somatic cell. Lines for *Tsc1* and *Tsc2* knockout mice have been established by gene targeting (Onda *et al* 1999; Kobayashi *et al* 2001). Heterozygous *Tsc1* mutant (*Tsc1*^{+/}/*Tsc1*⁻) mice developed renal and extra-renal tumours such as hepatic hemangiomas with the loss of wild-type *Tsc1* allele in tumours, and homozygous mutant (*Tsc1*⁻/*Tsc1*⁻) mice died *in utero* (Kobayashi *et al* 2001). *Tsc2* heterozygotes (*Tsc2*^{+/}/*Tsc2*⁻) demonstrate incidence of multiple bilateral renal cystadenomas, liver hemangiomas, and lung adenomas by 15 months of age, and similar to *Tsc1* null embryos *Tsc2* homozygotes died *in utero* (Onda *et al* 1999). Thus, both *Tsc1* and *Tsc2* have a role akin to that of classical tumour suppressor genes.

Mutation analysis of patients has revealed a total of 337 mutations in both genes: 105 in *TSC1* and 232 in the *TSC2* gene (Gilbert *et al* 1998; Cheadle *et al* 2000). Of these 337, 20 mutations in *TSC1* and 21 mutations in *TSC2* are recurrent and the rest are private mutations. Seventy-three (47%) mutations in the *TSC1* gene are single-base substitutions, and 82% of those are nonsense mutations. In the *TSC2* gene, 50% of the mutations are point mutations, and nonsense mutations account for 38% of the total. Protein truncating mutations (nonsense, splicing and frameshift) account for 98% and 77% of *TSC1* and *TSC2* mutations respectively. *TSC1* mutations have been identified in 10–15% of sporadic cases, whereas *TSC2* mutations make up for 70% of the cases (Cheadle *et al* 2000). A total of 33 and 81 different polymorphisms have been identified in *TSC1* and *TSC2* genes respectively (Cheadle *et al* 2000).

Orthologues of *TSC1* gene have been cloned and characterized in mouse, rat and *Drosophila*. The rat hamartin with 1163 amino acids shows approximately 86% identity with human hamartin. The *Drosophila Tsc1* protein encodes a protein of 1100 amino acid residues and is 22% identical (46% similar) to human hamartin (Cheadle *et al* 2000). The predicted coiled-coil and transmembrane domain, and a stretch of 133 amino acids close to the potential transmembrane domain are conserved in human, mouse, rat and *Drosophila*. Orthologues of *TSC2* gene have been cloned from rat, mouse, *Drosophila* and the Japanese pufferfish, *Fugu rubripes*. The *gigas* mutant in *Drosophila* results from mutation of the orthologue of the *TSC2* gene (Ito and Rubin 2001). Human and *Drosophila* tuberins are 26% identical (46% similar) with the highest level of conservation (53% identity) being in the GAP-related domain (Cheadle *et al* 2000).

Recently, two groups have reported independently that the *Drosophila* homologues of human *Tsc1* and *Tsc2* regulate cell growth, cell proliferation and organ size (Potter *et al* 2001; Tapon *et al* 2001). Tapon *et al* (2001) have demonstrated that inactivating mutations in *Drosophila Tsc1* or *Tsc2* genes cause an identical phenotype which is characterized by enhanced growth and increased cell size with no change in ploidy level. Co-expression of both wild-type *Tsc1* and *Tsc2* genes restricted tissue growth and reduced cell size and cell proliferation in mutant cells from wings and eyes, and over-expression of either protein alone in the wings and eyes had no effect (Potter *et al* 2001). This could account for why a mutation in only one of the *TSC* genes is enough to cause the disorder. This also explains why the clinical manifestations of *TSC1* and *TSC2* mutations are indistinguishable: both proteins work in the same pathway. Potter *et al* (2001) have shown that *Drosophila* eye cells mutant for *Tsc1* are dramatically increased in size yet differentiate normally. The individual facets of the eyes as well as the interommatidial bristles were significantly longer in mutant cells of the eyes

(Tapon *et al* 2001). Mutant clones of *Tsc1* have additional cell divisions and a shortened G₁ phase, and consistent with mammalian findings, *Tsc1* and *Tsc2* proteins bind to each other (Potter *et al* 2001). Further, Potter *et al* (2001) proposed a model in which *Tsc1* and *Tsc2* interact with each other to antagonize the insulin signalling pathway in regulating cell proliferation, cell growth and organ size.

Acknowledgements

Financial support from the Department of Biotechnology, New Delhi, is gratefully acknowledged.

