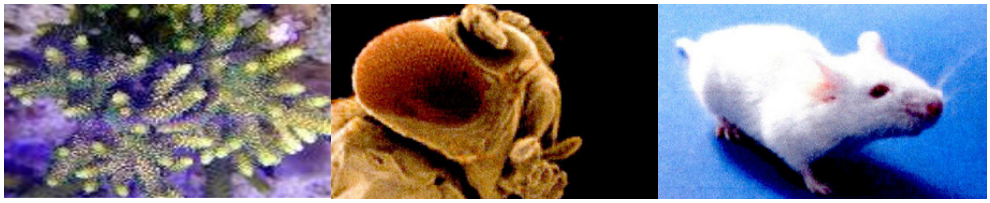
Characterisation of *Acropora* AmTPR1, *Drosophila* Dpit47 and mouse TTC4 – a tetratricopeptide (TPR) gene family involved in development and cell proliferation



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ABSTRACT

The tetratricopeptide repeat (TPR) is a protein-protein interaction motif present in a variety of functionally unrelated proteins. Recently, the first TPR gene was cloned from the coral *Acropora millepora*-AmTPR1. AmTPR1 has high similarity to human TTC4 and *Drosophila* Dpit47, genes implicated in tumorigenesis and cell proliferation. Using a comparative genomics approach, this thesis characterises the AmTPR1/Dpit47/TTC4 gene family in an attempt to understand the evolution of function.

Semi-quantitative PCR analysis indicates that AmTPR1 is expressed at low levels throughout the early development of *Acropora*. The AmTPR1 transcript was generally distributed in the early embryo, but by the end of gastrulation, transcripts were specifically associated with a subset of transectodermal cells. Treatment of *Acropora* embryos with the glycogen synthase kinase-3 (GSK-3)-specific inhibitor alsterpaullone resulted in increased expression of AmTPR1, suggesting a role of canonical Wnt/β-catenin signalling in regulating AmTPR1 expression. Consistent with this, several sequences showing 100 % homology to the core TCF/LEF-binding consensus sequence, were identified in the putative promoter regions of AmTPR1 and its closest relatives *Hydra* HmTPR1 and *Nematostella* NvTPR1 genes. Compared to AmTPR1, *Drosophila* Dpit47 showed different expression characteristics. Dpit47 transcripts could not be detected in early development of *Drosophila* (stages 6-11), while in late embryos (stages 13-16), strong and specific expression of Dpit47 was observed in the central nervous system. Furthermore, in contrast to AmTPR1, the expression of Dpit47 is likely to be regulated by Myb/E2F/DREF- and not by Wnt/β-catenin – dependent transcriptional regulation. Despite having different expression patterns, yeast 2-hybrid analysis indicates that both AmTPR1 and Dpit47 interact with Hsp90 and DNA polymerase α, suggesting the possibility of functional conservation.

The expression profile of mouse TTC4 differed from both AmTPR1 and Dpit47. In mouse neuroblastoma N2A cells, treatment with the GSK- 3 inhibitors kenpaullone and LiCl had no effect on TTC4 expression. Instead, the expression of TTC4 in N2A cells was downregulated in response to depolarizing stimuli, such as 85 mM KCl**.** Addition of 2.3 mM Ca^{2+} exacerbated the extent of depolarization-induced downregulation of TTC4,

indicating that TTC4 expression was Ca^{2+}-dependent. However, the mechanism of Ca^{2+}-dependent regulation of TTC4 expression under depolarizing conditions did not require extracellular Ca^{2+} influx through L-type Ca^{2+} channels or N-methyl-D-aspartate (NMDA) - receptor channels as treatment of N2A cells with nifedipine (L-type Ca^{2+} channel blocker), and NMDA did not affect the extent of TTC4 downregulation in response to 85 mM KCl. Instead, treatment with 20 mM tetraethylammonium chloride (TEA) greatly exacerbated the extent of TTC4 downregulation in response to 85 mM KCl in N2A cells. The K^+ channel opener mallotoxin and the mitogen bradykinin were both able to attenuate the effect of TEA on TTC4 expression under depolarizing stimuli, indicating that TTC4 expression was dependent on K^+ channel activity. Consistent with the involvement of Ca^{2+} in regulating TTC4 expression, four nuclear factor of activated T cells (NFAT) binding sequences were found in the 2 kb 5' region of the mouse TTC4 gene. Finally, TTC4 expression was higher in proliferating than in quiescent N2A cells and the greatest extent of upregulation was observed at the G1/S transition, suggesting TTC4 transcription was cell-cycle dependent.

In summary, despite lineage-specific differences in the expression patterns and regulatory characteristics, *Acropora* AmTPR1, *Drosophila* Dpit47 and mouse TTC4 each appear to function as developmental genes involved in the regulation of proliferation coupled to the cell cycle.

ACKNOWLEDGEMENTS

First and foremost I thank my supervisor Dr. David Miller, not only for his professional guidance but also for pushing me through all the hard times even those FEW when I thought all hope was gone. Doing a PhD in biochemistry is not just about science and research, it is much about character testing and stress resilience !

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Thanks to Margaret Jordan for unhesitant supply of RNA extraction columns, filtered tips, RNase free DNase kits and other "emergency" lab stuff that tends to run out in most inappropriate moments.

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I am very grateful to the Australian Government for providing me with an International Postgraduate Research Scholarship and I also wish to acknowledge the School of Pharmacy and Molecular Sciences at JCU for financial support.

Last but certainly not least, thanks to my parents Tanja and Ante Tomljenovic to whom I dedicate this work. Without your support, both financial and emotional I would not be the person I am today. In the end, I think I am happy with myself !!! Hell, I do not think of that as an arrogant statement !

In the end, if I had forgotten anyone, please forgive me, this is the last section of my thesis that I am writing, it is just after midnight and I just want to finish it, go home and sleep ☺

Just before I go, thank God for coffee, science research would not have come as far without it !!!

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INTRODUCTION

1.1. TPR-motif, a ubiquitous mediator of protein interactions

Protein-protein interactions control and integrate many essential biochemical functions in living cells. Among these, cell cycle control, transcription, splicing, signal transduction, protein folding, neurogenesis, mitochondrial and peroxisomal protein transport operate through inherently complex protein networks involving a common mediator: the tetratricopeptide repeat motif – TPR (Blatch and Lassle 1999, Becker *et al.* 1994, Carrigan *et al.* 2006, Chan *et al.* 2006, D'Andrea and Regan 2003, Das *et al.* 1998, Dolinski *et al.* 1998, Gatto *et al.* 2000, Goebl and Yanagida 1991, Groves and Barford 1999, Kumar *et al.* 2001, Lamb *et al.* 1995, Lee *et al.* 1994, Malek *et al.* 1996, Nakatsu *et al.* 2000, Prodromou *et al*. 1999, Ramarao *et al.* 2001, Scheufler *et al.* 2000, Schliebs *et al.* 1999, Sikorski *et al.* 1990, Sinclair *et al.* 1999, Swingle *et al.* 2004, Tzamarias and Struhl 1995, Urquhart *et al.* 2000, Yang *et al.* 2005, Zhang and Grishin 1999). TPR is a ubiquitous protein-protein interaction element that has persisted through evolution for over 3000 million years: from the ancient Archaea to Eukarya, more than 6000 TPR proteins have been discovered so far (Fig. 1.1).

Because of the widespread occurrence of TPRs much effort has been focused in elucidating the mechanisms of TPR-mediated protein interactions. Sequence analysis revealed that TPR proteins differ in the number and spatial arrangement of individual TPR motifs. Not surprisingly, the functions mediated by TPR proteins are as diverse as their structures (Fig. 1.2). An obvious question arose from these observations: what determines the specificity of TPR-mediated interactions ? It was shown by circular dichroism, X-ray crystalography and computational analysis that TPRs adopt helical conformations and even though individual motifs lack functional specificity, multiple TPRs create superhelical structures, with unique surface- interacting properties (Blatch and Lassle 1999, D'Andrea and Regan 2003, Das *et al.* 1998, Goebl and Yanagida 1991, Groves and Barford 1999, Hirano *et al*. 1990, Main *et al.* 2003, Sikorski *et al.* 1990).

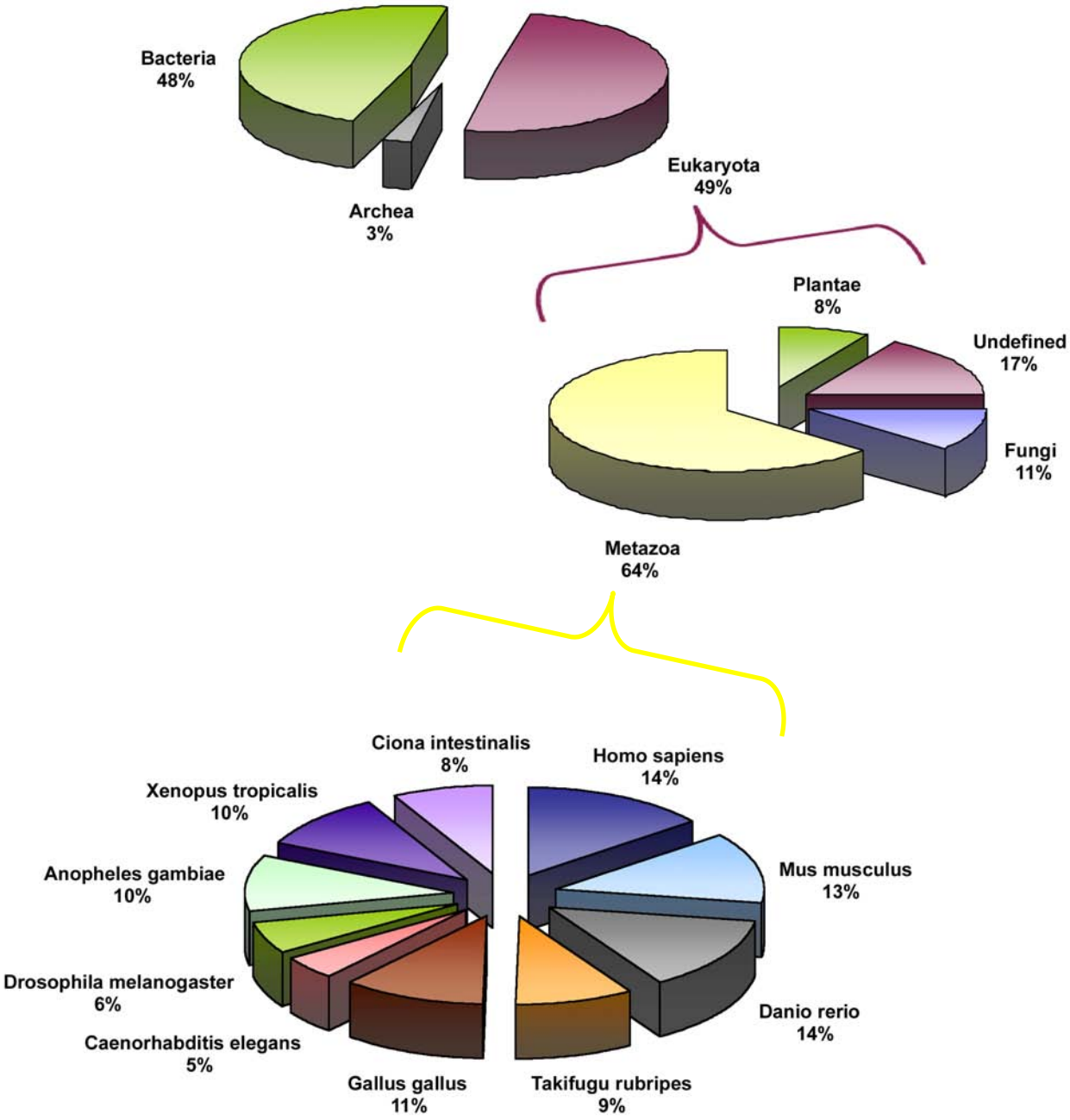


Fig. 1.1. The distribution of TPR proteins across phyla. 35061 TPR domains were detected in 6631 proteins in the SMART's non-redundant database NRDB, http://smart.embl-heidelberg.de): *Archea* (190 proteins), *Bacteria* (3158), *Eukaryota* (3257).

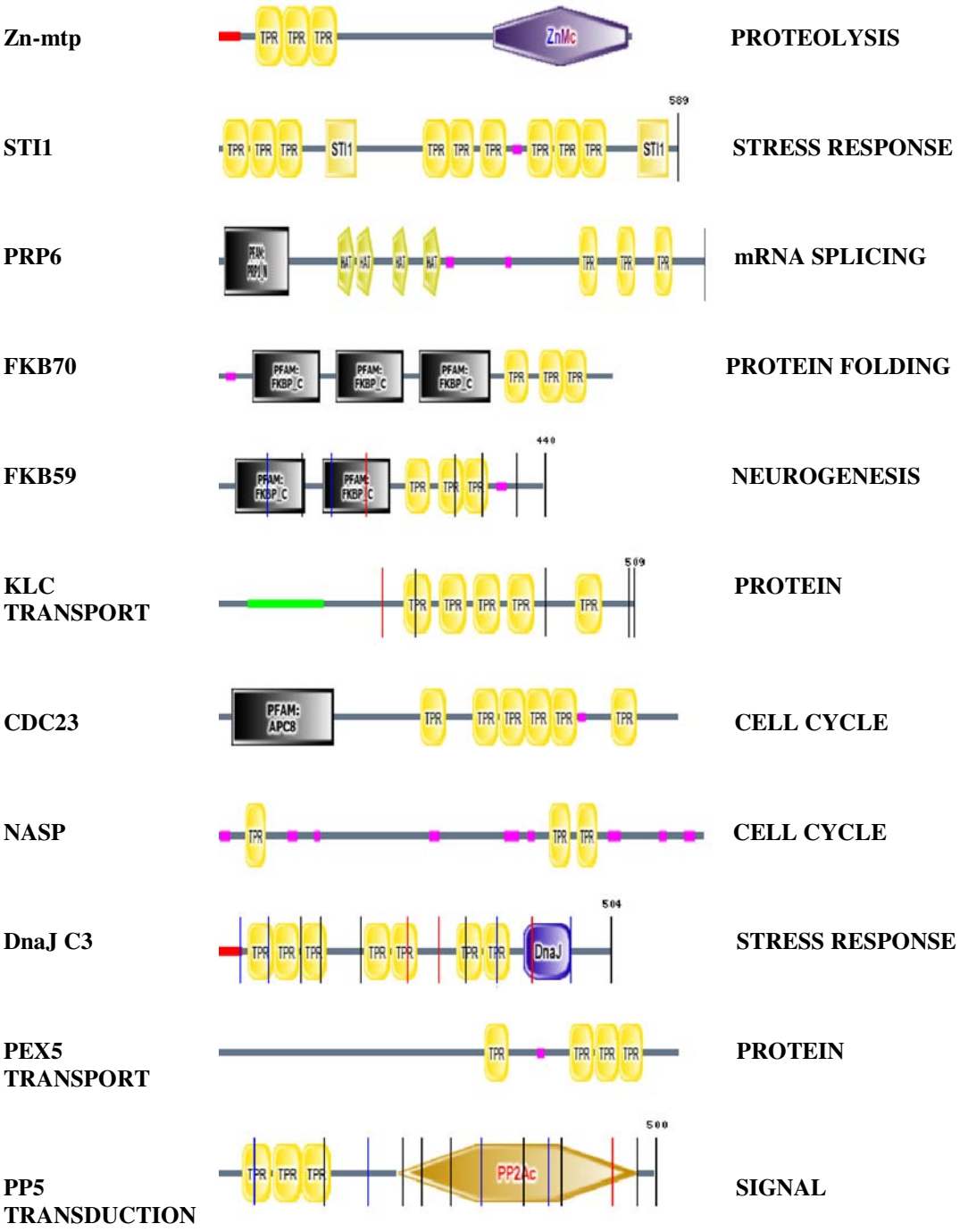


Fig. 1.2. Structural and functional diversity of TPR proteins. The proteins indicated were retrieved from the ExPASy proteomics server (http://au.expasy.org): Zn-mtp (*Archea*, Q977M9), STI1 (*S. cerevisiae*, P15705), PRP6 (*S. cerevisiae*, P19735), FKB70 (*A. thaliana*, Q38931), FKB59 (*D. melanogaster*, Q9VL78), KLC (*D. melanogaster*, P46824), CDC23 (*M. musculus,* Q8BGZ4), NASP (*M. musculus*, Q99MD9), DnaJ3 (*H. sapiens*, Q13217), PEX5 (*H. sapiens*, P50542) and PP5 (*H. sapiens*, P53041). Protein schematics were generated using SMART, protein domains are indicated in coloured boxes: TPR, ZnMc (Zinc-dependent metalloprotease domain), STI1 (Heat shock chaperonin-binding motif), PRP1 (mRNA splicing factor domain), HAT (Half-A-TPR repeat), FKBP_C (Peptidylprolyl cis-trans isomerise domain), APC8 (anaphase promoting complex subunit 8), DnaJ (DnaJ molecular chaperone homology domain), PP2Ac (protein phosphatase 2A catalytic domain).

1.2. TPR protein structure

TPR is a degenerate 34 amino acid motif found in many different proteins arranged in arrays of 2-16 repeats (Blatch and Lassle 1999, D'Andrea and Regan 2003, Goebl and Yanagida 1991, Lamb *et al.* 1995, Main *et al.* 2003). Each TPR consists of a pair of antiparallel α helices of equivalent length, helices A and B (Fig. 1.3, panel A). Adjacent TPR motifs are stacked in a parallel arrangement (Fig. 1.3, panel B). Within an array of TPR motifs the uniform angular and spatial arrangements of neighbouring TPRs results in the formation of a right-handed superhelix featuring an amphipathic groove. The surface of the amphipathic groove is predominantly formed by amino acid side chains of individual A helices (Fig. 1.3, panel C) and represents a functional interface for mediating protein interactions. (Blatch and Lassle 1999, D'Andrea and Regan 2003, Das *et al.* 1998, Groves and Barford 1999).

The helical repeats of TPR proteins arise from internal sequence repeats. Despite the lack of strict amino acid sequence conservation within the 34 residue TPR, multiple sequence alignments of individual motifs shows predominant occurrence of hydrophobic amino acids at specific positions (Blatch and Lassle 1999, D'Andrea and Regan 2003, Hirano 1990, Lamb *et al.* 1995, Main *et al.* 2003, Sikorski *et al.* 1990).The greatest sequence homology is observed in two clusters, corresponding to α-helix A: positions 4 (W/L/Y), 7 (L/I/M), 8 (G/A/S), 11 (Y/L/F) and α-helix B: 20 (A/S/E), 24 (F/Y/L) and 27 (A/S/L). A detailed analysis of the superstructure of TPR proteins provides a rationale for the observed consensus sequence. Namely, there is an intrinsic complementarity in the packing of small and large hydrophobic residues, such that small residues, (positions 8 and 20 and 27), are located at the closest contact between α helices A and B of each TPR while large hydrophobic residues (positions 4, 7, 11 and 24), form the interfaces between neighbouring α helices (Fig. 1.3, panels E and F). Thus, although overall the packing of the α helices seems relatively sequence independent, the key residues involved in stabilizing the unique conformation of the superhelix are well conserved.

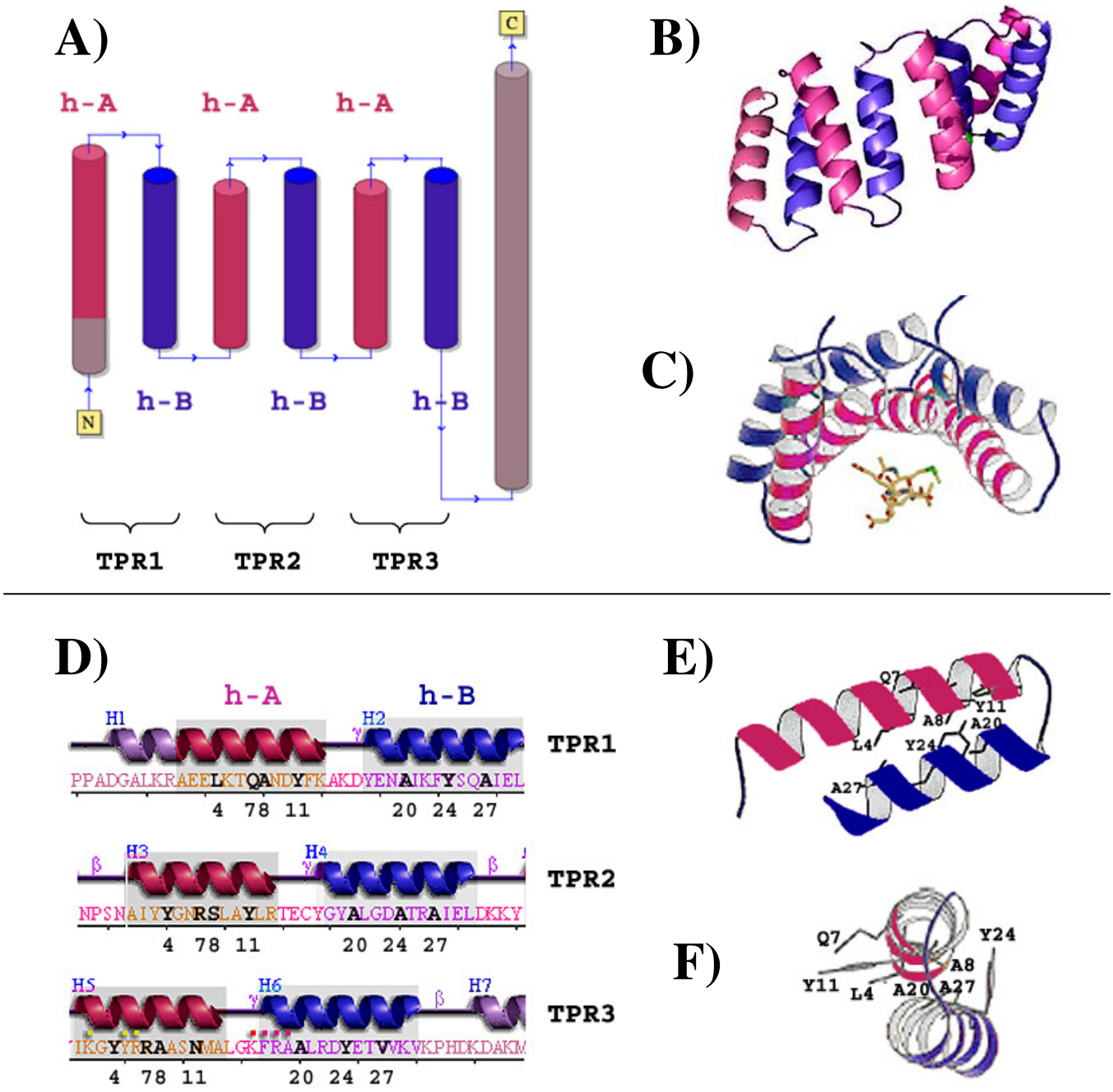


Fig. 1.3. Structural features of a three-motif TPR protein. A) Protein topology showing antiparallel orientation of α helices A and B. Grey shaded helical domain denotes the non-TPR region of the protein B) Parallel stacking of neighboring TPRs, C) Ligand-binding amphipathic groove, note that the interacting interface is formed exclusively from A α helices. D) Location of the TPR hydrophobic consensus residues in individual TPR motifs, note that Q7 (TPR1) and R7 (TPR2 and TPR3) are hydrophilic residues and represent a deviation from the hydrophobic consensus. E) and F) Topology of consensus residue packing in TPR1 motif. Large (L4, Y11, Y24), and small (A8, A20, A27), residues pack in an alternate pattern (E). Small residues are clustered at the closest contact between α helices A and B, large residues reside on the interface of the α helices (F). Schematics C), E) and F) were adapted from Blatch and Lassle (1999), A), B) and D) were retrieved from PDBSum database (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum), PDB codes 2fo7 (B), and 1a17 (A and D).

1.3. The anaphase promoting complex (APC)

The versatility of the TPR model has significant consequences in evolutionary terms: acquisition of novel functions has been greatly facilitated by the absence of a stringent selection pressure for a particular amino-acid sequence. Because of the degenerate nature of the TPR motif and differential combinatorial arrangements of repeating units, various conformations of the TPR superhelix are possible. As a result, an inventory of unique interacting interfaces is created that enables interaction with a large number of specific targets. In that respect, the pinnacle of TPR-mediated interaction versatility is observed in multimeric TPR protein complexes that elegantly exploit the multifunctional properties of TPR motifs (Fig. 1.4). A perfect example is the anaphase promoting complex (APC), a highly conserved protein complex important for the regulation of cell cycle events. By mediating ubiquitin-dependent proteolysis of G1 and mitotic checkpoint regulators (cyclins A and B, cdc6 and geminin), APC ensures proper cell cycle progression. Specifically, APC regulates timing of initiation of replication, separation of sister chromatids at metaphase/anaphase transition and exit from mitosis (Hirano *et al.* 1990, Irniger *et al.* 1995, Irniger and Nasmyth 1997, King *et al.* 1995, King *et al.* 1996, Nigg 1995, Sikorski *et al.* 1990, Sivaprasad *et al.* 2007, Visintin *et al.* 1997, Vodermaier *et al.* 2003, Wang *et al.* 2003). APC is an amazingly complex ubiquitin ligase composed of as many as 11 subunits, APC1-11 (Vodermaier *et al.* 2003). This structural complexity is not surprising given the vast repertoire of proteins that APC designates for destruction. The functional specificity of the APC depends on two activator proteins, CDC20 and CDH1, which function as adaptors in different phases of the cell cycle for recruitment of specific substrates to the APC. Namely, APC^{CDC20} is active early in mitosis, while APC^{CDH1} acts in late mitosis and G0/G1 (Sivaprasad *et al.* 2007, Visintin *et al.* 1997, Vodermaier *et al.* 2003). Critical to the assembly of APC^{CDH1} / APC^{CDC20} complexes are the TPR-domains of APC3 and APC7 (Vodermaier *et al.* 2003). Consequently, mutational or antibody-mediated interferences with TPR-subunits activity are detrimental for cell viability (Hirano *et al*. 1990, Lamb *et al.* 1994, Tugendreich *et al.* 1995, Wang *et al.* 2003). For example, substitution of a conserved G residue with a polar D residue in the 7^{th} TPR motif of *S. cerevisiae* APC3/APC7 homolog CDC27 results in metaphase arrest and failure to exit mitosis, indicating that TPRs are indispensable for APC function (Lamb *et al.* 1994). Other than APC3 and APC7, two other APC subunits contain TPR motifs,

APC6 and APC8 (human homologs of *S. cerevisiae* CDC16 and CDC23). In humans and yeast, APC6/APC8 (CDC16/CDC23) link together the different subunits of the APC complex and mutations of these genes leads to defects in cell cycle progression (Irniger *et al.* 1995, Irniger and Nasmyth 1997, Lamb *et al.* 1994, Visintin *et al.* 1997, Wang *et al.* 2003),. These results imply that in addition to APC3/APC7, APC6/APC8 also participate in adaptor-protein mediated APC functions. In conclusion, it appears that by the virtue of their TPR motifs, TPR subunits of the APC play dual roles, 1) they are responsible for the structural integrity of the APC and 2) they are used as modulators for interactions with different regulatory proteins, thereby allowing for multiple targeting of a single protein complex.

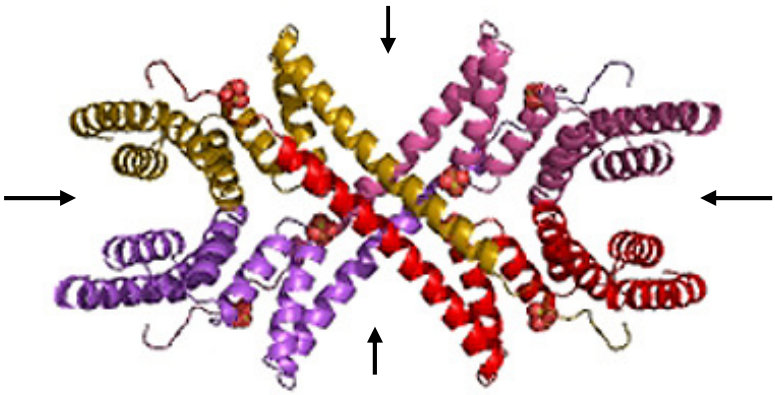


Fig. 1.4. Ribbon representation of a multimeric TPR protein complex. Coupling of distinct interacting surfaces allows for multifunctional properties of the complex. Sites of interactions are indicated with arrows. Schematics was retrieved from PDBSum database (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum), PDB code: 1a17.

1.4. The Hsp90/TPR co-chaperone complex

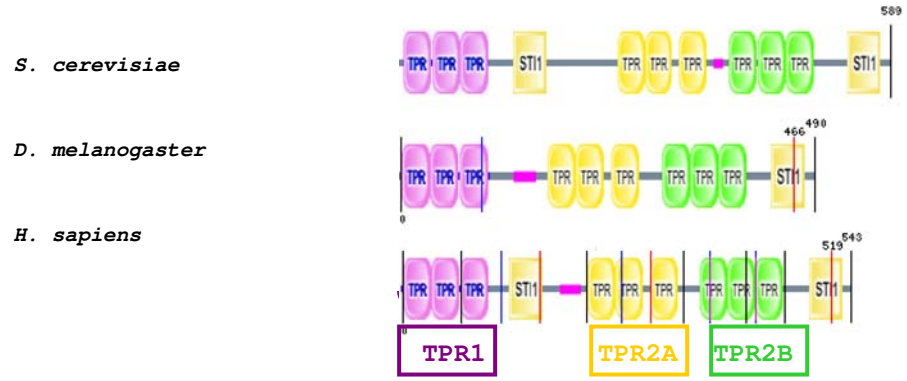
TPRs often occur in combination with other domains that confer functional specificity to the protein. For example, members of the serine/threonine phosphatase family such as Protein phosphatase 5 (PP5), are characterised by the presence of a C-terminal catalytic domain (PP2Ac, Fig. 1.2), responsible for catalysing dephosphorylation of phosphoserine and phosphothreonine residues (Das *et al.* 1998, Yang *et al.* 2005). However, many proteins contain only TPRs as functional domains and in such instances functional characterisation is only possible if specific TPR-related structural fingerprints are identified. Namely, 1) the number and spacing of TPR motifs, which defines the feature of the amphipathic groove and 2) amino acid composition of individual domains outside the consensus, which defines recognition surface properties and is thus responsible for substrate specificity. In support of this hypothesis, functionally related TPR proteins often show 1) the same number and arrangement of TPR motifs (Fig. 1.5, panel A) and 2) greater sequence homology between corresponding TPRs across species than the sequence homology between TPRs within a single specie (indicating homology above the eight TPR consensus residues (Fig. 1.5, panels B and C). In that context, the Hsp90/TPR co-chaperone complex is perhaps the best studied example.

Hsp90 is a ubiquitous molecular chaperone essential for folding and activation of a wide range of client proteins typically involved in cell cycle regulation and signal transduction pathways (Bohen *et al.* 1995, Carrigan *et al.* 2006, Grenert *et al.* 1999, Helmbrecht *et al.* 2000, Panaretou *et al.* 1998, Pearl and Prodromou 2000, Pratt *et al.* 1993, Pratt and Toft 2003, Prodromou *et al.* 1999, Song and Masison 2005, Yang *et al.* 2005). Hsp90 targets include steroid hormone receptors (Carrello *et al.* 1999, Dittmar *et al.* 1997, Dolinski *et al.* 1998, Nathan and Lindquist 1995, Pratt *et al.* 1993, Pratt and Toft 2003, Sanchez *et al.* 1985, Schuh *et al.* 1985), tyrosine and serine/threonine kinases (Aligue *et al.* 1994, Helmbrecht *et al.* 2000, Pratt and Toft 2003, Stancato *et al.* 1993), transcription factors (Helmbrecht *et al.* 2000, Pratt and Toft 2003, Shue and Kohtz 1994) and tumor suppressors (Chen *et al.* 1996, Helmbrecht *et al.* 2000). *In vivo* Hsp90 activity is often dependent on its association with Hsp70 and a number of co-chaperones, many of which are TPR proteins (Carrigan *et al.* 2006, Dittmar *et al.* 1997, Dolinski *et al.* 1998, Marsh *et al.* 1998, Pratt *et al.* 1993, Pratt and Toft 2003, Prodromou *et al.* 1999, Pearl and Prodromou 2000, Scheufler *et al.* 2000, Song and Masison 2005). By providing a bridging

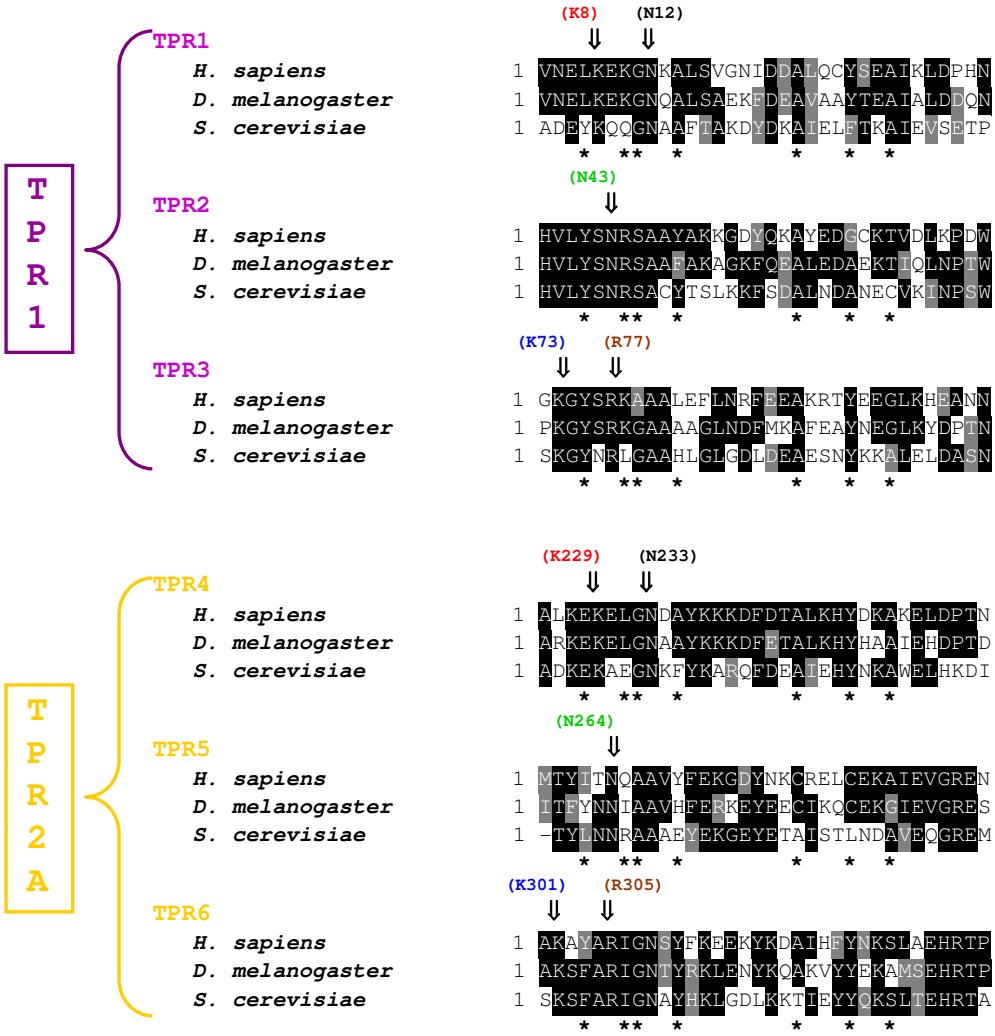
function between Hsp90 and client proteins, TPR co-chaperones are critical for the assembly of Hsp90 complexes. TPR co-chaperones may also act as scaffold proteins, physically coupling Hsp90 and Hsp70 via their TPR domains. For example, the interaction of Hsp90 with Hsp70 is mediated by the Hsp70/Hsp90 organizing protein Hop (Carrello *et al.* 1999, Carrigan *et al.* 2006, Chen and Smith 1998, Pearl and Prodromou 2000, Prodromou *et al.* 1999, Scheufler *et al.* 2000, Song and Masison 2005). Hop contains nine TPR motifs which form three separate TPR domains (each consisting of three TPRs), TPR1, TPR2A and TPR2B (Fig. 1.5, panel A). The TPR1 domain of Hop specifically interacts with Hsp70 while the TPR2A mediates the interaction with Hsp90 (Carrigan *et al.* 2006, Chen and Smith 1998, Scheufler *et al.* 2000, Song and Masison 2005). Both TPR1 and TPR2A domains interact with a C-terminal sequence that is remarkably conserved in Hsp90 and Hsp70, the EEVD motif (Scheufler *et al.* 2000). Consistently, the amino acids responsible for EEVD binding are identical in the two TPR domains (K8, N12, N43, K73 and R77 of the TPR1 domain and the corresponding K229, N233, N264, K301 and R305 of the TPR2A domain, Fig. 1.5, panel B). Most critical for EEVD binding by Hop is the interaction between the five residues of TPR1/TPR2A and the side chain carboxylate of the terminal Aspartate (D) of the Hsp proteins. However, the specificity of TPR-Hsp binding arises from additional contacts with sequences N-terminal to the EEVD motif, which are essential for the high affinity of peptide binding to the respective TPR domains. In particular, the Hsp70 octapeptide GPTIEEVD binds to TPR1 with a 20-fold higher activity than EEVD. Comparably, the Hsp90 pentapeptide MEEVD has almost 10-fold higher affinity for TPR2A domain of Hop than EEVD (Scheufler *et al.* 2000). The differential binding affinities of GPTIEEVD/MEEVD peptides for TPR1/TPR2A respectively result from distinct properties of interaction surfaces formed by the TPR1/TPR2A domains. Consequently, different hydrophobic pockets are available for interactions so that GPTIEEVD preferentially binds to TPR1 while MEEVD preferentially binds to TPR2A. Thus, steric compatibility appears to be a key factor in determining the specificity of substrate recognition. In conclusion, the Hsp70/Hsp90/Hop complex elegantly illustrates how architecture of TPR domains and invariant sequence conservation outside the eight residue TPR consensus defines a precise interaction complex.

The ubiquitous nature and the functional diversity of TPR proteins is remarkable. With so many TPR proteins involved in essential cellular functions, the starting point in functional analysis of discrete TPR families is to decipher structural determinants of TPR-mediated interactions. A large number of TPR proteins interact with Hsp90 and even though they belong to distinct functional groups, they all share the Hsp90 EEVD motif-binding consensus (Scheufler *et al.* 2000). In the course of the present study a highly conserved group of Hsp90 TPR co-chaperones was investigated: the coral AmTPR1, the fly Dpit47 and mammalian TTC4 protein. Gathered data indicates that AmTPR1/Dpit47/TTC4 constitute a peculiar family of developmental genes associated with cell-cycle regulation and cell proliferation.

A)


S. cerevisiae
D. melanogaster
H. sapiens
TPR
STI1
589
466
490
519
543
TPR1
TPR2A
TPR2B

B)


TPR1
TPR2
TPR3
TPR4
TPR5
TPR6
TPR1
TPR2A
H. sapiens
D. melanogaster
S. cerevisiae
(K8)
(N12)
(N43)
(K73)
(R77)
(K229)
(N233)
(N264)
(K301)
(R305)

C)

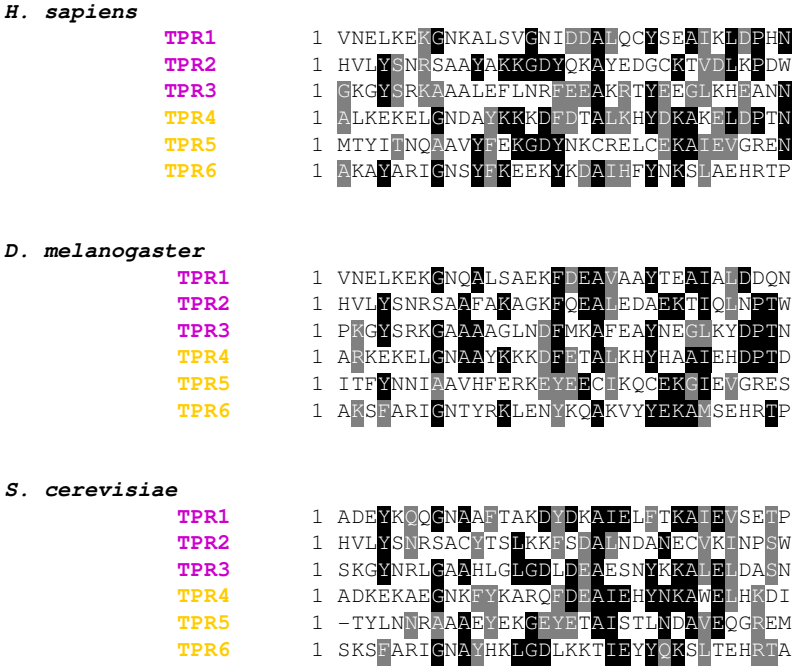


Fig. 1.5. TPR-related fingerprints in STI1/Hop proteins. A) Structural similarity of STI1/Hop homologs. Protein schematics were generated using SMART, the TPR motifs comprising the TPR1, TPR2A and TPR2B domains are indicated in purple, yellow and green respectively. Purple boxes outside of the TPR domains denote regions of low complexity B) and C) Comparison of sequence identity and similarity between TPR motifs of STI1/Hop homologs. Shaded boxes indicate regions of amino acid identity (black), and similarity (grey). B) Alignment of corresponding TPR motif sequences of STI1/Hop homologs (cross-species identity), the eight residue TPR hydrophobic consensus positions are indicated below the alignments. Hsp70 binding residues in TPR1 domain and the corresponding Hsp90 binding residues in TPR2A domain are indicated above the alignments in respective colour-codes. C) Sequence alignment of TPR motifs within individual species (intra-species identity). Note that cross-species identity is greater than intra-species identity (compare the extent of shaded residues between B) and C). All protein sequences were retrieved from the ExPASy proteomics server: Stress inducible phosphoprotein 1 (STI1), also known as Hsp70/Hsp90 organizing protein (Hop): *S. cerevisiae* (P15705), *D. melanogaster* (Q9VPN5), *H. sapiens* (P31948). Sequence alignments and box-shading of homologous residues were generated using ClustalW (http://www.ch.embnet.org/software/ClustalW.html) and BoxShade (http://www.ch.embnet.org/software/BOX_form.html), respectively.

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AmTPR1, a tetratricopeptide repeat protein from coral *Acropora millepora* is a homolog of *Drosophila* Hsp90 co-chaperone Dpit47 and the human cancer-related gene TTC4

2.1. INTRODUCTION

The TPR motif is a versatile protein-protein interaction element recruited by a large number of functionally different proteins (Blatch and Lassle 1999, D'Andrea and Regan 2003, Das *et al.* 1998, Dolinski *et al.* 1998, Gatto *et al.* 2000, Groves and Barford 1999, Kumar *et al.* 2001, Lamb *et al*. 1995, Malek *et al.* 1996, Nakatsu *et al.* 2000). Each TPR consists of 34 amino acids which form a pair of antiparallel α-helices of equivalent length, helices A and B. The arrangement of individual TPRs creates a right-handed superhelix, a well defined structural unit for mediating protein-protein interactions (Blatch and Lassle 1999, D'Andrea and Regan 2003, Das *et al.* 1998, Goebl and Yanagida 1991, Groves and Barford 1999, Hirano *et al*. 1990, Main *et al.* 2003, Sikorski *et al.* 1990). Even though the TPR motif is highly degenerate, sequence analysis of individual motifs shows a consensus of hydrophobic residues at specific positions in the two α-helices (Blatch and Lassle 1999, D'Andrea and Regan 2003, Hirano 1990, Lamb *et al.* 1995, Main *et al.* 2003, Sikorski *et al.* 1990). The residues constituting the hydrophobic consensus constitute the interface between the α helices A and B and are critical for stabilizing the conformation of the TPR superhelix (Blatch and Lassle 1999, D'Andrea and Regan 2003, Hirano 1990, Sikorski *et al.* 1990).

The TPR protein-protein interaction element is found in many protein complexes and it is indispensable for many cellular functions. By far, the most extensively studied TPR-protein complexes are: 1) the Hsp90 co-chaperone complex, essential for activation, folding and assembly of a wide range of client proteins involved in cell cycle regulation and signal transduction pathways (Bohen *et al*. 1995, Panaretou *et al*. 1998, Pearl and Prodromou 2000, Prodromou *et al*. 1999), 2) the anaphase promoting complex (APC), involved in ubiquitin-mediated proteolysis of cyclins and thus important for the regulation of the cell cycle, (Hirano *et al*. 1990, Irniger *et al.* 1995, King *et al.* 1995, Lamb *et al.* 1994, Nigg 1995, Tugendreich *et al.* 1995, Wang *et al.* 2003), 3) transcription repression complex, negative regulator of gene expression in

Saccharomyces cerevisiae (Lamb *et al.* 1995, Tzamarias and Struhl 1995), 4) protein transport complex, involved in trafficking of proteins across peroxisomal and mitochondrial membranes (Blatch and Lassle 1999, Goebl and Yanagida 1991, Lamb *et al*. 1995, Lithgow *et al.* 1995, Schliebs *et al.* 1999) and 5) serine/threonine protein phosphatase complex, associated with signal transduction pathways and cell growth (Becker *et al.* 1994, Das *et al.* 1998, Lee *et al.* 1994, Swingle *et al.* 2004)

The focus of the present study was functional characterisation of *Acropora millepora* AmTPR1, the first TPR protein identified in a coral species. AmTPR1 is a member of a distinct group of TPR proteins including *Drosophila* Dpit47 (Crevel *et al.* 2001), and human TTC4 protein (Su *et al.* 1999). Dpit47 is a Hsp90 co-chaperone and a DNA polymerase α interacting protein possibly involved in regulation of cell proliferation (Crevel *et al.* 2001). Little is known about the human TTC4 protein and the interpretation of the existing data is controversial. TTC4 maps to the region of chromosome 1p31 that is frequently deleted in melanoma and sporadic breast cancer and thus it has been hypothesised that TTC4 may be a tumor suppressor gene (Su *et al*. 1999, Poetsch *et al*. 2000). In contrast, there is other data which suggests that TTC4 may have a pro-proliferative role. In particular, in Burkitt lymphoma cells TTC4 expression appears to be regulated by the c-Myc oncogene (Li *et al.* 2003). Furthermore, in lung cancers TTC4 was identified in a minimally altered region of chromosomal gain (Kim *et al*. 2005). Taken together, these results imply that TTC4 may function as an oncogene rather than as a tumor suppressor.

The aim of this study was to characterise the cnidarian AmTPR1 gene in order to understand better the function and cellular roles of AmTPR1/Dpit47/TTC4 group of proteins. The rationale for this approach arises because cnidarians, being one of the most ancient metazoan phyla, are exceptionally informative in respect to ancestral gene function (Ball *et al.* 2002, Ball *et al.* 2004, Kortschak *et al.* 2003, Miller *et al.* 2000, Miller and Ball 2000, Technau *et al.* 2005). Although nominally classified as diploblastic animals, in the early embryogenesis Cnidaria undergo a process that is remarkably similar to gastrulation in higher metazoans. The embryo first undergoes a spiral-like holoblastic cleavage to form an irregular sphere (morula). Following, the embryo enters a bi-layered disc-shaped prawnchip stage during which a separation of the two layers occurs, leading to a depression on one side of the flattened disc (blastopore). The closure of the blastopore ("gastrulation"), ultimately

results in a formation of a second, inner tissue layer – gastroderm or endoderm at which stage the embryo resembles a sphere. The sphere gradually elongates and becomes a ciliated free-swimming planula, a dispersal stage (Miller and Ball 2000). The absence of a third layer mesoderm (which in triploblastic metazoans forms during gastrulation), with concomitant existence of a gastrulation-like process indicates that Cnidaria retained much of the genetic complexity that was thought to be associated with higher metazoans (Technau *et al.* 2005).

In addition to AmTPR1, further characterisation of *Drosophila* Dpit47 was carried out for comparative purposes. It will be shown by sequence analysis and investigation of expression pattern and protein interactions that AmTPR1 and Dpit47 are members of a unique family of TPR proteins and are likely to play important roles in development and cell proliferation.

2.2. MATERIALS AND METHODS

2.2.1. Reagents

General chemicals were analytical grade and obtained from the following companies: Ajax Chemicals (Australia), Clontech (Australia), GibcoBRL (USA), ICN Biomedicals (USA) and Sigma-Aldrich (Australia). Unless otherwise indicated, all buffers and solutions were prepared as described in Sambrook *et al*. (1989). The solutions were either sterilised by autoclaving or filtering with 0.22 ul filter (Millipore).

2.2.2. PCR and cDNA isolation

Partial *Acropora* AmTPR1 cDNA was amplified from *A.millepora* larval cDNA libraries with the following primers: forward pBluescript SK (-) T3, 5' - AATTAACCCTCACTA AAGGG - 3' and reverse AM1, 5' - CTCGAGTTCCAACAGCTT - 3'. Amplification was carried out at following conditions:

1. 95^0C -5 min

2. 95^0C - 1 min

3. 50^0C - 1 min

4. 72^0C - 2 min

5. 30 cycles 2-4

6. 72^0C - 3 min

The PCR mix contained 2.5 ul PCR buffer (10X, Promega), 2.5 ul dNTPs (2 mM, Promega), 2 ul $MgCl_2$ (25 mM, Promega), 0.5 ul of each of forward and reverse primer (25 uM, Sigma) and 0.12 ul Taq polymerase (5 U/µl, Promega) and DNase free water to a final volume of 25 ul per reaction. Taq polymerase was added after the 1^{st} step (hot start).

2.2.3. Library screening

The *A. millepora* larval and adult cDNA libraries were kindly provided by Dr. David Hayward (Research School of Biological Sciences at Australian National University). The libraries were constructed in the λ Uni-Zap system (Stratagene), using mRNA extracted from embryos at approximately 11-13 hrs post fertilization (prawnchip), 96 hrs post fertilization (post-settlement), and adult stages. XL1-Blue MRF' *E. coli* strain was used for propagation of the λ Uni-Zap phage. Culturing and phage infection of the XL1-Blue MRF' bacteria was carried out by using standard techniques (Sambrook *et al.* 1989). Following phage lysis, plaques were lifted onto 137 mm Hybond-N+ nylon membranes (Amersham Pharmacia Biotech), following the manufacturers instruction for colony and plaque lifts with the following exceptions: the phage were denatured for 5 min in 0.2 M NaOH, 1.5 M NaCl denaturing solution, followed by neutralisation for 7 min in 0.5 M TrisCl (pH=7.4), 1.5 M NaCl, 1 mM EDTA neutralisation solution, the lysis step was omitted. After washing in 2X SSC the membranes were allowed to dry at room temperature. The DNA was then fixed to the membranes by 2 min exposure to UV using a 312 nm UV transilluminator. Hybridisation was carried out in 300 X 35 mm bottles (Hybaid) at 42^oC in a Shake 'n' Stack hybridisation oven (Hybaid). The membranes were hybridised according to the Amersham Pharmacia Biotech protocol for hybridisation in tubes with the following exceptions: the membranes were not pre-wetted before use and they were prehybridised for at least two hours before adding the radiolabeled probe in 0.5 %

blotto, 2X SSPE, 1 % SDS prehybridisation/hybridisation solution. Following probe addition, the membranes were incubated for 12-16 hrs and then washed 2X 20 min in a low stringency wash (2X SSC, 0.1 % SDS). The probe for library screening was obtained by digestion of the AmTPR1 partial cDNA clone (amplified from *A. millepora* cDNA libraries) from the PCR-cloning vector pGEM-T (Promega). The AmTPR1 probe was radiolabeled using the oligonucleotide labeling method. Briefly, 30 uCi of (α-(32)P)-dATP (Gene Works) and Megaprime labeling kit (Amersham Pharmacia Biotech), were used to label 50 ng of denatured DNA according to manufacturer's instructions. Typically, 10-15 ul of purified radiolabeled probe was used for hybridisation. After screening approximately 700 000 plaques, a single positive clone was isolated and recovered from the λ Uni-Zap vector as outlined in the manufacturer's *in vivo* excision protocol using ExAssist helper phage and SOLR *E. coli* strain (Stratagene).