References

- Cheadle J P, Reeve M P, Sampson J R and Kwiatkowski D J 2000 Molecular genetic advances in tuberous sclerosis; *Hum. Genet.* **107** 97–114
- Gilbert J R, Guy V, Kumar A, Wolpert C, Kandt R, Aylesworth A, Roses A D and Pericak-Vance M A 1998 Mutation and polymorphism analysis in the tuberous sclerosis 2 (TSC2) gene; *Neurogenetics* **1** 267–272
- Ito N and Rubin G 2001 *Gigas*, a *Drosophila* homolog of tuberous sclerosis gene product-2, regulates the cell cycle; *Cell* **96** 529–539
- Kobyashi T, Minowa O, Sugitani Y, Takai S, Mitani H, Kobayashi E, Noda T and Hino O 2001 A germ-line *Tsc1* mutation causes tumor development and embryonic lethality that are similar, but not identical to, those caused by *Tsc2* mutation in mice; *Proc. Natl. Acad. Sci. USA* **98** 8762–8767
- National Tuberous Sclerosis Association, USA 1988 *Tuberous sclerosis: An illustrated brochure for physicians* Publication No. 001/85/88
- Onda H, Lueck A, Marks P W, Warren H B and Kwiatkowski D J 1999 *Tsc2*(+/-) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background; *J. Clin. Invest.* **104** 687–695
- Potter C J, Huang H and Xu T 2001 *Drosophila Tsc1* functions with *Tsc2* to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size; *Cell* **105** 357–368
- Tapon N, Ito N, Dickson B J, Treisman J E and Hariharan I K 2001 The *Drosophila* tuberous sclerosis complex gene homologues restrict cell growth and cell proliferation; *Cell* **105** 345–355

ARUN KUMAR*

S C GIRIMAJI[†]

*Department of Molecular Reproduction,
Development and Genetics,
Indian Institute of Science,
Bangalore 560 012, India*

[†]*Department of Psychiatry,
National Institute of Mental Health and Neurosciences,
Bangalore 560 029, India*

**(Email, karun@mrdg.iisc.ernet.in)*

Why are chillies pungent?

Early voyagers to the Americas, including Central America, Mexico, Peru, and Chile, found many forms of peppers, among them the 'hot' (pungent) ones. In Spain these hot peppers are called chili, meaning from Chile, and in India, chillies. When asked to guess the source of chillies, one might think of Mexico. However, despite the plant's popularity in that country, it is believed that chillies – or, to use the misleading but widely used name by which they are called in the United States, chili peppers – originated in South America, after which they spread to Central America. Most of the varieties of pepper referred to as chili peppers belong to *Capsicum annuum* L; some varieties with "chili" in their name are actually *C. frutescens* L. Precise categorization can be difficult because of the large number of varieties and the constant creation of new ones by hybridization. Their weedy nature, combined with the easy transportability of their seeds, made chili peppers among the first plants to be domesticated (Andrews 1984). Remains found in Tehuacan, Mexico, have been dated to approximately 7000 BC. Columbus mistook the chili pepper for a relative of black pepper, *Piper nigrum*, whence the term 'pepper' (Robbins 1992).

Dr Diego Alvarez Chanca, who accompanied Columbus on his second voyage, described chili peppers as the principal food of native Americans and compared them to the turnip (Andrews 1984). The seeds of the chili pepper were brought back to Spain and the plant was grown in monastery gardens. Portuguese traders spread it to many countries including Persia, India and Indonesia (Andrews 1984). The earliest reference to chillies in India is in a poem in Kannada by Purandara Dasa (1480–1564) that extols its praises; the *Bhojana Kutuhala*, a Marathi text on the enjoyment of food by Raghunatha (1650), also refers to chillies. But "... not a single recipe of over fifty given in the *Ain-i-Akbari* of 1590 used anything except black pepper to impart pungency" (Achaya 1994). While most people know the chili pepper as a food, it had other uses in ancient times. The pre-Columbian Indians used them as a medicine, as a punishment for children (inhalation of the smoke of burning chili peppers), and as a kind of tear gas during warfare (chili peppers were burned and the smoke blown by the wind over to enemy lines) (Andrews 1984). The basic sensation produced by chili peppers is extreme pungency or 'heat'.