2.2.4. Semiquantitative PCR

A 116 bp AmTPR1 and a 100 bp S-Adenosyl homocysteine hydrolase (ADH), fragments were amplified from double-stranded cDNA corresponding to egg, prawnchip (pch), donut (dnt), pear (per), planula (pln) and pre-settlement (prs) *Acropora* embryonic stages, using the following primer pairs: forward AM122, 5' - AGAAAGTCCCAGCCCTTAT - 3' and reverse AM238, 5' - CTGTCTTTCCTATAGCGTC - 3'; and forward ADH, 5'- AAGAAGACAAACATCAAGCCTCA - 3' and reverse ADH, 5'- CACATCCAAGGT TCACAAGACG - 3'. The cycling conditions were:

1. 95^0C -5 min
2. 95^0C - 1 min
3. 50^0C - 1 min
4. 72^0C - 20 sec
5. 26 cycles 2-4
6. 72^0C - 30 sec

Taq was added after the first denaturation step and the amplification mix was the same as above (PCR and cDNA isolation), the oligonucleotide primers were synthesized by Geneworks. Single-stranded cDNA was synthesized and kindly provided by Akira Iguchi. First strand cDNA was obtained using 1 ug of total RNA

and the Super SMART^{TM} PCR cDNA Synthesis Kit (Clontech Laboratories), in a 10 ul total reaction volume, according to manufacturer's instructions. Following synthesis, the cDNA was diluted 1:5 and 2 ul of the resulting dilution was used in semiquantitative PCR.

2.2.5. Alsterpaullone treatment of *A. millepora* embryos

Alsterpaullone treatment was carried out by Chuya Shinzato. Alsterpaullone (Calbiochem) dissolved in 0.025 % DMSO, was diluted in 0.22 um Millipore-filtered seawater (MPFSW) to final concentration 5 uM. *Acropora* embryos from 64-128-cell stage (6 hrs after fertilization), were exposed to 5 uM alsterpaullone for approximately 10 hrs, until early donut stage (16 hrs after fertilization). Embryos were then extensively washed in MPFSW and fixed using the procedure outlined below.

2.2.6. Fixation and storage of embryos

A. millepora embryos were fixed for 12 minutes in 3.7 % formaldehyde in MPFSW, buffered with Hepes to a pH 8.0. Following, embryos were repeatedly washed in MPFSW and gradually dehydrated through a methanol/ddH_2O series (20 %, 50 %, 70 %, 90 % and 100 % methanol). Embryos were stored in absolute methanol at -20^{0}C until further use. *Drosophila* embryos at different developmental stages were fixed using 4 % paraformaldehyde and kindly provided by Dr. David Hayward (RSBS, ANU). The embryos were stored in absolute methanol and kept at -20^{0}C. Prior hybridisation both *Acropora* and *Drosophila* embryos were returned to room temperature and then rehydrated through a methanol/PBT (PBT: 1X PBS, 0.1 % Triton-X-100), series (90 %, 70 %, 50 %, 20 % methanol in PBT). The embryos were then repeatedly washed in PBT and either placed in RIPA detergent (150 mM NaCl, 1 % Nonidet-P40, 0.5 % Na deoxycholate, 0.1 % SDS, 1 mM EDTA, 50 mM Tris pH 8.0), overnight (*Acropora*) or used directly for hybridisation (*Drosophila*). Following exposure to RIPA, *Acropora* embryos were rinsed in PBS and dehydrated in an ethanol/ddH_2O series (50 %, 70 %, 90 % and twice 100 % ethanol). Half volume of ethanol was then replaced with xylene, incubated for 10 min at room temperature, followed by 3 hrs incubation in 100 % xylene at room temperature. Xylene was removed by a series of ethanol washes (1:1 ethanol/xylene followed by three 100 % ethanol washes). Embryos were then gradually rehydrated to PBT (10 %, 30 %, 50 %, and 100 % PBT), washed three times in 100 % PBT and then hybridised to

Digoxigenin (DIG)- or Fluorescein (Flu)-labeled riboprobes. The RIPA/xylene treatment of *Acropora* embryos was carried out in order to remove the large amount of lipid content of embryos, a prerequisite for successful hybridisation.

2.2.7. In situ hybridisation

Acropora and *Drosophila* embryos in PBT were gradually exposed to hybridisation solution (1:1 PBT/hybridisation sol. followed by 100 % hybridisation sol., incubations were at room temperature, 10 min each wash; hybridisation sol.: 50 % formamide, 4X SSC, 50 ug/ul heparin, 1X Denhardt's, 5 % dextran sulphate, 0.1 % Tween, 500 ug/ml denatured salmon sperm DNA). Following, the embryos were incubated at 50 - 55^0C in 100 % hybridisation solution for 2 - 4 hrs. The embryos were then placed in 250 ul of hybridisation solution containing either DIG- or Flu-labeled riboprobe and incubated at 55^0C for either 72 hrs (*Acropora*), or 48 hrs (*Drosophila*). The riboprobes were hydrolised and diluted 1/125 for hybridisation purposes. Unbound probes were removed by extensive washing in hybridisation wash solution (50 % formamide, 4X SSC, 0.1 % Tween), at 55^0C (2X 15 min, 1X 12 hrs), which was then gradually replaced with PBT (1:1 PBT/hybridisation sol. followed by three washes in 100 % PBT for 15 min). Next, embryos were incubated either with anti-DIG-alkaline phosphatase Fab (Roche), or anti-Flu-alkaline phosphatase Fab antibody (Roche), in PBT (1:1600 dilution), for 2 hrs at room temperature with gentle agitation. Unbound antibodies were removed by a series of PBT washes (2X 10 min, 1X 12 hrs, 3X 15 min), followed by three 5 min washes in NTMT buffer (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 0.1 % Tween). NTMT wash solution was then replaced either with BCIP/NBT solution (Alkaline phosphatase substrate kit, Vector Laboratories Burlingame), for embryos that were incubated with anti-DIG antibodies, or SigmaFast^{TM} Fast Red solution (Sigma), for embryos incubated with anti-Flu antibodies, both according to manufacturer's instructions. The embryos were then incubated at room temperature until adequate color development occurred (typically between 2 - 4 hrs). Further color development was stopped by briefly washing the embryos in PBT (3X 5 min), followed by removal of background staining by gradual replacement of PBT by ethanol (1:1 PBT/ethanol, 3 – 5X 100 % ethanol, until the wash solution was clear of residual staining). Embryos were then gradually rehydrated in an ethanol/PBT wash series (90 %, 70 %, 50 %, 20 % ethanol in PBT, 3X PBT). Final PBT wash was replaced by 80 % glycerol after which the embryos were viewed

under a Leica MZ FLIII stereomicroscope and photographed using a SPOT digital camera. Images were processed using Adobe Photoshop 5.5. Cross sections of embryos were obtained by cutting the stained embryos using a fine razor attached to a short wooden skewer.

2.2.8. Riboprobe synthesis

Fluorescein-labeled Lipase riboprobe was synthesized and kindly provided by Chuya Shinzato. Riboprobes for AmTPR1 and Dpit47 were synthesized using run-off transcription. pGEM-T vectors containing AmTPR1 and Dpit47 cDNAs were digested with Nco I and Bgl II respectively, to produce 5' overhangs at the 5' ends of both cDNA inserts.1ug of the resulting linearised cDNA was then used for DIG-riboporobe synthesis in a reaction containing 1X DIG labeling buffer (Promega), 1X DIG-NTP mix (Roche), 9 mM DTT (Promega), 40 U RNasin (Promega) and 20 U of Sp6 RNA polymerase (Promega), in a total of 20 ul. The reactions were incubated at 37^0C for 3 hrs and then stopped by 20 mM EDTA. The riboprobes were purified by ethanol precipitation, resuspended in 50 ul RNase free H_20 and hydrolised by addition of 5.5 ul of sodium carbonate buffer (0.4 M $NaHCO_3$, 0.6 M Na_2CO_3, pH 10.2), and incubation at 60^0C for the times calculated using the following equation: $t = (L_0 – Lt) / 0.11\ L_0\ L_f$; where t - time of incubation, L_0 - initial length of RNA in kb, Lt - desired length of RNA in kb (0.5) and 0.11 - constant of hydrolysis efficiency. Further riboprobe hydrolysis was stopped by addition of 2 ul 3 M NaOAc. The riboprobes were then purified by ethanol precipitation, resuspended in 80 ul of riboprobe resuspension buffer (50 % formamide, 50 % TE pH 7.5, 0.1 % Tween), and stored at -20^0C until further use.

2.2.9. Cloning of AmTPR1 promoter

The 2 kb fragment corresponding to AmTPR1 promoter was obtained by PCR from *A. millepora* genomic library (50 ng), using an internal AM2 primer (reverse), 5' - CCTTGTCCAGCTCTTCTGC - 3', the λGEM-11 cloning primer sp6 (forward), 5' - ATTTAGGTGACACTATAGAA - 3' and the following conditions:

1. 95^0C -5 min

2. 95^0C - 1 min

3. 50^0C - 30 sec

4. 72^0C - 3 min, 45 sec

5. 35 cycles 2-4

6. 72^0C - 4 min, 45 sec

The hot start method of adding Taq was used and the PCR mix was same as above (PCR and cDNA isolation). The oligonucleotide primers were synthesized by Geneworks. *A. millepora* genomic library was constructed by Dr. David Hayward (RSBS, ANU). The high molecular weight genomic DNA was obtained from frozen sperm and pre-digested with MboI prior to cloning into λGEM-11 vector (Promega).

2.2.10. Plasmid constructs

The 630 bp AmTPR1 cDNA was cloned into the DupLex-A™ activation domain (AD)-fusion vector pJG4-5 as an EcoRI fragment, using standard cloning procedures (Sambrook *et al*. 1989). Correct orientation of the AmTPR1 insert was verified by DNA sequencing. Both pEG202-DNApolα and pEG202-Hsp90 DNA binding domain (DBD)-fusion protein constructs (containing *Drosophila* Hsp90 and *Drosophila* DNA polymerase α 180 kDa subunit coding regions respectively), were kindly provided by Dr. Sue Cotterill (Dept. of Basic Medical Sciences, St.Georges University London, UK)

2.2.11. Yeast two-hybrid assay

For the yeast two-hybrid experiment, yeast transformation was performed using the LiOAc method, as described in the DupLex-A™ Yeast Two-Hybrid System protocol for small-scale yeast transformation (OriGene Technologies Inc, 1998), with minor modifications. Briefly, the procedure was as follows. 5 ml overnight yeast culture of EGY48(pSH18-34) was grown at 30^oC with vigorous agitation (200 rpm) in SD dropout Ura medium (preparation of all media and solutions was according to the DupLex-A™ Yeast Two-Hybrid System User Manual). The 5 ml culture was used to inoculate 50 ml of the medium to an OD_{600} = 0.1. When the OD_{600} of the 50 ml culture reached 0.5-0.7 (3-6 hrs after inoculation), the cells were collected by centrifugation at 1500 g for 5 min at room temperature and resuspended in 20 ml of sterile deionised H_20. The centrifugation step was repeated, the cells were resuspended in 300 ul of 1X TE/LiOAc and the resulting suspension was aliquoted, 3X 100ul. 50 ng of denatured salmon sperm carrier DNA and 500 ng of each plasmid

construct were then added to the TE/LiOAc cell suspension and the contents mixed by inversion. Next, 300 ul of 1X TE/LiOAc/PEG was added to each transformation reaction followed by incubation at 30^{o}C for 30 min with moderate shaking (120 rpm). Following, 70 ul of DMSO was added to the transformation reactions which were then incubated at 42-45^{o}C for 15 min. The cells were recovered by centrifugation at 13.2 krpm for 30 sec, resuspended in 200 ul of sterile deionised H_20 and plated onto appropriate SD selective medium (see Appendix A). The plates were incubated at 30^{o}C for 2-4 days when transformed colonies started to appear. Positive transformants were replated onto fresh SD selective plates and tested for LacZ activity using the β–galactosidase filter assay. The assay was carried out as follows. A piece of Whatman No. 1 filter paper was cut and presoaked in 2 ml of Z-buffer/X-Gal (60 mM Na_2HPO_4 X $7H_20$, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ X $7H_20$, 50 mM β-mercaptoethanol, 1 mg/ml X-Gal; pH=7.0)**.** Using forceps, another cut piece of Whatman filter paper was placed on the surface of the plate containing the replated yeast transformants, ensuring that the colonies adhere to the filter. Using forceps, the filter with adherent yeast colonies was lifted and subjected to three freeze/thaw cycles (10 sec liquid nitrogen/room temperature), and then placed, yeast colonies side-up, onto the filter presoaked in Z-buffer/X-Gal solution. Following, the two filters were incubated at 30^{o}C and checked periodically for the appearance of the blue colour. In order to avoid false positives, incubation time was kept under 6 hrs.

2.3. RESULTS

2.3.1. *Acropora* AmTPR1 is a tetratricopeptide repeat protein, homolog of *Drosophila* Dpit47 and human TTC4 protein

As a part of an ongoing EST project, a total of 6021 unigenes corresponding to 5063 predicted peptides have been identified from the coral *Acropora millepora* (Technau *et al.* 2005). AmTPR1 was identified as an EST clone. BLAST search for homologous sequences revealed that the predicted protein sequence of AmTPR1 highly matched the sequence of an uncharacterised putative human tumor suppressor gene TTC4. Another significantly matching sequence was that of *Drosophila* Dpit47, an Hsp90 co-chaperone involved in DNA replication (Crevel *et al.* 2001). These intriguing matches prompted further investigations aimed at functional characterisation of AmTPR1. Initially, attempts were made to isolate the full length AmTPR1 cDNA clone. For this purpose, PCR-based cloning and library screening were employed. Based on the EST sequence, an internal reverse primer AM1 was designed and used in conjunction with the pBluescript cDNA vector forward primer T3 (Fig. 2.2). cDNA libraries from three different developmental stages of *A. millepora* were used as templates for the PCR: pre-settlement, prawnchip and adult cDNA library. As shown in Fig. 2.1, a single fragment of approximately 500 bp was amplified from both larval *A. millepora* cDNA libraries. In contrast, no products were obtained from the adult cDNA library. The 500 bp fragment obtained via PCR amplification was used to screen the *A. millepora* pre-settlement cDNA library. Of approximately 200 000 plaques screened, a single positive clone was obtained and selected for further analysis. Upon pBluescript phagemid rescue from the λ-UniZap cloning vector (for details see Materials and Methods), the putative clone was sequenced using pBuescript T3 and T7 vector primers. Sequencing results revealed that the clone isolated from the *A. millepora* pre-settlement cDNA library was 630 bp (Fig. 2.2). Subsequent analysis of the predicted peptide sequence, employing two independent programs which allow the identification and annotation of known protein domains, SMART (Simple Modular Architecture Research Tool) and TIGR (The Institute for Genomic Research), revealed that the 630 bp clone encoded a 210 amino acid residue tetratricopeptide protein featuring three repeats. The protein was termed *Acropora millepora* TPR1 (AmTPR1), the first tetratricopeptide protein identified

from the coral *Acropora*. It has to be noted that the deduced amino acid sequence of AmTPR1 is significantly shorter than that of *Drosophila* Dpit47 and human TTC4 protein. However, a search of cnidarian EST databases revealed both *Nematostella* and *Hydra* homologs of AmTPR1, termed NvTPR1 and HmTPR1 (*Nematostella vectensis* and *Hydra magnipapillata* TPR protein 1 respectively). The predicted amino acid sequences revealed that both NvTPR1 and HmTPR1 are similar in length to Dpit47 and TTC4 (Fig. 2.2). Given the close evolutionary relatedness between the three species of cnidarians (Ball *et al.* 2004, Technau *et al.* 2005), it is likely that a longer AmTPR1 clone may be present in *Acropora* cDNA libraries. However, additional attempts at library screening were not pursued due to time restrictions.

Further SMART search for proteins containing similar domain organization revealed several putative AmTPR1 homologs (Fig. 2.3). A more detailed sequence comparison showed that other than having the same basic structure, AmTPR1 and related proteins share a high degree of amino acid identity and similarity (Fig. 2.4). Significantly, apart from NvTPR1 (51 % identity, 69 % similarity) and HmTPR1 (46 % identity, 68 % similarity), AmTPR1 closest homologs are mammalian proteins: human TTC4 (46 % identity, 67 % similarity), and mouse TTC4 (41 % identity, 66 % similarity). Other AmTPR1 homologs include *D. rerio* TTC4 (44 % identity, 64 % similarity), *D. melanogaster* Dpit47 (38 % identity, 57 % similarity) and *C. elegans* C17G10.2 (35 % identity, 54 % similarity). As expected, the greatest extent of amino acid identity and similarity occurs between the corresponding TPRs across species (Fig. 2.5, panel A. It is important to note that in comparison, the sequence homology between TPRs within a single specie is much lower (Fig. 2.5, panel B).

A remarkable feature of AmTPR1-like proteins is a striking deviation from the hydrophobic TPR consensus. Specifically, at positions 7 and 11 of the first and the second TPR domain, polar hydrophilic charged and polar hydrophilic neutral substitutions are commonly observed (Table 2.1).

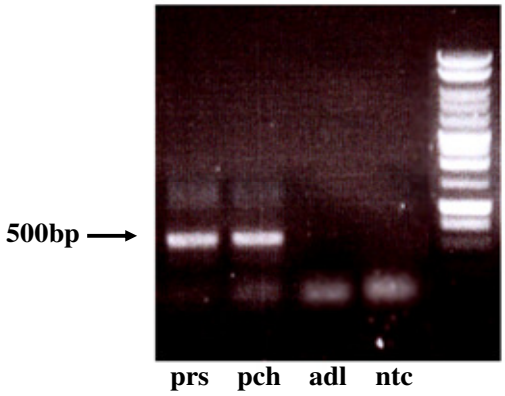


Fig. 2.1. A 500 bp fragment amplified from *A. millepora* cDNA libraries using AM1 reverse primer and pBluescript T3 forward primer. For the PCR, cDNA libraries corresponding to pre-settlement (prs), prawnchip (pch) and adult (adl) developmental stages of *A. millepora* were used in addition to the no template control (ntc) For primer sequences see Materials and Methods.

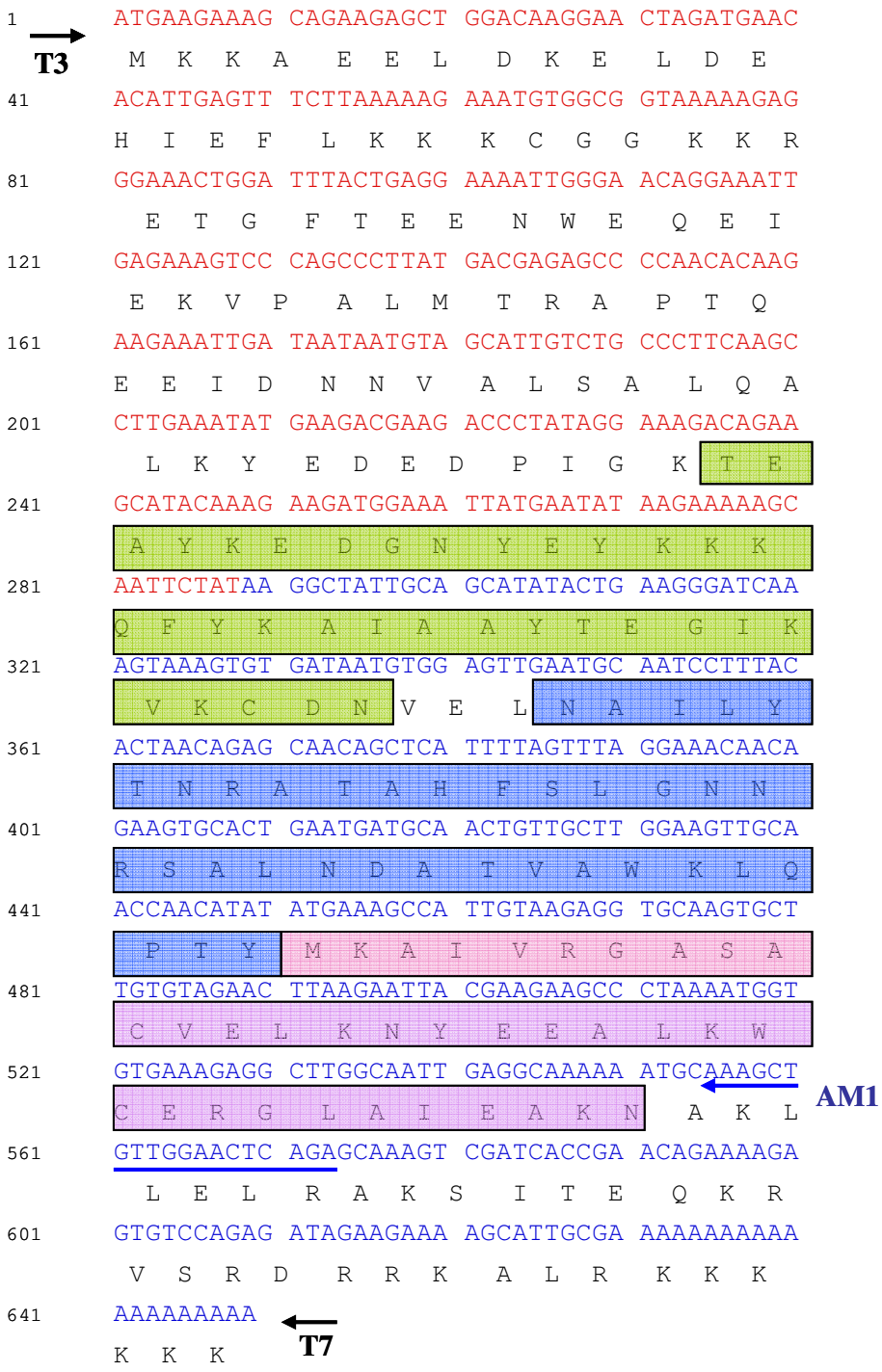


Fig. 2.2. Nucleotide and amino acid sequence of AM1PR1 clone isolated from *A. muepora* pre-settlement library. The three TPR motifs are indicated in green, blue and violet respectively; the portion corresponding to the EST sequence is denoted in blue (note that this sequence did not differ from the original EST sequence). pBluescript vector primers T3 and T7 (flanking the cloning site), and AM1 primer are indicated. For primer sequences see Materials and Methods.

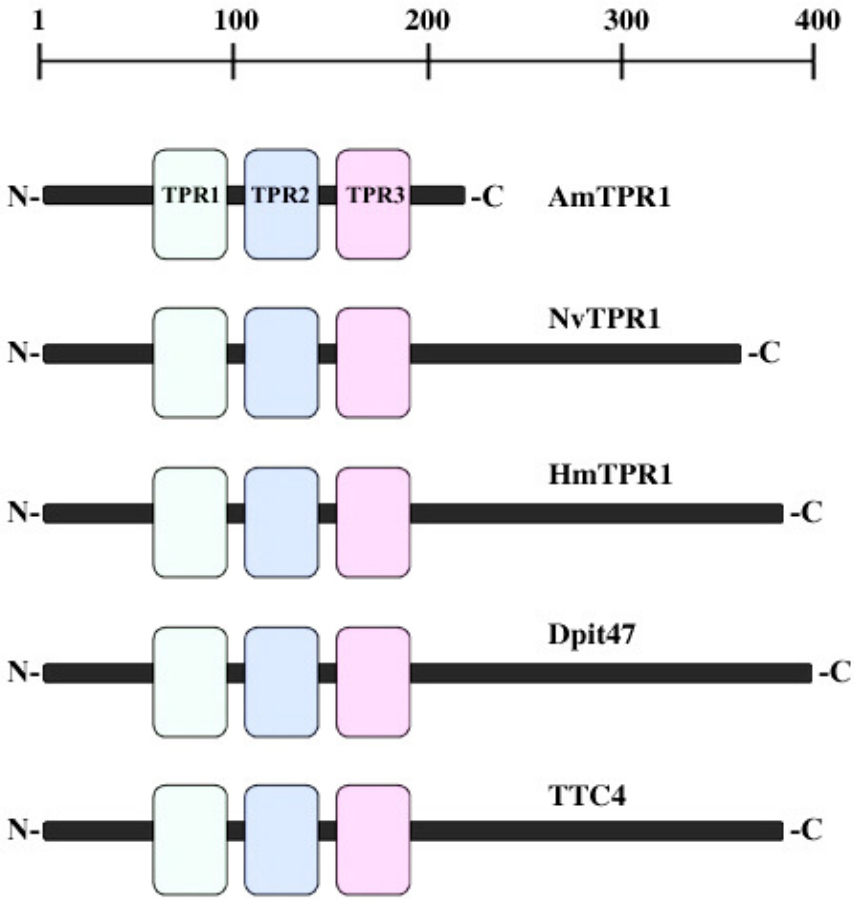


Fig. 2.3. Schematic representation of the structure of AmTPR1 and related tetratricopeptide proteins Dpit47 and TTC4. A*cropora* AmTPR1 (210 residues), *Nematostella* NvTPR1 (381 residues), *Hydra* HmTPR1 (387 residues), *Drosophila* Dpit47 (396 residues) and human TTC4 (387 residues).