The substance that produces all of the heat sensation is known as capsaicin (N-vanillyl-8-methyl-6-(E)-nonamide). Capsaicin is made by specialized gland cells found in the cross-walls or ribs of the pepper and is composed of several different alkaloids which vary in amounts depending on the species (Rowland *et al* 1983). Wilbur Scoville developed a scale in 1912 to measure the "heat levels" of chilli peppers. In the original Scoville test, a panel of volunteers would be asked to determine what dilution of the chilli pepper solution no longer cause burning discomfort in the mouth. Approximately one part per million of "heat" is equivalent to 1.5 Scoville units. Until recently, the hottest chilli pepper ever recorded was a Habanero which had 577,000 Scoville pungency units while in contrast the fiery Jalapeno has between 2,500 and 10,000 units, and in complete contrast the Sweet Italian Bell Pepper has a pungency of 0 units (Bellringer 2001). Indian scientists have recently claimed that a type of chilli grown in the country's northeast has the highest Scoville units of pure capsaicin. Called the Tezpur chilli, after the area where it is grown, scientists say the pepper has beaten Mexico's Red Savina Habanero. "The Tezpur chilli was rated having 855,000 Scoville units . . . the Mexican chilli contained 557,000 Scoville units of pure capsaicin," one of the scientists, who asked not to be identified, told Reuters (<http://www.cnn.com/2000/FOOD/news/09/04/india.chilli.reut/>). Pure capsaicin has a pungency of 16 million Scoville units. Thankfully for the tasters, the original Scoville taste test has given way to HPLC measurements.

The sensations of heat and pain in the mouth are the result of the stimulation of local heat receptors in the skin and mucous membranes by capsaicin, providing one answer to the question posed in the title. The capsaicin (vanilloid) receptor VR1 is a sensory neuron-specific ion channel that serves as a polymodal detector of pain-producing chemical and physical stimuli. Capsaicin is a trigeminal stimulant that is important in gustatory physiology (Liu and Simon 2000). Interestingly, capsaicin can also help in the mediation of pain: prolonged application of capsaicin is thought to cause the desensitization of sensory neurons responsible for pain. This might occur via the depletion of substance P, a peptide neurotransmitter in sensory "pain" fibres (Goettl *et al* 1997), the final outcome being the release of

B-endorphins which are endogenous opioids. Capsaicin can induce sweating, which is why chillies are popular in hot dry climates. Further, it stimulates the actions of the muscles of the stomach and intestine; this improves digestion and makes chili peppers an attractive condiment for a food that might upset the stomach (Andrews 1984). Most importantly, it appears that capsaicin was developed by plants as a way of preventing animals with digestive systems that can destroy chili pepper seeds from eating them, while allowing animals who will pass the seeds to eat them with no ill effects (Robbins 1992; Tewksbury and Nabhan, 2001). That may be the evolutionary explanation of why chillies are so pungent.

Surprisingly, most of the work on pain-induced by capsaicin had concentrated on mammals, with very little work on gustatory responses in birds. Recently Bryant *et al* (2000) cultured trigeminal nociceptors (pain receptors) from the Norway rat (*Rattus norvegicus*, laboratory strain), white leghorn chicken (*Gallus gallus*), coyote (*Canis latrans*), white-tailed deer (*Odocoileus virginianus*) and Canada goose (*Branta canadensis*) and then applied digital fluorescence microscopy to measure changes in intracellular calcium (an index of cellular activation) in response to applications of known and effective repellents. They found that capsaicin was a more effective stimulus for rat, coyote, and deer neurons than cells from chicken. Does this mean that birds do not have capsaicin-stimulated or vanilloid-receptors in their oral linings, or do they have potent antagonists?

The limited effect of capsaicin on birds appears to be why capsaicin is now believed to cause directed toxicity or directed deterrence of potential mammalian seed predators in the chiltepine chilli plant (*Capsicum annuum* var. *glabriusculum*) in southern Arizona, while having no effect on seed-dispersing birds, the curve-billed thrashers (*Toxostoma curvisrostre*) (Tewksbury and Nabhan 2001). To find out whether small fruit-consuming mammals such as cactus mice (*Peromyscus eremicus*) and packrats (*Neotoma lepida*) avoid chillies because of their capsaicin content, the authors presented these mammals as well as desert thrasher birds with the pungent chilli fruit (*C. annuum*), fruit of a non-pungent mutant variety of *C. chacoense* which is similar in all other aspects to the fruit of *C. annuum* except in lacking pungency, as well as desert hackberry fruit (*Celtis pallida*) as a control. They found that while the birds consumed all three types of fruits equally, the mammals consumed no pungent chilli fruit, an intermediate amount of the non-pungent chilli fruit and all the hackberry fruit. It appears that the capsaicin in the chiltepine chilli fruit deters consumption by mammals. Furthermore, germination trials of *C. chacoense* fruit (non-pungent chilli) showed that there was zero germination following gut passage through the mammals, while germination levels following gut passage of chiltepine seeds and non-pungent chilli seeds through the birds were excellent and comparable with that of control seeds taken directly from the fruit and planted. If seeds can germinate just as well with and without passage through bird guts, what is the advantage to being consumed by the birds? Tewksbury and Nabhan (2001) found that birds that consumed chilli fruit are more likely to deposit these seeds in shaded sites suitable for germination. This fact coupled with the zero germination on passage through mammalian guts can explain why the chiltepine chilli plant “wants to encourage consumption” by the thrasher birds and to deter consumption by mammals.