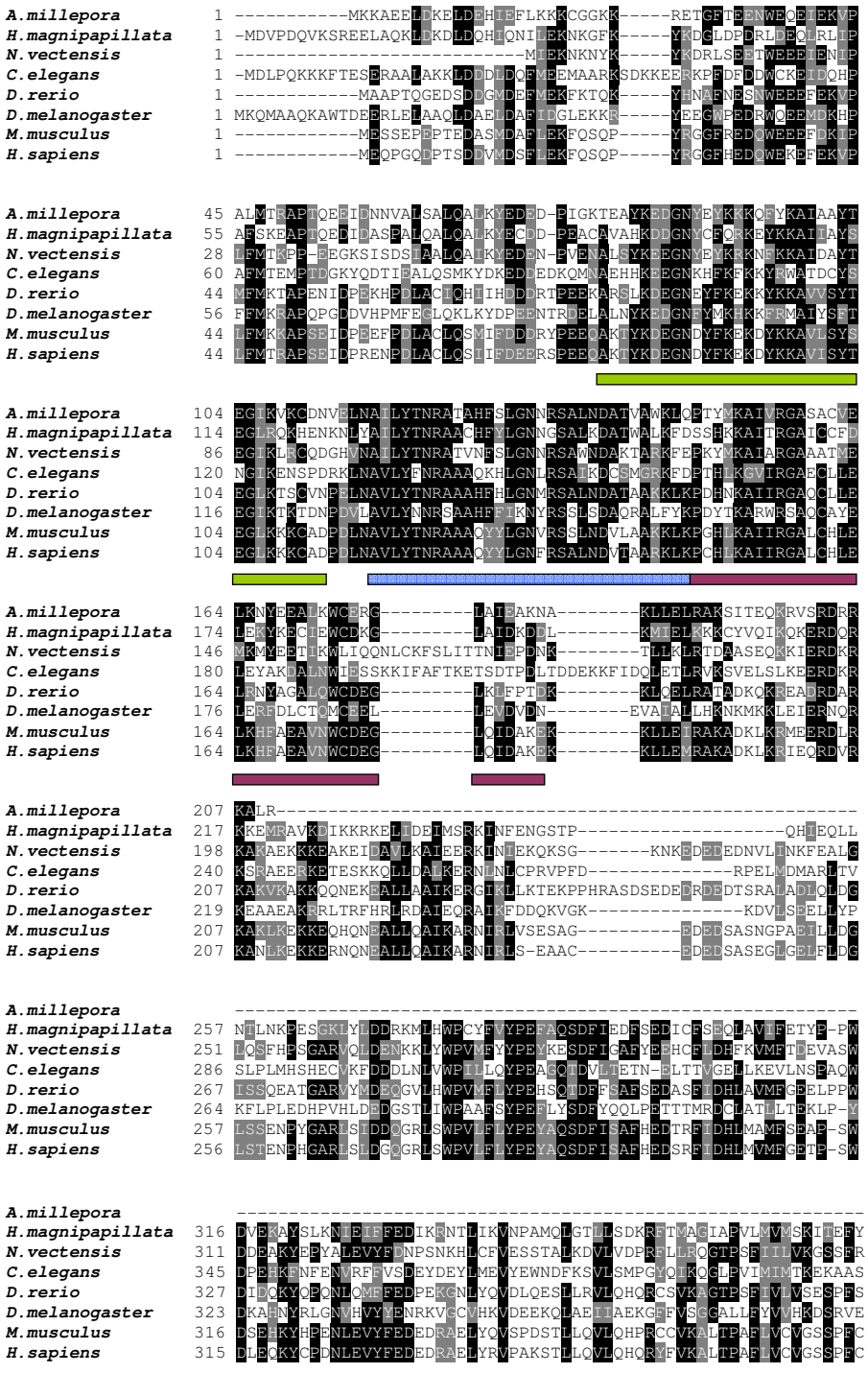


Fig. 2.4. Sequence alignment of AmTPR1 and related proteins. Shaded boxes indicate regions of amino acid identity (black), and similarity (grey). The three TPR motifs are indicated in green, blue and violet respectively. Sequences of related TPR proteins were retrieved from the NCBI database (www.ncbi.nlm.nih.gov): *H. sapiens* TTC4 (NP_004614.2), *M. musculus* TTC4 (NP_082485.1), *D. rerio* TTC4 (NP_001002122.1), *C. elegans* C17G10.2 (NP_495087.1), *D. melanogaster* Dpit47 (NP_525106.2), *N. vectensis* (XP_001637204) and COMPAGEN database (http://compagen.zoologie.uni-kiel.de): *H. magnipapillata* (CL1354 Contig1). Alignment of sequences and box-shading of homologous residues were generated using ClustalW (www.ch.embnet.org/software/ClustalW.html) and BoxShade (www.ch.embnet.org/software/BOX_form.html), respectively.

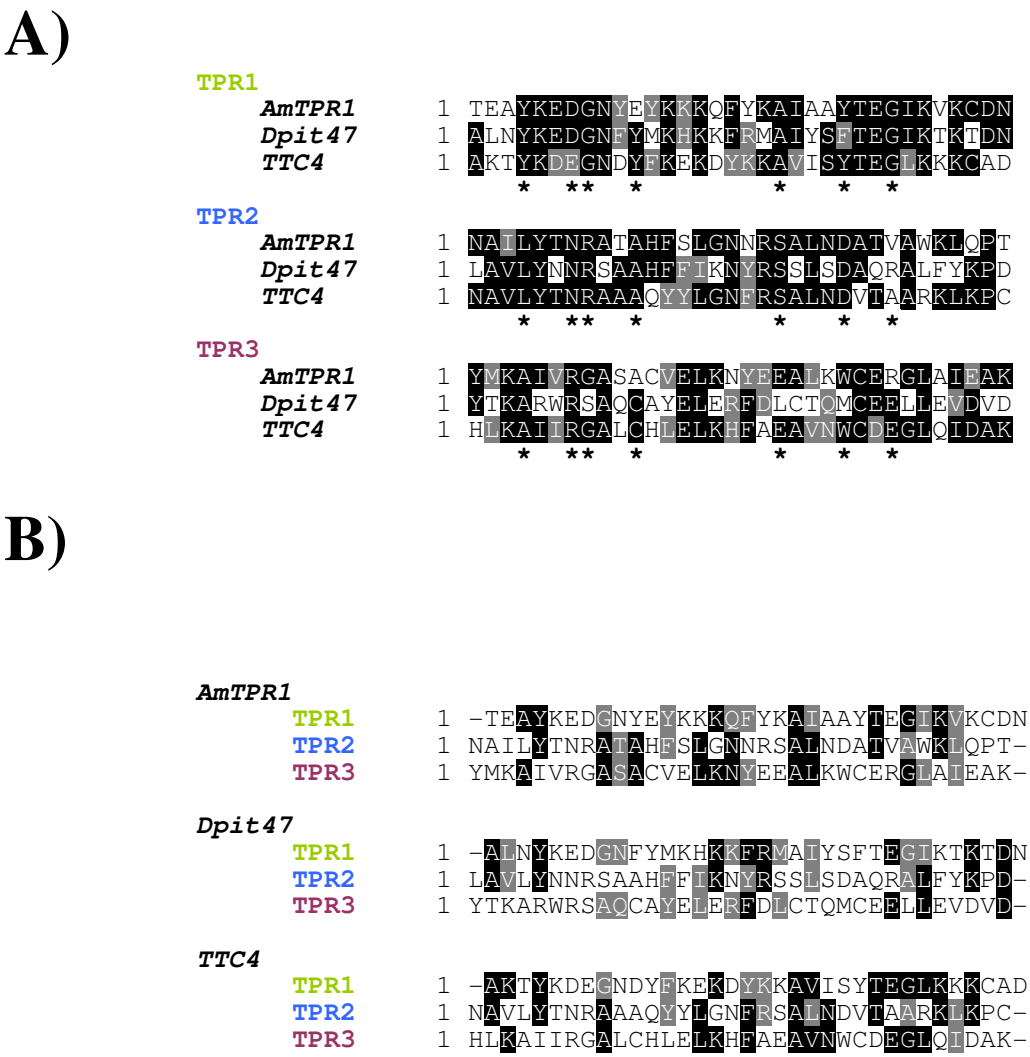


Fig. 2.5. Comparison of sequence identity and similarity between TPR motifs. Shaded boxes indicate regions of amino acid identity (black), and similarity (grey). A) Alignment of corresponding TPR motif sequences of *A. millepora* AmTPR1, *D. melanogaster* Dpit47 and *H. sapiens* TTC4 (cross-species identity), the eight residue TPR hydrophobic consensus positions are indicated below the alignments. B) Sequence alignment of TPR motifs within individual species (intra-species identity). Note that cross-species identity is greater than intra-species identity.

Table 2.1. Deviation from the hydrophobic TPR consensus in AmTPR1 and related proteins. The residues at positions 4, 7, 8, 11 and 20, 24, 27, constitute the hydrophobic interface between α helices A and B respectively. Polar hydrophilic charged and polar hydrophilic neutral residue substitutions in the first two TPR motifs of AmTPR1 homologs are depicted in blue and light blue respectively. The TPR consensus is shown below the table. Note that S, even though hydrophilic, is permitted at positions 8 and 20 (see TPR consensus).

TPR1	**helix A**					**helix B**		
position	**4**	**7**	**8**	**11**		**20**	**24**	**27**
A. millepora	Y	D-	G	E-		A	Y	G
H. magnipapillata	H+	D-	G	C		A	Y	G
N. vectensis	Y	E-	G	E-		A	Y	G
D. melanogaster	Y	D-	G	Y		A	F	G
D. rerio	L	D-	G	Y		A	Y	G
H. sapiens	Y	E-	G	Y		A	Y	G
M. musculus	Y	E-	G	Y		A	Y	G
C. elegans	H+	E-	G	H+		A	Y	G

TPR2	**helix A**					**helix B**		
position	**4**	**7**	**8**	**11**		**20**	**24**	**27**
A. millepora	Y	R+	A	H+		A	A	A
H. magnipapillata	Y	R+	A	H+		A	A	A
N. vectensis	Y	R+	A	N		A	A	A
D. melanogaster	Y	R+	S*	H+		S*	A	A
D. rerio	Y	R+	A	H+		A	A	A
H. sapiens	Y	R+	A	Q		A	V	A
M. musculus	Y	R+	A	Q		S*	V	A
C. elegans	Y	R+	A	Q		A	C	G
TPR consensus:	**W** **L** **Y**	**L** **I** **M**	**G** **A** **S**	**Y** **L** **F**		**A** **S** **E**	**F** **Y** **L**	**A** **S** **L**

2.3.2. *Acropora* AmTPR1 is expressed in secretory-like cells and may be a target of the Wnt/β-catenin signalling pathway

To determine the cellular function of AmTPR1, it was necessary to examine the expression pattern and regulation of AmTPR1 in *Acropora* embryos. First, using semi-quantitative PCR analysis, it was established that, relative to expression of the housekeeping gene S-Adenosyl homocysteine hydrolase (ADH), low levels of AmTPR1 transcript were present throughout the embryonic development of *Acropora* (Fig. 2.6). Subsequent in situ hybridisation of *Acropora* embryos revealed that AmTPR1 expression was ubiquitous in the early embryo, at the onset of gastrulation. In contrast, at the end of gastrulation, AmTPR1 transcript became restricted to a specific population of cells, as indicated by the appearance of a discrete punctate staining (Fig. 2.7, panels C-E). This pattern of cell-specific expression of AmTPR1 persisted in later stages of *Acropora* development (Fig. 2.7). Other than AmTPR1, several well characterised *Acropora* genes show cell-specific punctate pattern of expression. Predominantly, they are either neuronal markers like Emx, Cnox and Pax, or markers of secretory cells such as Lipase (Ball *et al.* 2002, Ball *et al.* 2007, Hayward *et al.* 2001, Miller *et al.* 2000). To reveal the identity of the cells expressing AmTPR1, in situ hybridisation was carried out in order to compare AmTPR1, Emx and Lipase expression patterns. As shown in Fig. 2.8, AmTPR1 expression appeared to be more resemblant of the expression pattern of Lipase, thus indicating that AmTPR1 may be expressed in secretory cells rather than in neurons. Although the morphology of the AmTPR1 positive cells cannot be easily discerned at a magnification shown, higher power observations confirmed much broader appearance of these cells, consistent with the notion of a secretory cell-like phenotype. In contrast, Emx stained cells showed morphology typical of neurons, with thin long bodies projecting across the ectoderm.

Based on the available data on *Drosophila* Dpit47, it was proposed that Dpit47 may be a protein involved in cell proliferation (Crevel *et al.* 2001). Thus, it was of interest to determine whether AmTPR1 may also have a proliferation-associated function. One of the major pathways that regulates the transcription of genes involved in development, growth and cell fate in multicellular organisms is the canonical Wnt/β-catenin pathway (Golan *et al.* 2004, Loureiro and Peifer 1998, Moon *et al.* 1997, Moon *et al.* 2002, Okubo and Hogan 2004, Sato *et al.* 2004, Uren *et al.* 2000, Willert

et al. 2002, Wodarz and Nusse 1998, Zechner *et al.* 2003). Canonical Wnt signalling cascade results in stabilization and nuclear accumulation of β-catenin, a process which then leads to β-catenin – mediated TCF/LEF-dependent transcription of target genes (Fig. 2.9, panel A). Central to the regulation of the canonical Wnt/β-catenin pathway is glycogen synthase kinase-3 (GSK-3). Phosphorylation of β-catenin by GSK-3 targets β-catenin for proteosomal degradation and hence prevents the accumulation of β-catenin in the nucleus (Ikeda *et al.* 1998, Liu *et al.* 2002, Meijer *et al.* 2004, Moon *et al.* 2002, Salic *et al.* 2000, Yost *et al.* 1998). Consequently, inhibition of GSK-3 results in activation of β-catenin and TCF/LEF-dependent transcription (Dihlmann *et al.* 2005, Huber *et al.* 1996, Hurlstone and Clevers 2002, Meijer *et al.* 2004, Moon *et al.* 2002, Patel *et al.* 2004, Tetsu and McCormick 1999, Wetering *et al.* 1997, Willert *et al.* 2002). To examine whether Wnt/β-catenin pathway may be regulating the expression of AmTPR1, *Acropora* embryos were treated with alsterpaullone, a potent inhibitor of GSK-3 (Meijer *et al.* 2004). As shown in Fig. 2.9 (panel B), 5 uM alsterpaullone dramatically increased the expression of AmTPR1 in *Acropora* embryos, suggesting that AmTPR1 may be a target of the Wnt/β-catenin pathway. To confirm this hypothesis, a search for TCF/LEF-binding sites was carried out a in a 2 kb AmTPR1 putative promoter region, cloned from the *Acropora* genomic library (for cloning details see Materials and Methods). As shown in Fig. 2.9 (panel C), three sequences showing 100 % homology to the core TCF/LEF-binding consensus (Dihlmann *et al.* 2005, Tetsu and McCormick 1999), were found in the 2 kb AmTPR1 promoter. Significantly, TCF/LEF binding sites were also found in the 2 kb putative promoter regions of AmTPR1 closest relatives *Hydra* HmTPR1 and *Nematostella* NvTPR1 genes (Fig. 2.9, panel C).

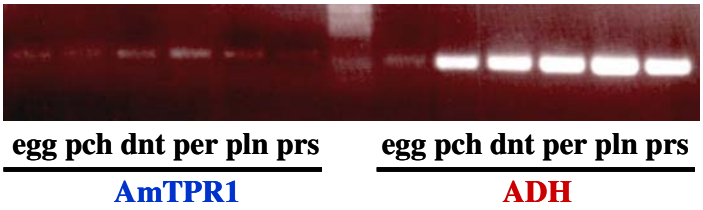


Fig. 2.6. Expression levels of AmTPR1 in different developmental stages of *Acropora*. A 116 bp AmTPR1 and a 100 bp ADH fragments were amplified from single-stranded cDNA corresponding to egg, prawnchip (pch), donut (dnt), pear (per), planula (pln) and pre-settlement (prs), stage. Amplification was carried out for 26 cycles. For details on PCR conditions and primer sequences see Materials and Methods.

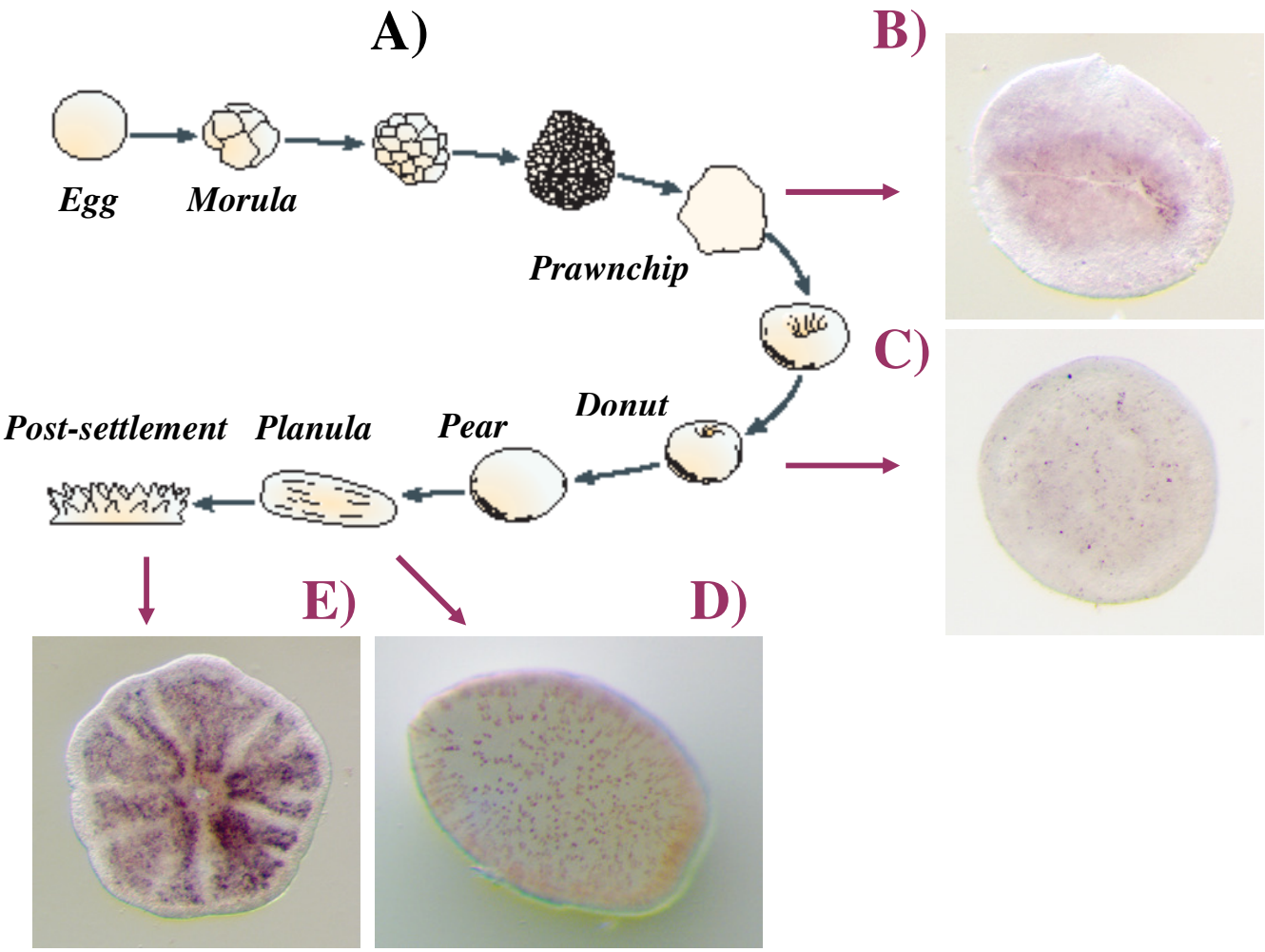
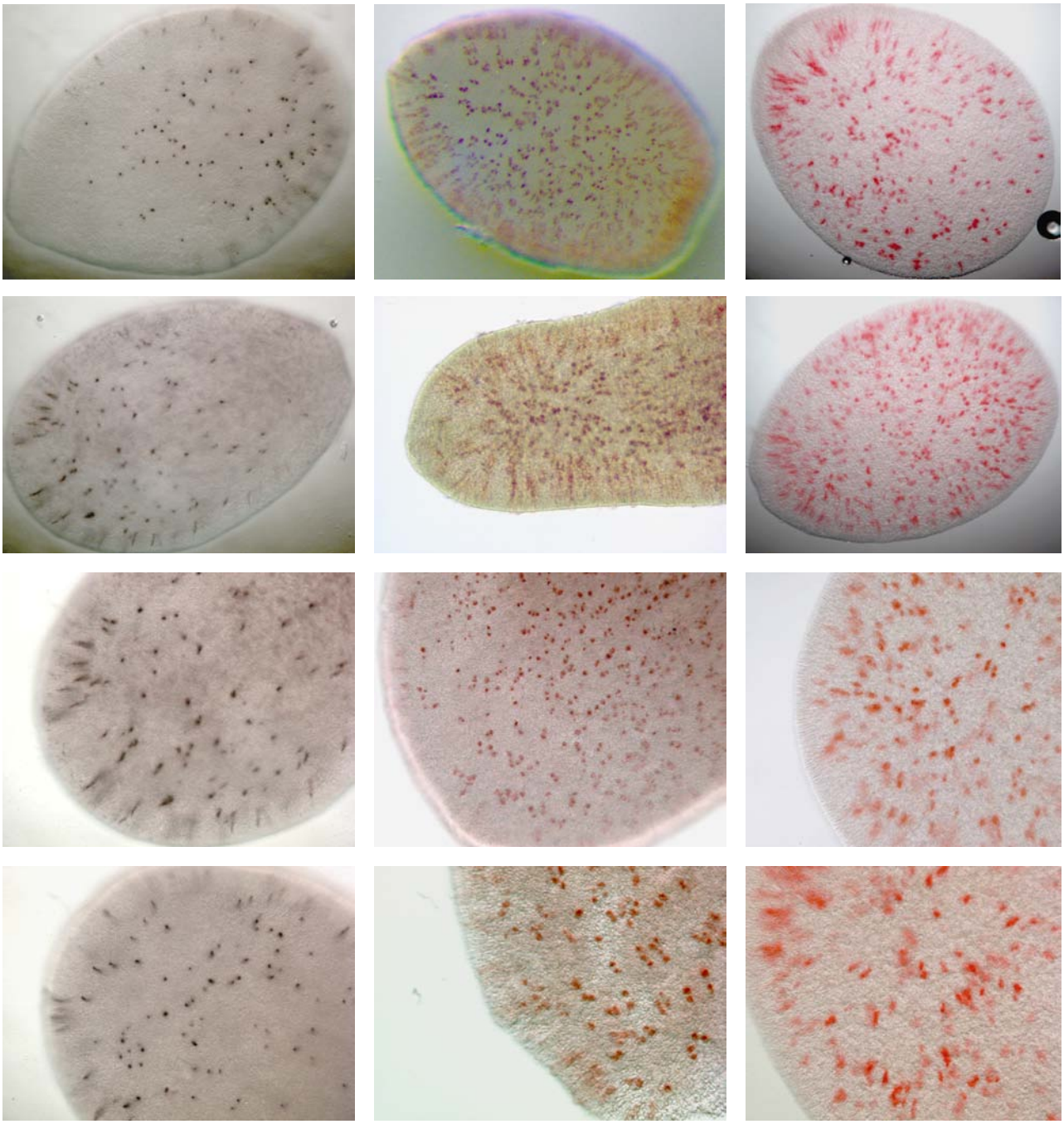


Fig. 2.7. Expression pattern of AmTPR1 in different developmental stages of *Acropora*. A) Schematic representation of *Acropora.* embryonic development, following fertilization the egg undergoes extensive cell divisions (morula), resulting in the formation of a disc-shaped flattened bilayer (prawnchip), at the onset of gastrulation. Gastrulation is characterised by shrinking and thickening of the disc as the edges of the disc fold inward to produce a blastopore. Blastopore closure features the end of gastrulation, when the embryo forms a sphere (donut), which subsequently elongates into a spindle-shaped, free-swimming planula. The planula eventually settles and undergoes extensive morphogenetical changes during the post-settlement stage to become an adult coral. B-E) In situ hybridisation of whole-mount *Acropora* embryos showing expression of AmTPR1.