Some years ago, Cipollini and Levey (1997a, b) proposed two sets of hypotheses to address patterns of secondary metabolites in ripe fruit pulp. The first set of hypotheses dealt with toxicity of the metabolites. The compounds could either have directed deterrence i.e. be targeted towards specific harmful consumers, or have general toxicity, wherein retention of toxins in ripe fruit is a manifestation of a trade-off between seed defense and frugivore attraction. The second set of hypotheses addressed whether fruit removal rates and fruit pulp nutrient content could explain the secondary metabolite patterns. Several workers have since then attempted to test these sets of hypotheses. Tewksbury and Nabhan (2001) believe they have for the first time shown directed toxic deterrence in a fruit, and how this may influence the interaction between plants and their fruit-eating visitors.

Have we finally understood why chillies are so pungent? Have mammalian seed predators and their VR1 nociceptive oral receptors been the driving force behind capsaicin evolution? Interestingly, an ultrapotent analogue of capsaicin, resiniferatoxin, is found in the latex of the succulent spiny shrub *Euphorbia resinifera*, and its use in pain mediation has been known since Roman times (Appendino and Szallasi 1997). Resiniferatoxin has been shown to have acute emetic effects in the house musk shrew (Andrews *et al* 2000). It would be worth investigating whether a similar selective pressure may explain the origin and maintenance of this potent vanilloid too.

References

- Achaya K T 1994 *Indian food – A historical companion* (Oxford: Oxford University Press)
- Andrews J 1984 *Peppers* (Austin: University of Texas Press)
- Andrews P L R, Okada F, Woods A J, Hagiwara H, Kakaimoto S, Toyoda M and Matsuki N 2000 The emetic and anti-emetic effects of the capsaicin analogue resiniferatoxin in *Suncus murinus* the house musk shrew; *Br. J. Pharmacol.* **130**, 1247–1254
- Appendino G and Szallasi A 1997 Euphorbium: Modern research on its active principle resiniferatoxin revives in ancient medicine; *Life Sci.* **60** 681–696
- Bellringer M 2001 *Capsaicin. The molecule of the month, April 2001* wwwchmbrisacuk/motm/chilli/scovillehtm
- Bryant B P, Savchenko A, Clark L and Mason J R 2000 Potential for cell culture techniques as a wildlife management tool for screening primary repellents; *Int. Biodeterior. Biodegrad.* **45**, 175–181
- Cipollini M L and Levey D J 1997a Why are some fruits toxic? Glykaloids in *Solanum* and fruit choice by vertebrates; *Ecology* **78**, 782–798
- Cipollini M L and Levey D J 1997b Antifungal activity of *Solanum* fruit glykoalkaloids: implications for frugivory and seed dispersal; *Ecology* **78**, 799–809
- Goettl V M, Larson D L, Portoghese P S and Larson A A 1997 Inhibition of substance P release from spinal cord tissue after pretreatment with capsaicin does not mediate the antinociceptive effect of capsaicin in adult mice; *Pain* **71**, 271–278
- Liu L and Simon S A 2000 Capsaicin, acid and heat-evoked currents in rat trigeminal ganglion neurons: Relationship to functional VR1 receptors; *Physiol. Behav.* **69**, 363–378
- Robbins J 1992 *It feels like your lips are going to fall off* (Washington DC: Smithsonian) pp 42–51
- Rowland B J, Villalon B and Burns E E 1983 Capsaicin production in Sweet Bell and punjent Jalapeno peppers; *J. Agric. Food Chem.* **31** 484–487
- Tewksbury J J and Nabhan G P 2001 Directed deterrence by capsaicin in chillies; *Nature (London)* **412**, 403–404

RENEE M BORGES
Centre for Ecological Science,
Indian Institute of Science,
Bangalore 560 012, India,
(renee@ces.iisc.ernet.in)