A)

Emx ***AmTPR1*** ***Lipase***



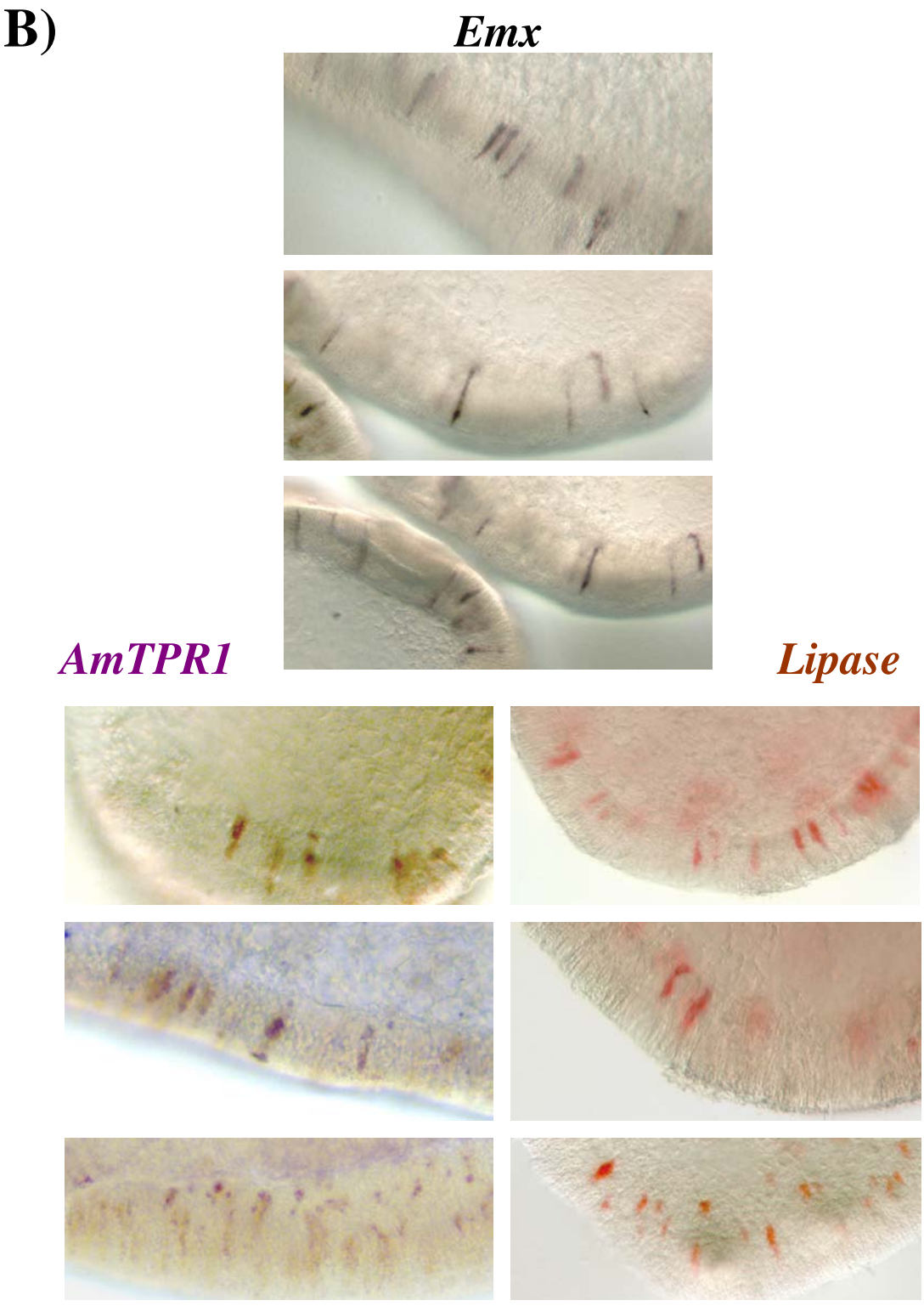
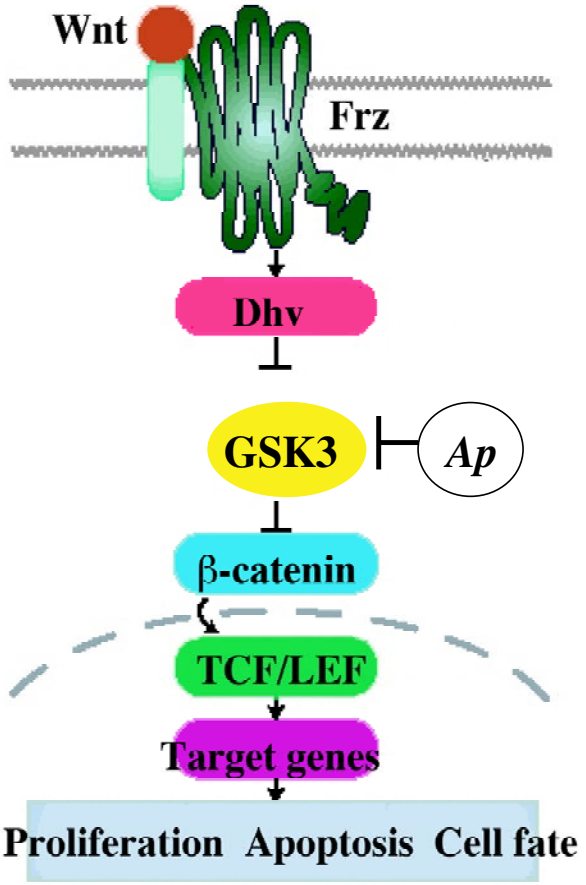


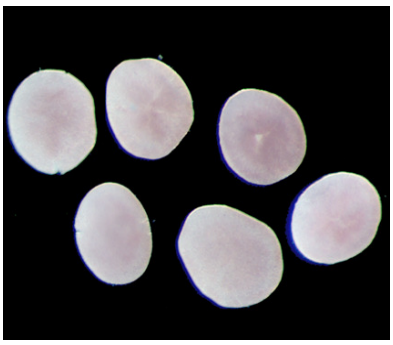
Fig. 2.8. Expression patterns of Emx, Lipase and AmTPR1 in *Acropora* planulae as determined using in situ hybridisation. Images of whole (A), and sectioned embryos (B).

A)

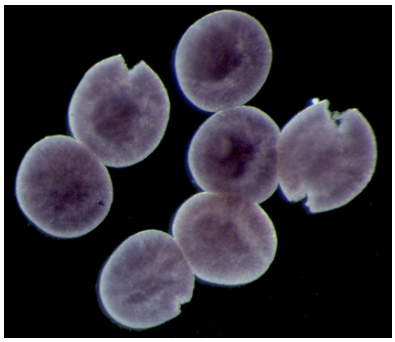


B)

DMSO



DMSO*+*Ap



C)

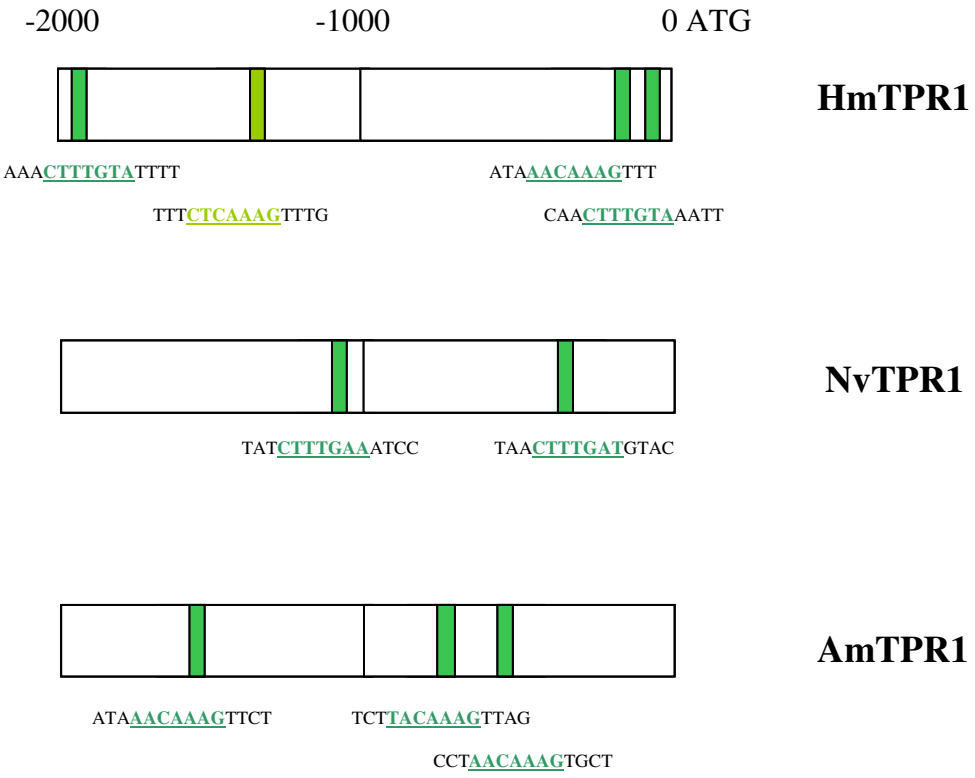


Fig. 2.9. AmTPR1 expression is regulated by the Wnt/β-catenin pathway. A) Schematic representation of canonical Wnt/β-catenin signaling. In the absence of Wnt, β-catenin is targeted for proteosomal degradation by GSK-3. Activation of the Frizzled receptor (Frz) by Wnt binding leads to GSK-3 inhibition by Dishvelled (Dhv). This results in stabilization of the cytosolic pool of β-catenin which then translocates to the nucleus and binds to TCF/LEF transcription factors to initiate the transcription of Wnt/β-catenin target genes and consequently, Wnt/β-catenin – mediated cellular processes (adapted from Moon *et al.* 2002). Alsterpaullone (Ap), induces expression of Wnt/β-catenin target genes by antagonizing GSK-3 activity, B) AmTPR1 expression determined using in situ hybridisation of donut stage *Acropora* embryos treated with either 0.025 % DMSO (solvent control), or 0.025 % DMSO + 5 mM alsterpaullone, C) Location of TCF/LEF-binding sites in the 2000 bp sequence upstream of the initiating ATG codon of AmTPR1, NvTPR1 and HmTPR1 genes. The sites indicated in dark green are sequences matching 100 % the core TCF/LEF-binding consensus (A/T A/T CAAAG and CTTTG A/T A/T, forward and reverse orientation respectively), and in light green are sequences diverging in the terminal A/T nucleotide from the consensus. The promoter sequences were retrieved from the JGI genome database (http://genome.jgi-psf.org/Nemve1/Nemve1.home.html): *N. vectensis* (CL3824) and Metazome Hydra genome browser database (http://hydrazome.metazome.net/cgi-bin/gbrowse/hydra): *H.magnipapillata* (Contig 37986). NFAT and TCF/LEF binding sites were identified by manual inspection.

2.3.3. *Drosophila* Dpit47 is specifically expressed in the central nervous system

Thus far, Dpit47 expression has only been examined semi-quantitatively on a protein level (Crevel *et al.* 2001). It was shown that relatively high levels of Dpit47 protein are present in the early *Drosophila* embryos (0-8 hrs post fertilization) and pupae. Interestingly, Dpit47 protein was barely detectable in late embryos and females (8-20 hrs post fertilization) and undetectable in larvae and males (Crevel *et al.* 2001). Cumulatively, this data indicated that Dpit47 may be involved in cell proliferation (Crevel *et al.* 2001). To further elucidate the function of Dpit47, in situ hybridisation of *Drosophila* embryos was used to examine the tissue-specific expression of Dpit47 mRNA. Surprisingly, in early embryos (stages 6-11, 3-7 hrs post fertilization), Dpit47 transcript was not detectable above background levels (Fig. 2.10, panel A). In contrast, in late embryos (stages 13-16, 9-15 hrs post fertilization), strong and specific expression was associated with the central nervous system (CNS, Fig. 2.10, panel B). No staining was observed using a control, Dpit47 sense probe (2.10, panel C). Thus, the levels of Dpit47 protein and mRNA during *Drosophila* embryogenesis differed dramatically. Early in development, protein levels are high and mRNA levels are low, while later in development the opposite holds. Notably, the CNS-specific localization of the transcript suggests that Dpit47 may be important for CNS development. Both in vertebrates and invertebrates, the Wnt/β-catenin pathway plays a prominent role in nervous system development (Loureiro and Peifer 1998, Zechner *et al.* 2003). For example, *Drosophila* β-catenin homolog Armadillo is highly expressed in the embryonic CNS where it directs cell-fate determination (Loureiro and Peifer 1998). To examine whether CNS-specific expression of Dpit47 was mediated by the Wnt/β-catenin pathway, a search for TCF/LEF-responsive elements in Dpit47 promoter was carried out. As shown in Fig. 2.11, there were no TCF/LEF-binding sites in the 2 kb promoter region of Dpit47. Instead, two DNA replication-related element binding factor (DREF) sites in addition to a cluster consisting of two partially overlapping Myb-binding sites and one E2F-binding site were found in Dpit47 promoter. Thus, contrary to AmTPR1, the expression of Dpit47 is unlikely to be mediated by the Wnt/β-catenin pathway, rather it appears to Myb/E2F/DREF-dependent.

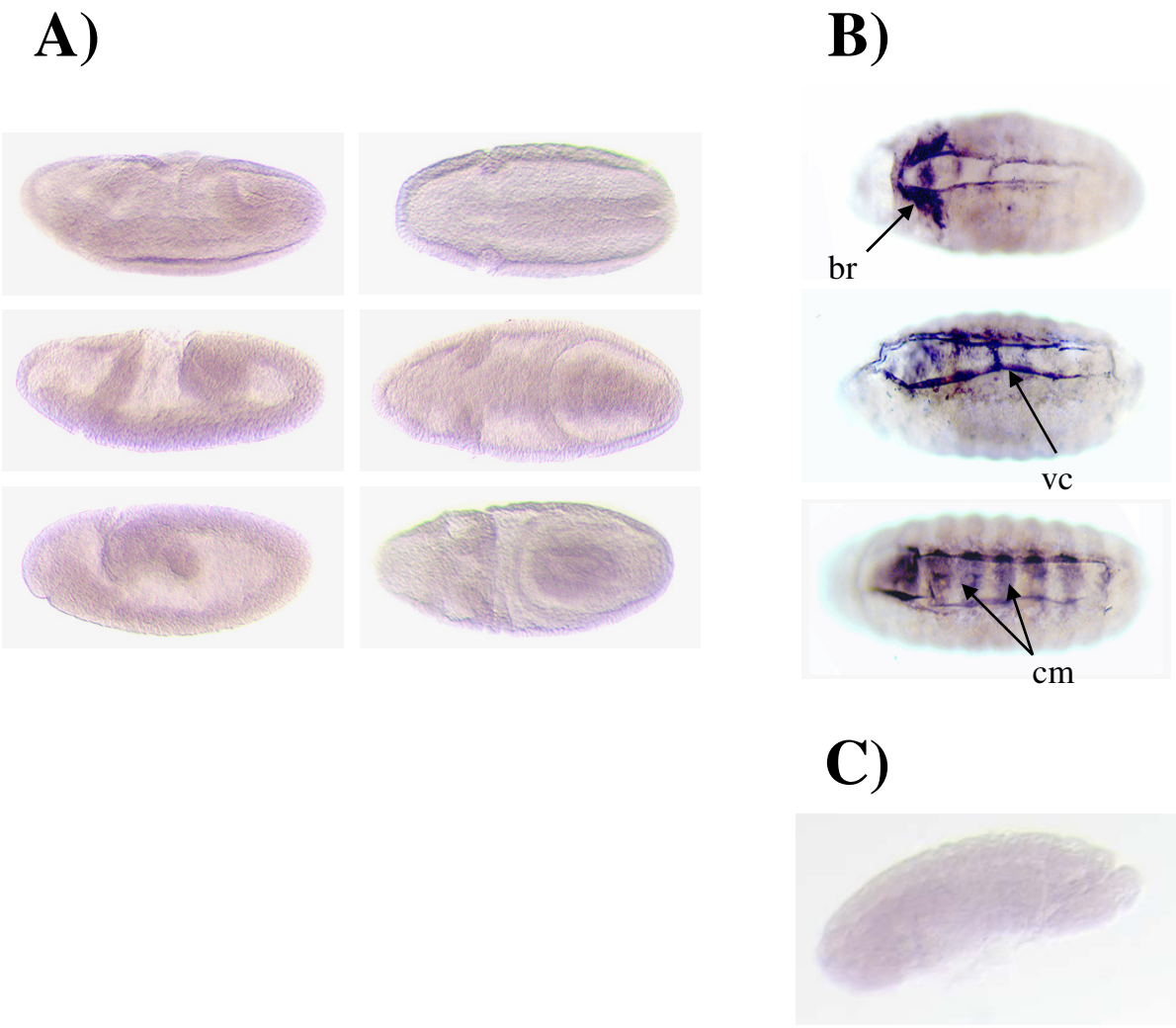


Fig. 2.10. Expression of Dpit47 in different stages of *Drosophila* embryonic development. A) and B) Antisense Dpit47 probe staining, A) Background expression detected in stage 6-11 embryos, left: lateral view, right: dorsal view, B) CNS-specific expression of Dpit47 mRNA in stage 13-16 embryos. Transcript is detected in the ventral nerve cord (vc), brain (br) and commissures (cm). C) Sense Dpit47 control probe staining in stage 13-16 embryo.

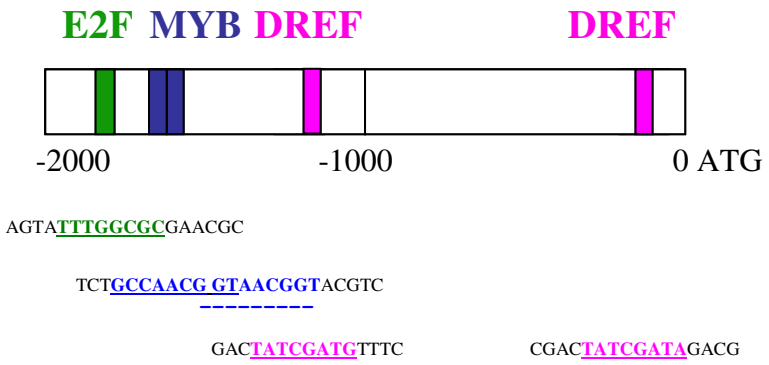


Fig. 2.11. Location of Myb, E2F and DREF binding sites in the 2000 bp sequence upstream of the ATG codon in Dpit47 promoter. The promoter sequence of Dpit47 was retrieved from the ENSEMBL genomic browser database (www.ensembl.org): *D.melanogaster* (CG3189). EF2- and Myb-binding sites were identified using the TFSEARCH software (http://www.cbrc.jp/research/db/TFSEARCH.html). The sequences highly homologous to the DREF-binding element TATCGATA (Matsukage *et al.* 1995), were identified by manual inspection.

2.3.4. *Acropora* AmTPR1 interacts with Hsp90 and DNA polymerase α *in vivo*

Drosophila Dpit47 was originally identified in a yeast two hybrid screen for proteins that interact with DNA polymerase α, a key enzyme involved in eukaryotic DNA replication (Crevel *et al.* 2001, Steitz 1999). Subsequently, it was shown by immunoprecipitation that other than interacting with DNA polymerase α, Dpit47 was also associated with Hsp90 (Crevel *et al.* 2001). Given that significant differences were found in AmTPR1 and Dpit47 expression profiles (namely AmTPR1 transcript seemingly associated with secretory-like cells while Dpit47 was prominent in the CNS), it was of particular interest to determine whether the Hsp90 and DNA polymerase α - protein interactions were conserved between the two TPR homologs. The LexA yeast two-hybrid system was employed to investigate AmTPR1 interaction with Hsp90 and DNA polymerase α (Fig. 2.12). First, EGY48 LacZ yeast strain was co-transformed with pJG4-5-AmTPR1 (prey), and either pEG202-DNApolα or pEG202-Hsp90 (bait) constructs (containing *Drosophila* 180 kDa DNA polymerase α subunit and *Drosophila* Hsp90 cDNAs respectively, Fig. 2.12, panel B). The interactions were then assessed on the basis of the LacZ phenotype of the EGY 48 cells that were positive for both, the bait and the prey constructs. As shown in Fig. 2.13, both EGY48 cells carrying pJG4-5-AmTPR1/pEG202-DNApolα and EGY48 cells carrying pJG4-5-AmTPR1/pEG202-Hsp90 constructs, were $LacZ^+$ in the presence of galactose but not in the presence of glucose. In contrast, empty prey vector EGY48 cells co-transformants (pJG4-5/pEG202-DNApolα and pJG4-5/pEG202-Hsp90), were $LacZ^-$ irrespective of the carbon source. Given that the expression of the AmTPR1 prey construct is galactose-inducible (Fig. 2.12, panel B), it can be concluded that the $LacZ^+$ phenotypes resulted from specific interactions of AmTPR1 with Hsp90 and DNA polymerase α, and not from leaky expression or autoactivation of the LacZ reporter by either of the two bait constructs.

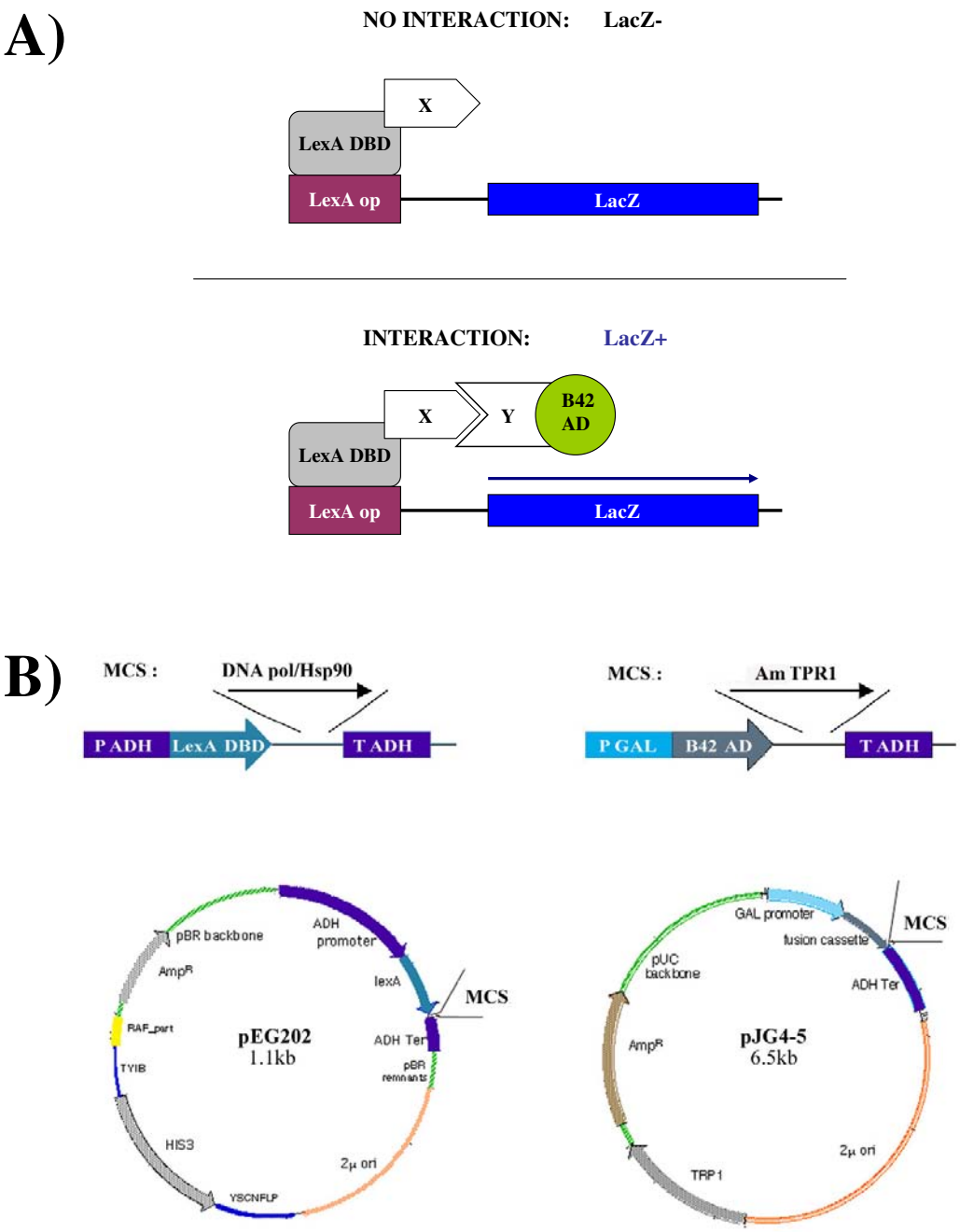
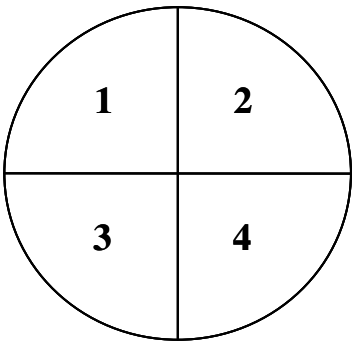


Fig. 2.12. Principle of the Lex A yeast two hybrid system. A) In the LexA system, the expression of the LacZ reporter gene is based on the interaction between two fusion proteins: DBD-fusion ("bait"), and the AD-fusion (prey) protein. The DBD and the AD domains are derived from *E. coli* proteins, LexA and the acid blob B42 respectively B) Schematics of LexA bait and prey fusion protein construct: pEG202 (DBD-Hsp90/DNApolα), and pJG4-5 (AD-fusion protein AmTPR1). The expression of the DBD-fusion protein is under the control of the constitutive ADH promoter and the expression of the AD-fusion protein is driven by the galactose-inducible GAL promoter . DBD – DNA-binding domain, AD – activation domain, P ADH - alcohol dehydrogenase promoter, P GAL – galactose promoter, T ADH - alcohol dehydrogenase terminator, MCS – multiple cloning site

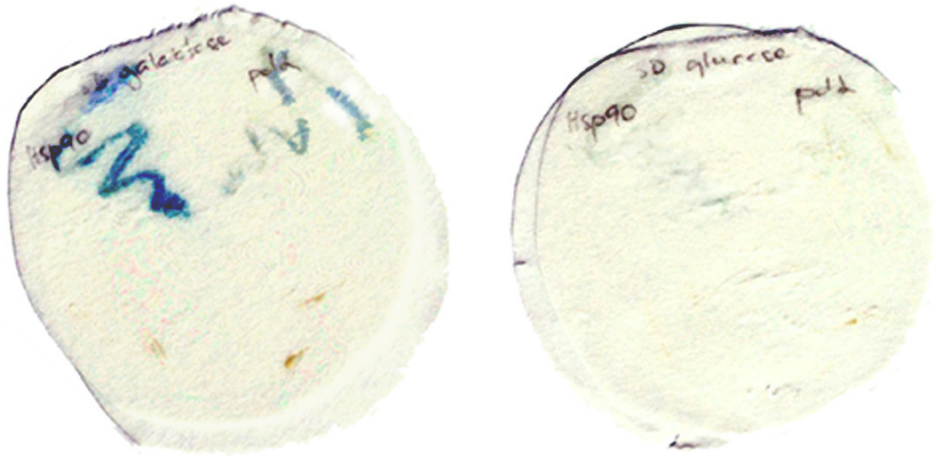
A)

EGY 48
co-transformants: pEG202 + pJG4-5



1	Hsp90	AmTPR1
2	DNApolα	AmTPR1
3	Hsp90	-
4	DNApolα	-

B)



+ galactose **+ glucose**

Fig. 2.13. Interaction of *Acropora* AmTPR1 with *Drosophila* proteins Hsp90 and DNA polymerase α. A) Schematics of co-transformation of EGY48 cells with indicated pEG2 and pJG4-5 constructs. B) The activity of the LacZ reporter following the colony-lift β-galactosidase assay (see Materials and Methods). The blue colour of $LacZ^+$ colonies was detectable within 4 hours.

2.4. DISCUSSION

This study identified a TPR-encoding gene from the coral *Acropora millepora*, AmTPR1, a homolog of the putative human tumor suppressor gene TTC4 and *Drosophila* Dpit47. Like Dpit47, AmTPR1 is a Hsp90- interacting protein that also associates with DNA polymerase α, a key enzyme mediating DNA replication in eukaryotes (Crevel *et al.* 2001, Steitz 1999). Based on their respective expression patterns and interaction with the polymerase, both AmTPR1 and Dpit47 are likely to be important developmental genes with a specific role in cell proliferation.

Apart from TPR motifs, a large number of TPR proteins contain other domains which specify their function. For example, *Schizosaccharomyces pombe* nuc2+, which is required for mitotic segregation of chromosomes, contains a DNA-binding domain (Hirano *et al*. 1990). $p150^{TSP}$, a nuclear phosphoprotein and a protein tyrosine kinase substrate (PTK), contains a specific kinase binding site - SH2 domain (Malek *et al.* 1996), whereas Hsp90 associated immunophilins are distinguished by a peptidyl-prolyl isomerase (PPI) domain (Dolinski *et al.* 1998, Marsh *et al.* 1998, Pearl and Prodromou 2000). When proteins are exclusively composed of TPRs, their functional characterisation becomes problematic. Nevertheless, the fact that AmTPR1 and related proteins share specific structural fingerprints suggests that there may be functional similarities among the members of the AmTPR1 protein family. In particular, AmTPR1-like proteins show: 1) the same domain organization (three TPR motifs identically spaced, Fig. 2.3), 2) a specific deviation from the hydrophobic consensus (Table 2.1) and 3) sequence homology that is greater between corresponding TPRs across the species than the sequence homology between TPRs within a species (Fig. 2.5). Since the hydrophobic consensus is critical for stabilizing the structure of the TPR superhelix (Blatch and Lassle 1999, D'Andrea and Regan 2003, Hirano 1990, Sikorski *et al.* 1990), the presence of polar substitutions in AmTPR1 homologs could result in a novel conformation of the superhelix, thus conferring unique functional properties to the protein. Of particular significance is the observation that, based on the amino acid sequence, AmTPR1 is more closely related to the human TTC4 than to Dpit47 (67 % versus 57 %, similarity respectively). Considering the degeneracy of the TPR motif and the evolutionary distance between corals and humans, the extent of AmTPR1 and TTC4 protein homology is

remarkable. Importantly, this further emphasizes the usefulness of cnidarians as model organisms (Ball *et al.* 2002, Ball *et al.* 2004, Kortschak *et al.* 2003, Miller *et al.* 2000, Miller and Ball 2000, Technau *et al.* 2005).

Examination of *Drosophila* Dpit47 expression lead to the hypothesis that Dpit47 may have a role in cell proliferation. Namely, Dpit47 protein was found to be highly expressed in early embryos and pupae and expressed at much lower or undetectable levels in late embryos and larvae (Crevel *et al.* 2001). Comparably, AmTPR1 transcript was only detected in larval and not in the adult stages of *Acropora* (Fig. 2.1 and 2.6), indicating that AmTPR1 expression is likely to be associated with proliferative tissues. Albeit the relative abundance of AmTPR1 transcript was low, its ubiquitous presence during *Acropora* embryogenesis suggests that AmTPR1 may be essential for the development of *Acropora* (Fig. 2.6). In that respect, it has to be noted that transcript abundance does not necessarily correlate with the abundance of the corresponding protein. This is neatly illustrated by the fact that high levels of Dpit47 mRNA were detected throughout the CNS of late *Drosophila* embryos, 9-15 hrs post fertilization (Fig. 2.10, panel B), when Dpit47 protein was barely detectable (see Fig. 2.5, Crevel *et al.* 2001). Taken together, these results indicate that the expression regulation of both AmTPR1 and Dpit47 occurs on multiple levels. This is a feature common for genes that participate in fundamental cellular processes. For example, the activity of the Cdc2 gene is regulated on the level of transcription and protein synthesis and degradation. Cdc2 is important for several cell cycle-related events such as chromosome condensation, initiation of DNA synthesis, formation of the mitotic spindle and breakdown of the nuclear envelope (Li *et al.* 2004, Weingartner *et al.* 2001, Welch and Wang 1992). While relatively constant levels of Cdc2 protein are maintained by co-ordination of protein synthesis and degradation throughout the cell cycle, the levels of mRNA show cell cycle-dependent fluctuations. The expression of the Cdc2 mRNA is highest at the G1/S transition and at the end mitosis the transcription is turned off (Welch and Wang 1992).

Apart from the semi-quantitative data on AmTPR1 expression, two other lines of investigation produced results consistent with its proposed involvement in cell proliferation. First, as shown by in situ hybridisation, after gastrulation AmTPR1 mRNA appears to be associated with secretory-like cells that cross the ectoderm-endoderm boundary, the so-called trans-ectodermal cells. In cnidarians, both neurons and nematocytes, together with secretory cells, represent three distinct subpopulations

found in the trans-ectoderm (Bode 1996, Fautin and Mariscal 1991). However, morphologically there are significant distinctions between the three cell types (Ball *et al.* 2002, Fautin and Mariscal 1991, Hayward *et al.* 2001, Miller *et al.* 2000). Although in morphology, the cells expressing AmTPR1 are more similar to secretory cells than neurons, the exact origin of AmTPR1 transcript is somewhat debatable until co-localization of AmTPR1 with either Emx and/or Lipase is confirmed. Nevertheless, it is clear from the present in situ data that AmTPR1 transcript locates to the trans-ectoderm. In that respect, it has to be noted that Anthozoan trans-ectodermal cells are most likely to be analogous to the interstitial cells of Hydra (Bode 1996). Most significantly, it has been shown that Hydra interstitial cells represent a proliferative compartment with stem cell-like properties. They are self-renewing cells that give rise to several different cell lineages, including neurons, nematocytes and secretory cells (Fig. 2.14). Second, AmTPR1 may be a target of the canonical Wnt/β-catenin pathway, as suggested by the increased expression of AmTPR1 mRNA following treatment of *Acropora* embryos with the GSK-3 - specific inhibitor alsterpaullone (Fig. 2.9, panel B). Interestingly, the fact that all three cnidarian TTC4-like genes, *Acropora* AmTPR1, *Hydra* HmTPR1 and *Nematostella* NvTPR1 contain putative TCF/LEF binding sites in their promoters (Fig. 2.9, panel C), implies that regulation by Wnt/β-catenin pathway may be a conserved feature of cnidarian TTC4 homologs. Canonical Wnt/β-catenin signalling plays a major role in development of multicellular organisms. By controlling gene expression, members of the Wnt/β-catenin family are fundamental to many cellular processes such as cell proliferation and differentiation, cell adhesion, axial patterning and organogenesis (Moon *et al.* 1997, Moon *et al.* 2002, Okubo and Hogan 2004, Sato *et al.* 2004, Uren *et al.* 2000, Willert *et al.* 2002, Wodarz and Nusse 1998, Zechner *et al.* 2003). Consequently, aberrations of Wnt signalling are often a hallmark of cancer development (Liu *et al.* 2002, Meijer *et al.* 2004, Moon *et al.* 2002, Morin *et al.* 1997, Okubo and Hogan 2004, Patel *et al.* 2004, Polakis 1999, Rubinfeld *et al.* 1997, Tetsu and McCormick 1999, Willert *et al.* 2002).

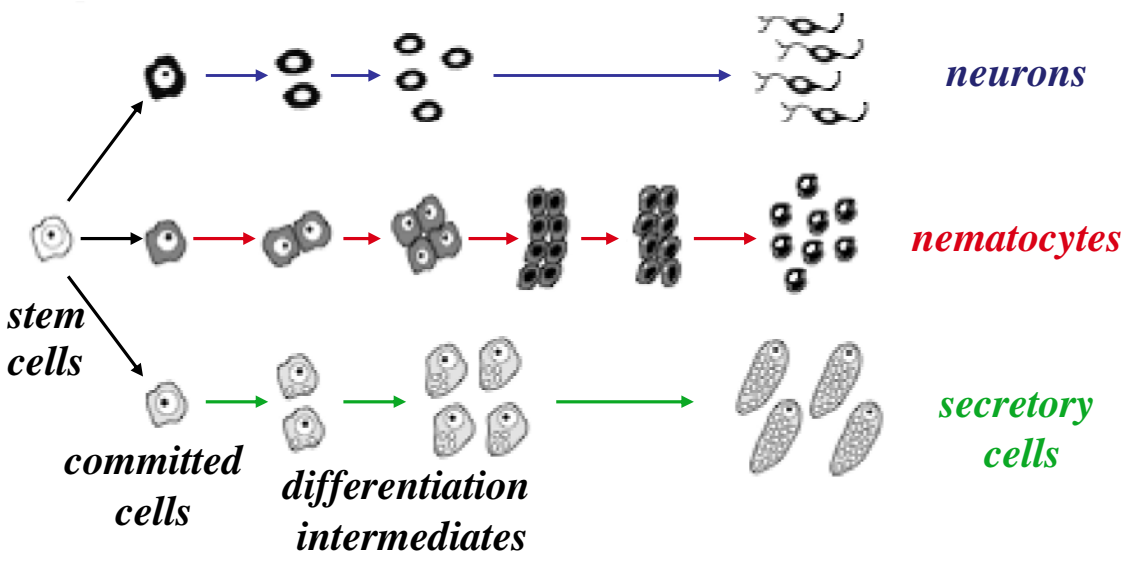


Fig. 2.14. Interstitial stem cells of Hydra and their differentiation pathways. Adapted from Bode (1996).

In contrast to AmTPR1, Dpit47 appears to be independent of the Wnt/β-catenin pathway, as suggested by the absence of TCF/LEF binding sites within the 2 kb region of Dpit47 promoter. Nevertheless, the 2 kb Dpit47 promoter contains two putative Myb and one putative EF2 binding site in close proximity (Fig. 2.11). Like Wnt/β-catenin members, both E2F and Myb are highly conserved transcription factors required for normal development of different organisms (Golay *et al.* 1994, Hao *et al.* 1995, Hlaing *et al.* 2004, Ito 2005, Johnson and Schneider-Broussard 1998, Katzen and Bishop 1996, Lewis *et al.* 2004, Li *et al.* 2003a, Li *et al.* 2003b, Mucenski *et al.* 1991, Muller *et al.* 2001, Sitzmann *et al.* 1995, Toscani *et al.* 1997, Trauth *et al.* 1994, Turque *et al.* 1997, Vara *et al.* 2003). Accordingly, similar to the impairment of Wnt pathway, mutations that affect E2F and Myb-mediated processes are also associated with tumorigenesis (Fung *et al.* 2003, Johnson and Schneider-Broussard 1998, Kauraniemi *et al.* 2000, Loop *et al.* 2004, Manak *et al.* 2002, Shepard *et al.* 2005, Zhu *et al.* 2001). E2F and Myb are involved in many aspects of cell growth and proliferation (Beall *et al.* 2004, Duronio *et al.* 1995, Hao *et al.* 1995, Muller and Helin 2000, Muller *et al.* 2001, Sawado *et al.* 1998). In particular, prominent E2F and Myb targets are genes involved in G1/S progression of the cell cycle, including members of the DNA replication machinery (Duronio *et al.* 1995, Fitzpatrick *et al.* 2002, Hao *et al.* 1995, Muller *et al.* 2001, Ohtani and Nevins 1993, Thacker *et al.* 2003, Zhu *et al.* 2004). Dpit47 interaction with DNA polymerase α is consistent with the hypothesis that it may function as a G1/S-specific E2F/Myb target. Furthermore, Myb is abundantly expressed in all mitotically active tissues throughout *Drosophila* embryonic development (Katzen and Bishop 1996). Interestingly, high levels of Myb transcript are uniformly distributed in early *Drosophila* embryos, while in late embryos, Myb mRNA is highly expressed only in the CNS (Katzen and Bishop 1996). In parallel, Dpit47 protein is highly abundant in early embryogenesis (Crevel *et al.* 2001). Most significantly, like Myb, Dpit47 is strongly expressed in the CNS of the late fly embryos. In particular, Dpit47 mRNA becomes prominent at stage 13 (Fig. 2.10, panel B), which marks the end of germ band retraction and the beginning of CNS and PNS development. Accordingly, at this stage of *Drosophila* embryogenesis, the nervous system is the predominant proliferative compartment (Hao *et al.* 1995).

In summary, the expression pattern of Dpit47 strikingly resembles the expression pattern of Myb and it is thus consistent with the hypothesis that Myb may be

regulating Dpit47 expression. Not only is this indicative of Dpit47 role in cell proliferation but also, it suggests that Myb-mediated Dpit47 transcription may be specifically required for CNS development. This finding has important implications in terms of understanding the mechanisms involved in *Drosophila* neurogenesis given that thus far, the role of Myb in *Drosophila* CNS development remains elusive (Katzen and Bishop 1996, Loop *et al.* 2004).

In addition to E2F and Myb, Dpit47 promoter contains two putative DREF binding sites. Remarkably, DREF sites have been identified in over 60 *Drosophila* genes that are functionally associated with cell proliferation (Hochheimer *et al.* 2002, Hyun *et al.* 2005, Matsukage *et al.* 1995, Ohno *et al.* 1996, Seto *et al.* 2006). Notably, Dpit47 interacting partner DNA polymerase α, and Dpit47 putative transcriptional regulators E2F and Myb also appear to be regulated by DREF-dependent transcription (Hyun *et al.* 2005). Curiously, Crevel *et al.*'s (2001), report on Dpit47 promoter analysis negates the presence of E2F and DREF binding sites. This is particularly surprising given the close proximity of the first, 100 % matching DREF binding consensus sequence to the ATG initiating codon. The second DREF site (situated close to E2F and Myb sites), diverges from the 100 % DREF consensus sequence site by one base and thus may be less relevant in the context of Dpit47 expressional regulation (Fig. 2.11). In the case of E2F, the putative binding sequence identified by TFSEARCH software appears to match 100 % the E2F binding sequence of human and mouse E2F-1. Nevertheless, members of the E2F gene family are highly conserved between flies and vertebrates (Hao *et al.* 1995, Ohtani and Nevins 1994). In particular, *Drosophila* E2F-1 is not only structurally homologous to the human E2F-1, but also binds to similar sequences (Ohtani and Nevins 1994). Furthermore, one of the first identified E2F-1 targets in *Drosophila*, DNA polymerase α, was identified based on searching *Drosophila* gene libraries for mammalian-like E2F-consensus sites (Ohtani and Nevins 1994). In context of the present study, this finding not only supports the hypothesis of Dpit47 being an E2F-regulated gene but is also consistent with its interaction with DNA polymerase α .

Through investigations aimed at characterisation of AmTPR1, this study has revealed that AmTPR1 interacts with Hsp90 chaperone and DNA polymerase α, thus indicating that AmTPR1 and Dpit47 share functional conservation. Quite a large number of TPR proteins are thought to be Hsp90 interactors, for example human Hop

(Scheufler *et al.* 2000), *S. cerevisiae* immunophilins FKBPs, Cpr6 and Cpr7 (Marsh *et al.* 1998). Even though they do not belong to the Dpit47/AmTPR family, they all share the Hsp90 binding consensus sequence. Not surprisingly, alignment of sequences shows that residues K229, N233, N264, K301 and R305 within the C-terminal TPR domain of human Hop, critical for binding the C-terminal MEEVD motif of Hsp90 (Scheufler *et al.* 2000), are also present in AmTPR1 homologs (Fig. 2.15). Thus, given that Dpit47 and AmTPR1 interact with Hsp90, it is likely that this function extends to the uncharacterised members of the AmTPR1 family including the human TTC4 protein.

One of the essential roles of the Hsp90 chaperone is to establish active conformation of client proteins by stabilizing specific transient conformations required for protein function For example, in the case of steroid hormone receptors, Hsp90 establishes and maintains the state competent for hormone binding (Carrello *et al.* 1999, Dittmar *et al.* 1997, Dolinski *et al.* 1998, Nathan and Lindquist 1995, Pratt *et al.* 1993, Prat and Toft 2003). Comparably, the initiation of DNA replication by DNA polymerase α may be dependent on a specific conformation of the polymerase. In that respect, Dpit47 may function as a negative regulator of DNA polymerase α, given that the enzyme is inactive when complexed with Dpit47 (Crevel *et al.* 2001). Furthermore, there is evidence to suggests that Dpit47 and DNA polymerase α form a trimeric complex with Hsp90 (Crevel *et al.* 2001). Thus, it is possible that the interaction of Hsp90 with Dpit47(AmTPR1)/DNA polymerase α complex establishes a replication permissive conformation of the polymerase. Hsp90s are highly conserved from bacteria to humans (Grenert *et al.* 1999, Panaretou *et al.* 1998), therefore the processes mediated by Hsp90 are likely to be conserved. Indeed, there is solid evidence in support of a role of Hsp90 in controlling replication in prokaryotes (Konieczny and Zylicz 1999), and in eukaryotic viruses (Helmbrecht *et al.* 2000, Hu and Seeger 1996). However, Dpit47(AmTPR1)/DNA polymerase α /Hsp90 chaperone complex is the first to implicate Hsp90 in regulation of DNA replication in eukaryotes (Crevel *et al.* 2001).

In comparison to Hsp90, AmTPR1 is weakly associated with DNA polymerase α, as indicated by the low staining intensity of the LacZ reporter (Fig. 2.13, panel B). It is unlikely that the weak interaction was due to the fact that heterologous *Drosophila* DNA polymerase α was used in the two-hybrid test, since DNA polymerases are highly conserved across phyla (Steitz 1999). A more plausible alternative is that

Dpit47/DNA polymerase α interaction may be transient and/or require other interacting partners.

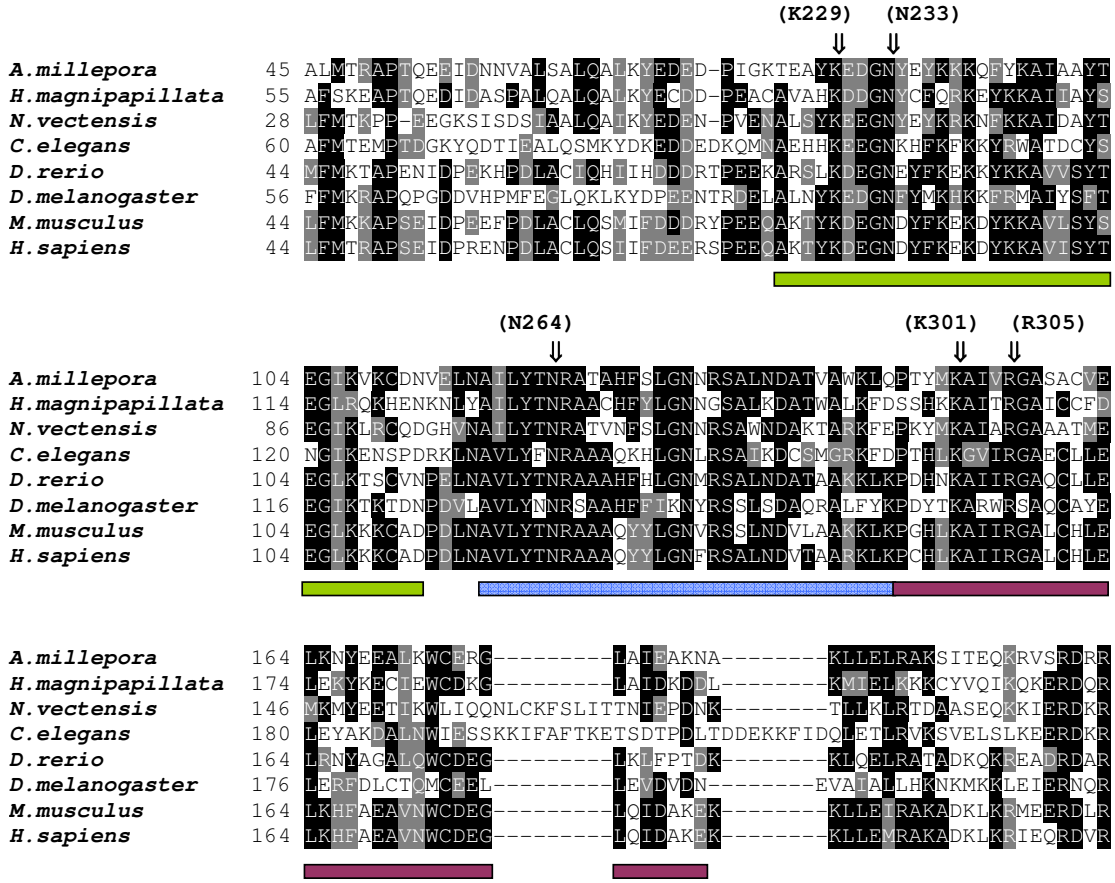


Fig. 2.15. Partial sequence alignment of AmTPR1 and related proteins showing the Hsp90-binding consensus. Residues corresponding to human Hop Hsp90-binding sites (K229, N233, N264, K301 and R305), are indicated with arrowheads and the three TPR motifs are highlighted in green, blue and violet respectively.

In conclusion, the results of this study show that AmTPR1 and Dpit47 belong to a unique group of Hsp90 co-chaperones that interact with DNA polymerase α. Interestingly, the regulation of AmTPR1 and Dpit47 expression appears to be different. While AmTPR1 expression may be regulated by the canonical Wnt/β-catenin pathway, Dpit47 expression may depend on the synergistic action of E2F, Myb and DREF. Nevertheless, both Wnt/β-catenin and E2F/Myb are important regulators of developmental processes involved in various aspects of cell growth and proliferation (Hlaing *et al.* 2004, Ito 2005, Johnson and Schneider-Broussard 1998, Katzen and Bishop 1996, Lewis *et al.* 2004, Moon *et al.* 2002, Okubo and Hogan 2004, Sato *et al.* 2004, Uren *et al.* 2000, Willert *et al.* 2002). It is well known that alterations in developmental pathways often lead to cancer development (Dean 1998), consequently, developmental genes constitute strong candidates for cancer research. At present it is unknown whether the human TTC4 gene functions as an oncogene or a tumor suppressor. However, considering its high homology to invertebrate AmTPR1 and Dpit47 genes, it is likely to also have a developmental role. Thus, the findings of the present study are relevant in elucidating its potential involvement in tumorigenesis.

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Mitogens and modulators of potassium channel activity regulate the expression of a cancer-related gene TTC4 in mouse neuroblastoma cells

3.1. INTRODUCTION

K^+ channels are perhaps the most diverse of all ion channels and are ubiquitously distributed in various cells (Pardo 2004). By regulating membrane potential, they influence numerous cellular processes in excitable and non-excitable cells. Thus, K^+ channel activity has been associated with neuronal excitability (Hu *et al.* 2001, Luscher *et al.* 1997, Neusch *et al.* 2003, Selyanko *et al.* 1999, Vogalis *et al.* 2003), secretion (Lotshaw 1997, Mackenzie *et al.* 2003, Schmid-Antomarchi *et al.* 1990), muscle contraction (Liu *et al.* 2007), regulation of cell volume (Rouzarie-Dubois and Dubois 1998), apoptosis (Lang *et al.* 2005, Wang *et al.* 1999, Wang 2004, Yu *et al.* 2001), cell proliferation (Coiret *et al.* 2007, Guo *et al.* 2005, Jensen *et al.* 1999, Knutson *et al.* 1997, Kodal *et al.* 2000, Pardo 2004, Pillozzi *et al.* 2002, Ransom and Sontheimer 2001) and differentiation (Arcangeli *et al.* 1997, Arcangeli *et al.* 1998, Pancrazio *et al.* 1999). Transient ionic fluxes that occur as a consequence of altered membrane potential activate signalling cascades that trigger differential gene expression and associated cellular processes. In particular, Ca^{2+} transients are key messengers in these signalling cascades (Carrasco and Hidalgo 2006, Cohan 1992, Fukuchi *et al.* 2005, Juretic *et al.* 2005, Lnenicka *et al.* 1998, Wellman *et al.* 2001).

Different modes of regulation of K^+ channels are essential to their functional diversity. For example, voltage-gated K^+ channels, are critical regulators of cell proliferation and differentiation (Ghiani *et al.* 1999, Knutson *et al.* 1997, Pardo 2004, Wang 2004). Importantly, activation of voltage-gated K^+ channels positively correlates with cell cycle progression and cell proliferation. K^+ channel activity causes hyperpolarization of the membrane potential and increases the driving force for Ca^{2+} entry, a condition required for G1/S progression (Ghiani *et al.* 1999, Kahl and Means 2003, Lang *et al.* 2005, MacFarlane and Sontheimer 2000, Santella 1998, Wang 2004, Wonderlin and Strobl 1996). The fact that tumor cells of different origins commonly show selective upregulation of K^+ channel activity, puts a strong emphasis on the mitogenic properties of K^+ channels. An outstanding example is that of the human

EAG-related K^+ channel (HERG) which is specifically expressed in a large number of tumor-derived cells, including myeloid leukemia, neuroblastoma and breast cancer, but not in their non-tumor derived counterparts (Bianchi *et al.* 1998, Pillozzi *et al.* 2002, Wang *et al.* 2002). Consistently, K^+ channel openers promote cell proliferation whereas K^+ channel blockers attenuate cell proliferation in both, tumor and non-tumor derived cells (Basrai *et al.* 2002, Coiret *et al.* 2007, Ghiani *et al.* 1999, Huang and Rane 1994, Jensen *et al.* 1999, Pardo 2004, Wonderlin and Strobl 1996). Furthermore, both growth factors and mitogenic stimuli have been strongly associated with K^+ channel activation (Guo *et al.* 2005, Huang and Rane 1994, Kodal *et al.* 2000, Lang *et al.* 2005, Xu *et al.* 1999).

The processes that lie between K^+ channel activation and cell proliferation are intrinsically complex. They involve cross-talk of signalling networks through second messengers and our knowledge on the way these processes work is very limited (Pardo 2004, Wang 2004). Thus, elucidating the downstream components of K^+ channel activation is imperative to understand better the events that are involved in both pathological and non-pathological cell proliferation. The present study reveals a putative downstream target of K^+ channel activity: TTC4, a gene that may also play a role in cell cycle and cell proliferation. TTC4 is a poorly characterized mammalian gene originally assigned a tumor suppressor role, based on its location in a region on 1p31 that shows frequent aberrations in many cancers including breast cancer, neuroblastoma and melanoma. Su *et al.* (1999) identified TTC4 as a gene in a region of loss of heterozygosity (LOH) in breast cancer. Further studies excluded TTC4 locus to be affected by LOH (Hey *et al*. 2000). To add more to the controversy on the putative tumor suppressor role of TTC4, Poetch *et al.* (2000) found frequent mutations of TTC4 in malignant melanoma in contrast to Irwin *et al.* (2002) who found no mutations. Despite these discrepancies, there is more data which indicates that TTC4 may be a cancer-related gene involved in cell proliferation. Namely, 1) TTC4 promoter is occupied by c-myc oncogene in Burkitt lymphoma cells (Li *et al.* 2003), 2) Screening of genomic alterations in lung cancers identified TTC4 in a minimally altered region of chromosomal gain (Kim *et al*. 2005), 3) *Drosophila* Dpit47 and *Acropora* AmTPR1, invertebrate homologs of TTC4, are both Hsp90 co-chaperones that interact with, DNA polymerase α, suggesting that TTC4-like genes may be an integral part of the eukaryotic DNA replication machinery (Crevel *et al.*

2001, Tomljenovic *et al*. unpublished) and 4) Dpit47 expression is upregulated in brat^{k06028} neoplastic tissue (Loop *et al.* 2003). The aim of the present study was to investigate the function of TTC4 and to clarify its involvement in cell proliferation. The results shown indicate that growth factors, mitogenic-stimuli, membrane depolarization and modulation of K^+ channel activity may regulate the expression of TTC4 in mouse neuroblastoma N2A cells. This confirms our hypothesis that TTC4 may be a cancer-related gene.

3.2. MATERIALS AND METHODS

3.2.1. Reagents

All chemicals were obtained from Sigma Aldrich. For preparing stock solutions, chemicals were dissolved in autoclaved water unless otherwise stated. Following stock solutions were used for cell culture manipulations: 4 M KCl, 4 M NaCl, 1 M kenpaullone (in DMSO), 100 mM N-methyl-D-aspartic acid (NMDA), 0.1 M nifedipine (in DMSO), 0.1 M ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA), 0.25 M tetraethylammonium chloride (TEA), 10 mM mallotoxin (in DMSO), 1 mM bradykinin acetate, 1 mM staurosporine (in DMSO) and 1 mg/ml aphidicolin (in DMSO). The working concentrations of all chemicals used are indicated in the Result section.

3.2.2. Cell culture and manipulations

N2A mouse neuroblastoma cells were grown in RPMI medium with glutamine (Invitrogen), supplemented with 2 % fetal bovine serum (FBS, Invitrogen) and 10 ml/L antibiotic-antimycotic solution (Sigma), under humidified conditions and 5 % CO_2 at 37^{o}C. Cells were seeded at 40-50 % confluency in 90 mM Petri dishes and allowed to grow for 12-24 hrs before beginning of treatments. At the time of treatments, cells were typically ~70 % confluent. For cell synchronization in G0/G1 phase, cells were deprived of serum for 49 hrs. For G1/S synchronization, G0/G1 cultures were incubated in the growth medium containing 10 % FBS and 5 ug/ml aphidicolin for 24 hrs. To allow cell cycle progression beyond the G1/S phase, the medium with aphidicolin was removed, cells washed twice in the growth medium containing 10 % FBS and then incubated in the same medium for either 2 hrs, 4 hrs or 6 hrs. For all other experiments, cells were deprived of serum for 24 hrs before

addition of chemicals and then incubated with the chemicals in serum-free medium for either 4.5 hrs or 8 hrs. Each treatment was replicated at least twice. Following treatment, cells were rinsed twice with 5 mls of 1X PBS and harvested using a cell scraper. The cell suspension was transferred to a 10 ml Falcon tube and the cells were pelleted at 1500 rpm for 3 min.

3.2.3. Flow-cytometric analysis

The distribution of cells in different phases of the cell cycle was deter mined by flow cytometry. Following cell harvest, cells were fixed in 70 % EtOH. Briefly, cell pellets were resuspended by vortexing in ice-cold 70 % EtOH and then incubated at 4^{o}C for at least 2 hrs. EtOH fixed cells were washed twice with 5 mls of 1X PBS and resuspended in 1 ml of propidium iodide (PI) solution (0.1 %(v/v) Triton X-100 in 1X PBS, 0.2 mg/ml DNase-free RNase A and 0.02 mg/ml propidium iodide). The cells in the PI solution were then incubated for 15 min at 37^{o}C in the dark. Following PI incubation, the cells were rinsed with 5 mls of 1X PBS, resuspended in 400-500 ul of 1X PBS and then analysed using a FACSCalybur cytometer and the CellQuest software (BD Biosciences). At least 100 000 events were analysed per sample.

3.2.4. RNA extraction and cDNA synthesis

Cell pellets were resuspended in 600 ul RLT lysis buffer (QIAGEN) and total RNA extracted using the RNEasy Mini Kit (QIAGEN) according to manufacturer's instructions. The concentration and purity of RNA samples were analyzed using a nanodrop and measuring absorption at 260 nm (nucleic acid) and the 260/280 nm ratio (nucleic acid/priotein), respectively. First-strand cDNA was synthesized using 1-5 ug of total RNA, oligo(dT)$_{20}$ primers and SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions and subsequently diluted in DNase-free water to 30-80 ng/ul final concentration.

3.2.5. Real-time (RT) PCR

RT-PCR amplification mix (20 ul), contained 3 ul first-strand cDNA, 10 ul Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and either 100 nM 18S RNA forward and reverse primer, 85 nM TTC4 forward and reverse primer or 100 nM Cdc2 forward and reverse primer (Table 3.1). Duplicate reactions were run for each sample on the Rotorgene 3000 (Corbett) and the cycling conditions were as follows:

50°C for 2 min (UDG incubation), 95°C for 2 min (polymerase activation) and 40 cycles at 95°C for 15 sec and 60°C for 30 sec. ΔΔCt method (Livak and Schmittgen 2001), was used to analyse changes in gene expression. Ct is arbitrarily set as the number of cycle at which gene amplification is in the exponential phase. The fold-change in TTC4 gene expression was presented as $2^{-\Delta\Delta Ct}$, where:

$\Delta\Delta Ct = (Ct\ TTC4 - Ct\ 18S\ RNA)_{treatment\ x} - (Ct\ TTC4 - Ct\ 18S\ RNA)_{nontreated\ control}$ or

$\Delta\Delta Ct = (Ct\ TTC4 - Ct\ 18S\ RNA)_{time\ x} - (Ct\ TTC4 - Ct\ 18S\ RNA)_{time\ 0}$

Table 3.1. Nucleotide sequences of primers used in RT PCR. The 18S RNA primer sequence is according to Schmittgen and Zakrajsek (2000)

Gene	Forward primer 5'→3'	Reverse primer 5'→3'
18S RNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
TTC4	GTGCAGACCCTGATTTGAATGCTG	GGCACCTCTTATGATGGCTTTCAG
Cdc2	TGTCCATGGACCTCAAGAAGTACC	GGAGTGGCAAAACACAATTCCCTG

3.3. RESULTS

3.3.1. Depolarizing concentrations of K^+ downregulate TTC4 expression in N2A cells

Since the expression of the invertebrate homolog of TTC4 *Acropora* AmTPR1 appears to be regulated by the Wnt/β-catenin pathway (Tomljenovic *et al.* unpublished), it was of particular interest to determine whether Wnt/β-catenin also regulates the expression of the mouse TTC4 gene. For that purpose, glycogen synthase kinase-3 (GSK-3) inhibitors LiCl and kenpaullone were used. GSK-3 is a central regulator of the Wnt/β-catenin pathway, phosphorylation of β-catenin by GSK-3, designates β-catenin for proteosomal degradation (Meijer *et al*. 2004, Moon *et al.* 2002, Patel *et al.* 2004, Willert *et al.* 2002). Therefore, inhibition of GSK-3 is necessary for the Wnt/β-catenin signalling to occur. Mouse N2A cells were treated either with 10 mM kenpaullone or with 20 mM LiCl to inhibit GSK-3 and with 85 mM KCl. Cells were collected for RT-PCR analysis at different time points over a 24 hr period. Surprisingly, neither LiCl nor kenpaullone treatment altered TTC4 expression. On the other hand, depolarizing concentrations of KCl resulted in dramatic downregulation of TTC4 expression. The effect of KCl was apparent within

3 hrs of treatment (6-fold downregulation), and it peaked at 8 hrs when TTC4 was 33-fold downregulated compared to the beginning of the treatment (Fig. 3.1).

To investigate further the effect of elevated K^+ on TTC4 expression, N2A cells were treated with 10 mM, 30 mM and 85 mM KCl for 8 hrs. NaCl was used in the same range of concentrations as a negative control. As expected, 85 mM KCl downregulated the expression of TTC4 (4.4-fold), whereas 85 mM NaCl had no appreciable effect on TTC4 expression. The variation in the extent of TTC4 downregulation between the two experiments (33-fold vs 4.4-fold) may have been due to minor differences in cell culture conditions such as number of cell passages and degree of culture confluence. Furthermore, lower concentrations of KCl did not alter the expression of TTC4 (Fig. 3.2). High concentrations of KCl also affected the morphology of N2A cells. N2A cells in serum-deprived growing conditions have a distinct morphology with extending neurites (Fig. 3.3, panels A,B,D,E and F). Increasing the concentration of KCl to 85 mM caused neurite retraction and rounding of cells (Fig. 3.3, panel C). In contrast, increasing NaCl to 85 mM did not have a drastic effect on cell morphology (Fig. 3.3, panel F).

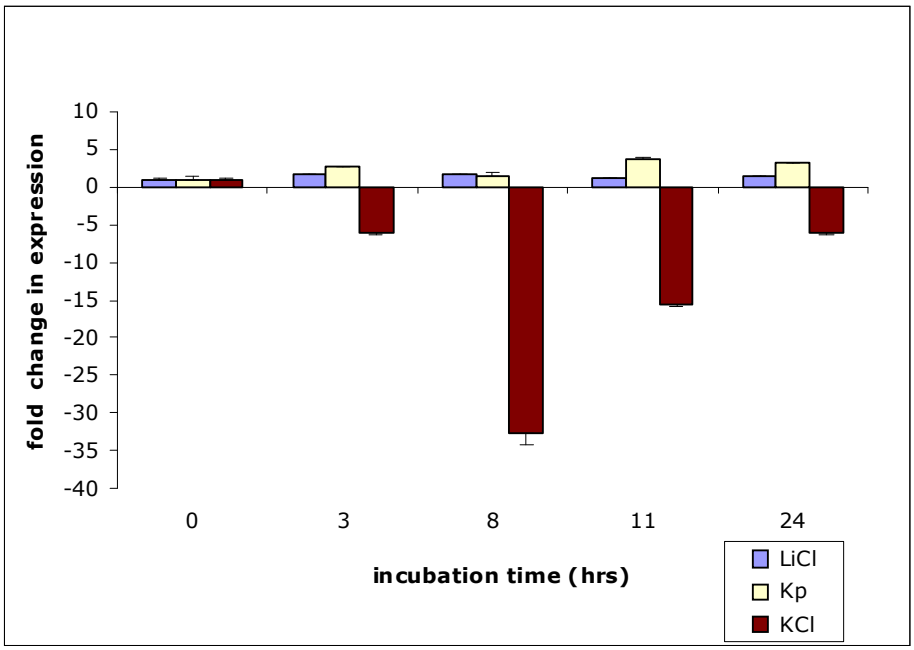

Fig. 3.1. The effect of LiCl, kenpaullone and KCl treatment on TTC4 expression in N2A cells – TTC4 is downregulated by 85 mM KCl. N2A cells were serum starved for 24 hrs and then treated with 20 mM LiCl, 10 uM kenpaullone (Kp) and 85 mM KCl in serum-free medium for the indicated time points. Cells were harvested, RNA was extracted and RT-PCR analysis was performed as described in Materials and Methods. The following applies to all result figures: consistent results were obtained between treatment replicates and the results derived from a typical single-replicate treatment are presented. Calculated standard error values were small given that replicate RT-PCRs showed very little variation in Ct values.

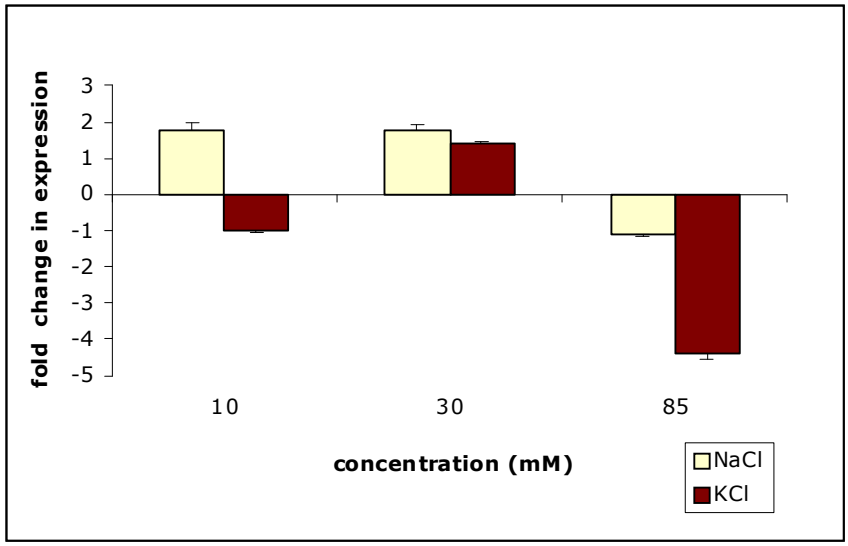

Fig. 3.2. The effect of KCl and NaCl treatment on TTC4 expression in N2A cells – TTC4 is downregulated by 85 mM KCl but not by 85 mM NaCl. N2A cells were serum starved for 24 hrs and then treated with the chemicals in indicated concentrations in serum-free medium for 8 hrs.

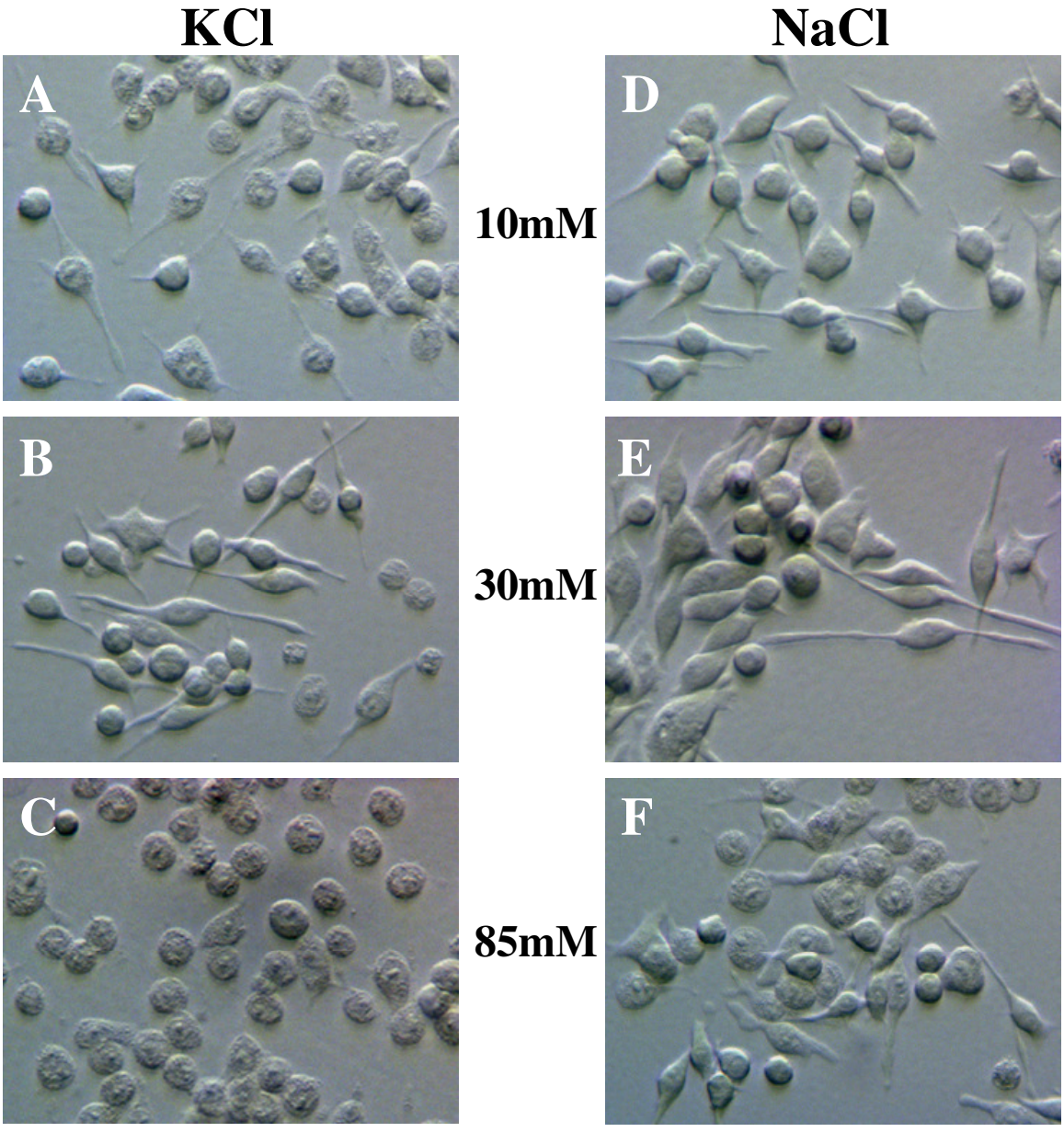

Fig. 3. 3. The effect of KCl and NaCl treatment on N2A cell morphology - 85 mM KCl causes neurite retraction in N2A cells. Cells from the same treatment as in Fig. 3.2. Photographs were taken with a Spot digital camera.

3.3.2. Downregulation of TTC4 under depolarizing conditions is not affected by modulation of extracellular Ca^{2+} influx through L-type Ca^{2+} channels or NMDA receptor channels

Membrane depolarization does not only occur as a consequence of elevated concentrations of extracellular K^+ but also as a consequence of increasing extracellular Ca^{2+} and subsequent influx of Ca^{2+} across the plasma membrane (Katzung 1975, Kumura *et al.* 1999, Limbrick *et al.* 2003, Schwaninger *et al.* 1993). Ca^{2+} can enter the cells via three main routes: 1) through voltage-operated Ca^{2+} channels (VCCs), 2) store-operated Ca^{2+} channels (SOCCs) and 3) receptor-operated Ca^{2+} channels (RCCs). To investigate the role of extracellular Ca^{2+} on TTC4 expression, N2A cells were treated with 10 mM, 30 mM and 85 mM KCl alone or in the presence of 2.3 mM $CaCl_2$. As a result of this treatment, in the presence of 2.3 mM $CaCl_2$, TTC4 was markedly downregulated even at the lowest concentration of KCl (10 mM). Ca^{2+} also greatly exacerbated the extent of 85 mM KCl-induced TTC4 downregulation (Fig. 3.4)

It is known that depolarization stimuli trigger the opening of voltage-gated Ca^{2+} channels (Catterall 2000, Charles *et al.* 1998, Dolphin 1995, Vallano *et al.* 2006). Subsequent influx of Ca^{2+}, particularly through L-type channels has been linked to transcriptional events in various cell types (Fukuchi *et al.* 2005, Guerini *et al.* 1999, Rubing *et al.* 2007, Wellman *et al.* 2001). To investigate whether interfering with Ca^{2+} influx affects TTC4 expression, N2A cells were treated with 7.5 mM, 10 mM and 85 mM KCl alone and in combination with either 10 uM nifedipine, L-type Ca^{2+} channel blocker, or with 5 mM EGTA to chellate extracellular Ca^{2+}, in the medium containing 2.3 mM $CaCl_2$. As shown in Fig. 3.5, neither nifedipine nor EGTA affected the extent of TTC4 downregulation in response to 85 mM KCl. Furthermore, the basal expression of TTC4 at lower concentrations of KCl was unaffected by both, nifedipine and EGTA treatments.

Other than through voltage-gated Ca^{2+} channels, Ca^{2+} entry into the cytoplasm can also occur via receptor-mediated stimuli (Berridge *et al.* 1998). Stimulation with N-methyl-D-aspartate (NMDA) promotes Ca^{2+} entry through NMDA receptor-channels (Isaacson and Murphy 2001, Schiller *et al.* 1998, Vallano *et al.* 1996). Similar to nifedipine and EGTA treatments, administration of 140 uM NMDA failed to affect the extent of TTC4 downregulation at 85 mM KCl in the presence of 2.3 mM $CaCl_2$

in N2A cells. The basal expression of TTC4 at 10 mM and 30 mM KCl was also unaffected by NMDA receptor stimulation (Fig. 3.6).

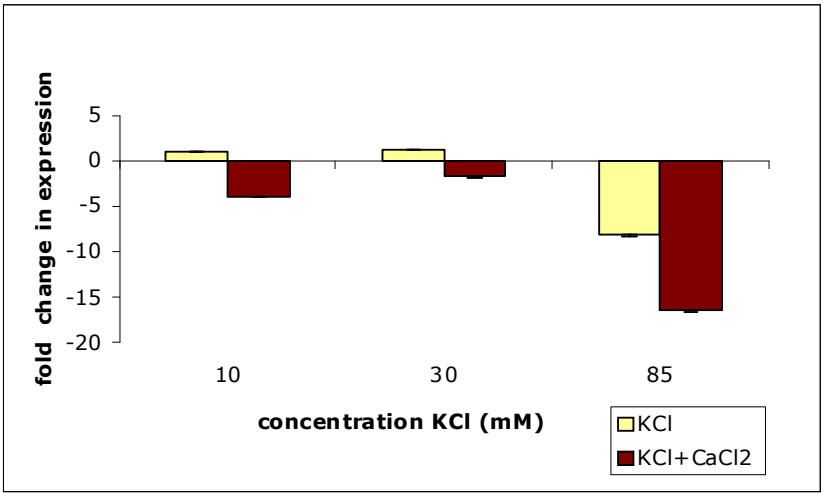

Fig. 3.4. The effect of elevated extracellular Ca^{2+} on TTC4 expression in N2A cells – TTC4 downregulation at 10 mM and 85 mM is exacerbated by the addition of Ca^{2+}. N2A cells were serum starved for 24 hrs and then treated with KCl in serum-free medium for 8 hrs. $CaCl_2$ was added to the medium to the final concentration of 2.3 mM.

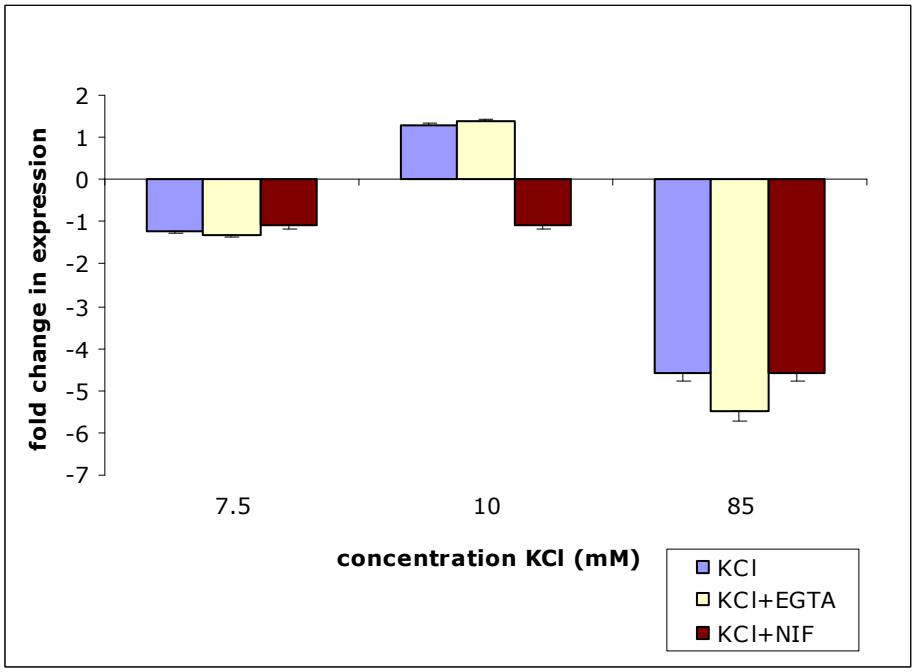

Fig. 3.5. The effect of KCl, EGTA and nifedipine treatment on TTC4 expression in N2A cells - TTC4 downregulation at 85 mM KCl is not affected by EGTA and nifedipine. N2A cells were serum starved for 24 hrs and then treated with KCl, KCl+5 mM EGTA and KCl+10 uM nifedipine in serum-free medium containing 2.3 mM $CaCl_2$ for 8 hrs.

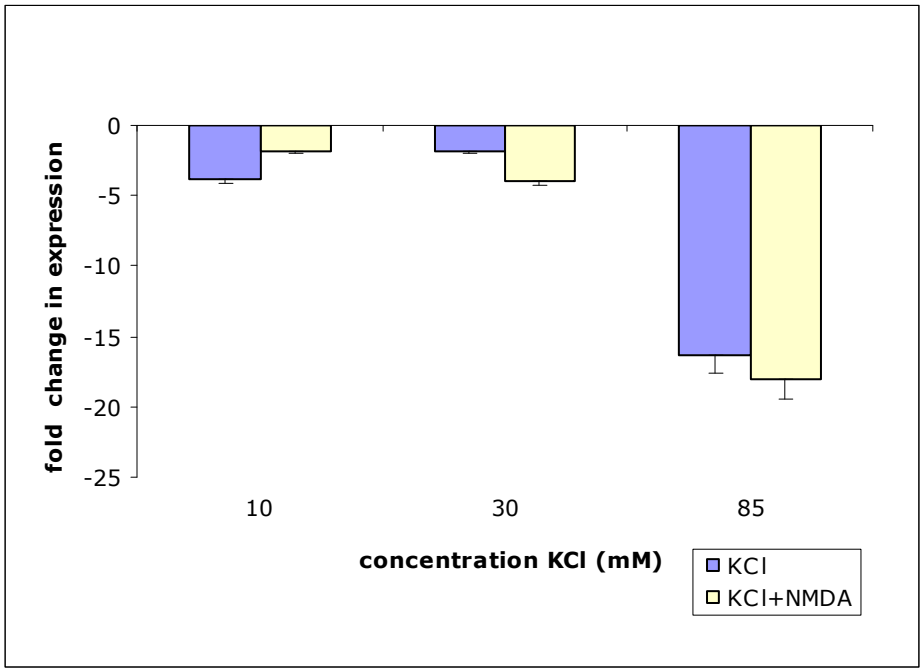

Fig. 3.6. The effect of NMDA receptor stimulation on TTC4 expression in N2A cells - TTC4 downregulation at 85 mM KCl is not affected by NMDA. N2A cells were serum starved for 24 hrs and then treated with KCl and KCl+140 uM NMDA in serum-free medium containing 2.3 mM $CaCl_2$ for 8 hrs.

3.3.3. K^+ channel activity affects TTC4 expression in N2A cells

Increase in intracellular Ca^{2+} required for initiating Ca^{2+} - dependent gene transcription does not only occur as a consequence of membrane depolarization and opening of voltage-operated Ca^{2+} channels. It can also occur as a result of K^+ channel activity and consequent membrane hyperpolarization (Fanger *et al.* 2001, Hess *et al.* 1993, Wulff *et al*. 2004, Zweifach and Lewis 1993). Like voltage-gated Ca^{2+} channels, voltage-gated K^+ channels are also influenced by changes in membrane potential. Voltage-gated K^+ channels open upon depolarization and by promoting K^+ efflux repolarize the membrane potential (Raffaelli *et al.* 2004, Wickenden 2002). Thus, activation of K^+ channels leads to hyperpolarization, whereas inhibition of K^+ channels has the opposite effect-depolarization of the membrane potential (Frieden *et al.* 1999, Lotshaw 1997, Wang 2004). It is important to know that K^+ channel-induced membrane hyperpolarization provides an electrochemical driving force for sustained Ca^{2+} entry and consequently, leads to an increase of intracellular Ca^{2+} concentration, required for Ca^{2+}-mediated regulation of gene transcription (Fanger *et al.* 2001, Hess *et al.* 1993, Wulff *et al*. 2004).

Given that membrane depolarization negatively affects the expression of TTC4, it was intriguing to test whether modulation of K^+ channel activity may be involved in this process. For this purpose, N2A cells were treated with K^+ channel blocker tetraethylammonium chloride (TEA) in the presence of 10 mM, 30 mM and 85 mM KCl. 20 mM TEA greatly exacerbated the extent of TTC4 downregulation in response to 85 mM KCl: TTC4 was 28-fold downregulated as a result of the treatment with TEA and KCl and only 16-fold as a result of KCl treatment alone. The basal expression of TTC4 at lower concentrations of KCl was not appreciably affected by 20 mM TEA (Fig. 3.7). The lack of effect of TEA on TTC4 expression at lower concentrations of KCl strongly suggested the involvement of voltage-gated K^+ channels. To confirm this hypothesis, the effect of TEA on TTC4 expression was examined under non-depolarizing conditions. As expected, in the absence of KCl-induced depolarization, TEA had no effect on TTC4 expression (Fig. 3.8).

If blocking K^+ channels exacerbates downregulation of TTC4 in response to depolarization, it is possible that by opening K^+ channels depolarization-induced downregulation of TTC4 would be attenuated. Indeed, treatment of N2A cells with 20 uM mallotoxin, a K^+ channels opener (Wu *et al.* 2007, Zakharov *et al.* 2005), dramatically attenuated TEA-induced downregulation of TTC4 under depolarizing

concentrations of KCl (Fig. 3.9). Furthermore, mallotoxin was also able to attenuate 85 mM KCl-induced downregulation of TTC4 in the absence of TEA in a dose-dependent manner (Fig. 3.10).

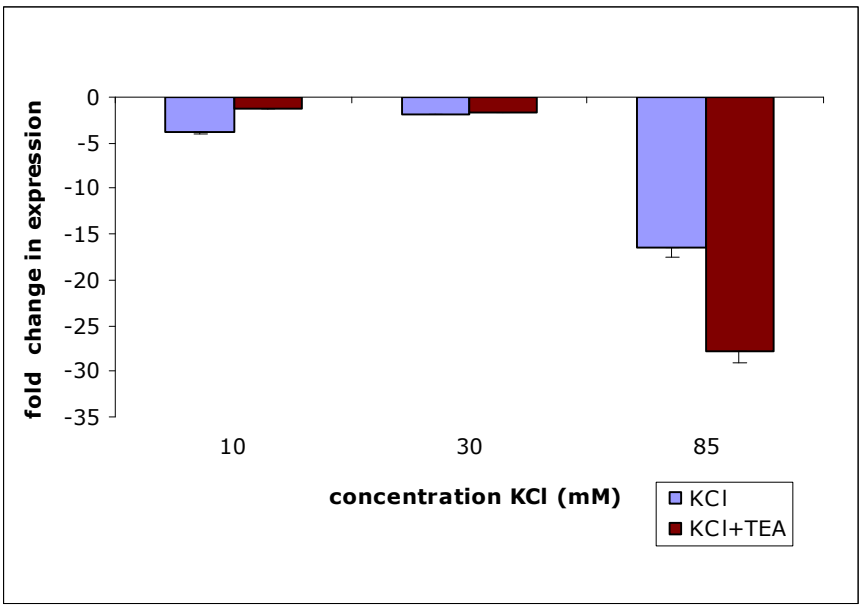

Fig. 3.7. The effect of TEA treatment on TTC4 expression in N2A cells - TTC4 downregulation at 85 mM KCl is exacerbated by TEA. N2A cells were serum starved for 24 hrs and then treated with KCl and KCl+20 mM TEA in serum-free medium for 8 hrs.

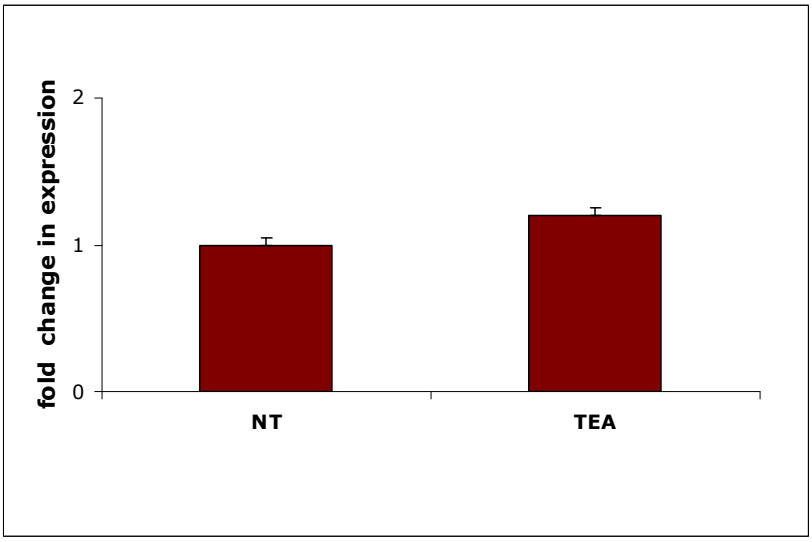

Fig. 3.8. The effect of TEA treatment on TTC4 expression in N2A cells - TTC4 basal expression under non-depolarizing conditions is not affected by TEA. N2A cells were serum starved for 24 hrs and then treated with 20 mM TEA in serum-free medium for 8 hrs. Non-treated control cells (NT), were cultured in serum-free medium.

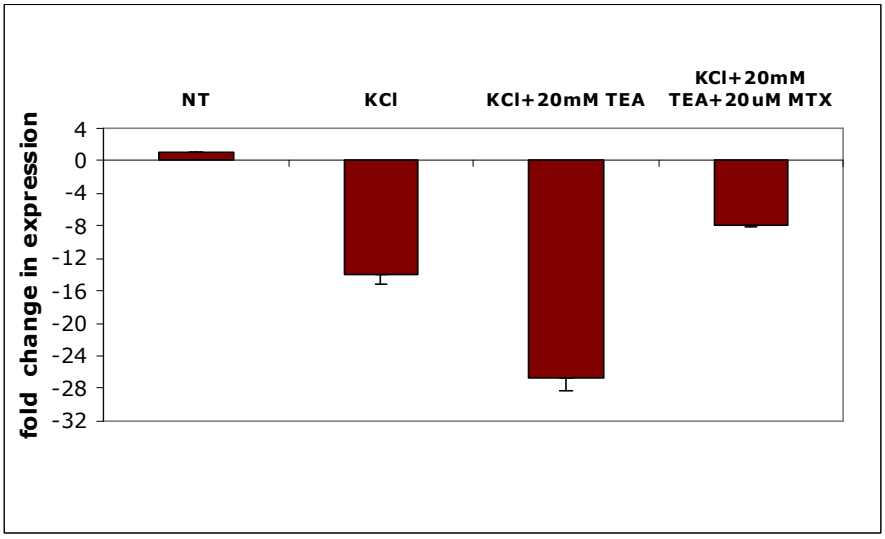

Fig. 3.9. The effect of KCl, TEA and mallotoxin treatment on TTC4 expression in N2A cells - TTC4 downregulation in response to 85 mM KCl and TEA is attenuated by mallotoxin. N2A cells were serum starved for 24 hrs and then treated with 85 mM KCl and 85 mM KCl in combination with TEA or TEA+mallotoxin (MTX), in serum-free medium for 4.5 hrs. Non-treated control (NT) cells were cultured in serum-free medium.

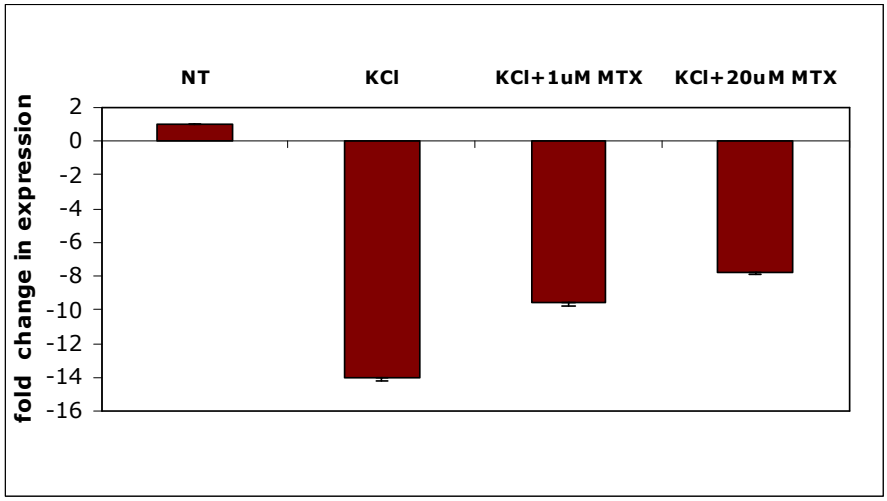

Fig. 3.10. The effect of KCl and mallotoxin treatment on TTC4 expression in N2A cells - TTC4 downregulation in response to 85 mM KCl is attenuated by mallotoxin in a dose–dependent manner. N2A cells were serum starved for 24 hrs and then treated with 85 mM KCl and 85 mM KCl+mallotoxin (MTX), in serum-free medium for 4.5 hrs. Non-treated control (NT) cells were cultured in serum-free medium.

3.3.4. TTC4 expression is not regulated by protein kinase C but may involve intracellular Ca^{2+}

Mallotoxin is a compound that can also inhibit enzymes of the protein kinase C (PKC) family, especially at concentrations >3 uM (Gschwendt *et al.* 1994). Several observations rule out that the effect of mallotoxin on TTC4 expression was due to inhibition of PKC. Firstly, mallotoxin increases the open probability of K^+ channels (Wu *et al.* 2007, Zakharov *et al.* 2005). This means that in the presence of mallotoxin more K^+ channels would be opened than in the absence of mallotoxin. TEA blocks K^+ channel activity by occupying the K^+ channel pore and thus sterically interferes with the transport of K^+ ions (Kutluay 2005). Thus, the ability of mallotoxin to activate K^+ channels would be greater at lower concentration of TEA than at higher concentrations of TEA. Accordingly, the effect of mallotoxin on TTC4 expression is greater at lower concentrations of TEA whilst being appreciably reduced at higher concentrations of TEA (Fig. 3.11). Secondly, in support of the hypothesis that mallotoxin affects TTC4 expression via modulating K^+ channel activity as opposed to PKC inhibition, PKC-specific inhibitor staurosporine failed to attenuate depolarization-induced downregulation of TTC4 (Fig. 3.12). Interestingly, 10 uM bradykinin attenuated depolarization-induced downregulation of TTC4 to the same extent as mallotoxin. Bradykinin is a mitogen known for its ability to stimulate Ca^{2+} -dependent voltage-gated K^+ channels (Greco *et al.* 2005, Liu *et al.* 2007).

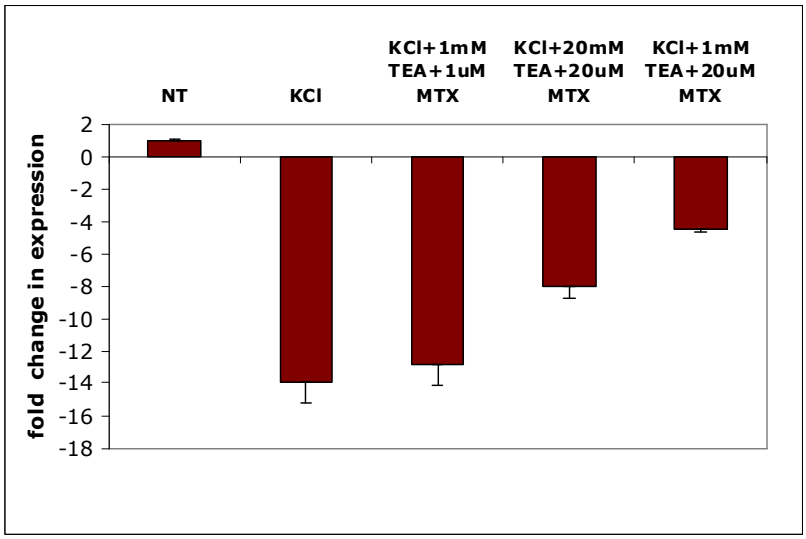

Fig. 3.11. The effect of KCl, TEA and mallotoxin treatment on TTC4 expression in N2A cells – attenuation of TTC4 downregulation in response to 85 mM KCl by mallotoxin is greater at lower concentrations of TEA. N2A cells were serum starved for 24 hrs and then treated with 85 mM KCl and 85 mM KCl in combination with TEA+mallotoxin (MTX) in serum-free medium for 4.5 hrs. Non-treated control (NT) cells were cultured in serum-free medium.

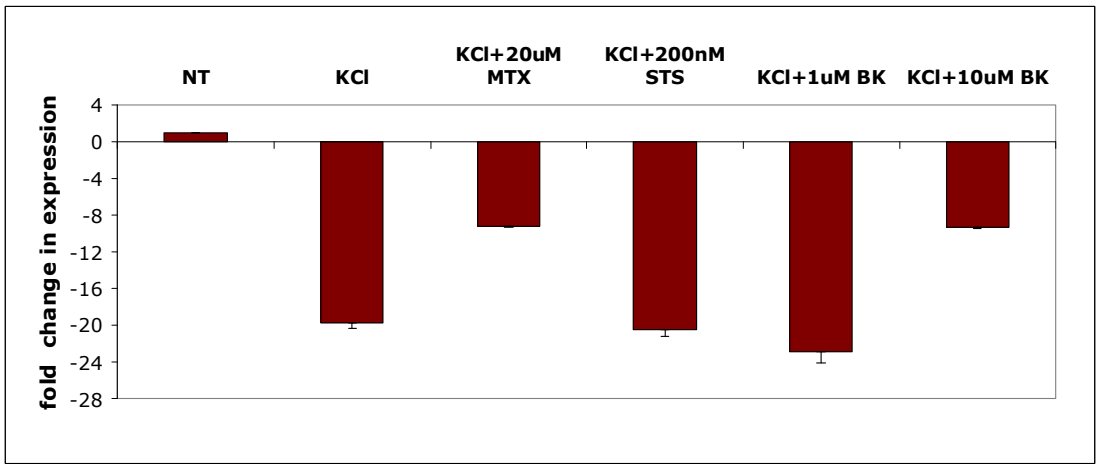

Fig. 3.12. The effect of KCl, mallotoxin, staurosporine and bradykinin treatment on TTC4 expression in N2A cells – TTC4 downregulation in response to 85 mM KCl is attenuated by mallotoxin and bradykinin but not by PKC-specific inhibitor staurosporine. N2A cells were serum starved for 24 hrs and then treated with 85 mM KCl and 85 mM KCl in combination with either mallotoxin (MTX), staurosporine (STS) or bradykinin (BK) in serum-free medium for 4.5 hrs. Non-treated control (NT) cells were cultured in serum-free medium.

3.3.5. Promoters of TTC4 homologs contain nuclear factor of activated T cells (NFAT) binding consensus sequences

The two main executors of Ca^{2+} - dependent regulation of gene transcription are Ca^{2+} - cyclic AMP response element binding protein (CREB) and nuclear factor of activated T cells (NFAT). The specificity of CREB- and NFAT-mediated gene transcription is dependent on two factors: 1) a selective preference for a particular source of Ca^{2+} signal and 2) the duration of the Ca^{2+} signal (Barlow *et al.* 2006, Berridge *et al.* 1998). If TTC4 expression is Ca^{2+} - dependent, its transcription may be regulated by either CREB- or NFAT. As shown in Fig. 3.13, sequences analysis of the first 2000 base pairs (bp) upstream of the starting ATG codon revealed that TTC4 genes carry between three and five putative NFAT-binding sites GGAAA (Barlow *et al.* 2006, Buchholz *et al.* 2006). Notably, on a random basis a five bp sequence (such as the NFAT-binding consensus), would be expected only once every 1024 bp whilst the 2000 bp promoter interval of most TTC4 genes contained four putative NFAT-binding sites (Fig. 3.13).

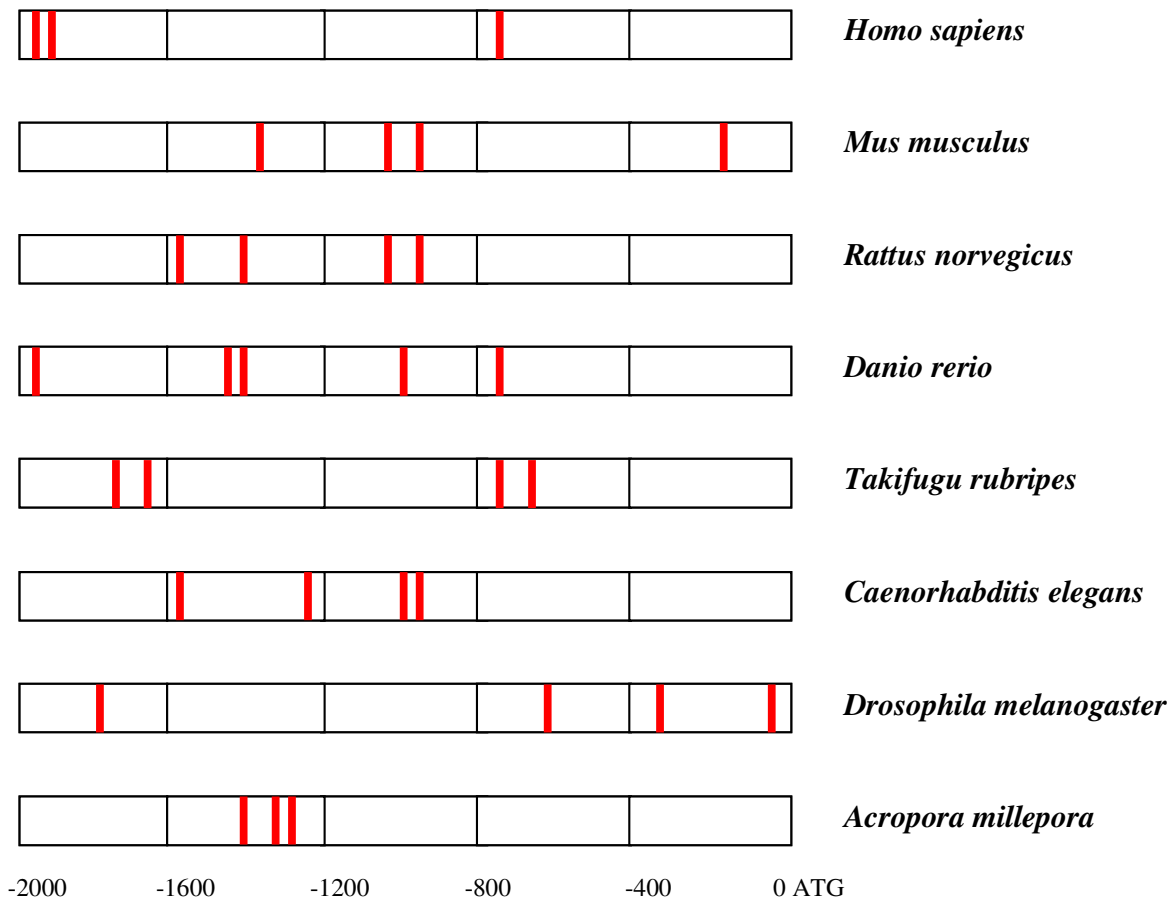

Fig. 3.13. Location of NFAT binding sites (GGAAA) in the 2000 bp sequence upstream of the ATG codon in TTC4 homologs. The promoter sequences were retrieved from the ENSEMBL genome browser database: *H.sapiens* (ENSG00000184313*), M.musculus* (OTTMUSG00000008164), *R.norvegicus* (ENSRNOG00000022624), *D.rerio* (ENSDARG00000044405), *T.rubripes* (SINFRUG00000147013), *C.elegans* C17G10.2 (C17G10.2) and *D.melanogaster* Dpit47 (CG3189).

3.3.6. TTC4 expression is cell cycle-dependent

Proliferative properties of K^+ channels are largely mediated through growth factor-dependent activation of K^+ channels (Huang and Rane 1994, Kodal *et al.* 2000, Lang *et al.* 2005, Xu *et al.* 1999). Thus far, the data indicates that modulating K^+ channel activity may be affecting TTC4 expression in N2A cells. To investigate whether growth factors directly influence TTC4 expression, proliferating N2A cells were brought to quiescence by serum starvation for 49 hrs and then stimulated with 10 % FBS to allow cell cycle progression. Time-course analysis of TTC4 expression revealed that TTC4 was differentially expressed during the cell cycle in N2A cells. Remarkably, the pattern of TTC4 expression closely resembled the pattern of expression of Cdc2 (Fig. 3.14), a gene that is critical for the regulation of eukaryotic cell cycle (Welch and Wang 1992). The highest expression of both TTC4 and Cdc2 was observed 4 hrs after FBS stimulation, 6 hrs after FBS stimulation, the expression of both genes was low and then it increased after 12 hrs of FBS stimulation. After 24 hrs of FBS stimulation, the expression of TTC4 and Cdc2 was again low (Fig. 3.14, panels A and B). This repeated cycle of upregulated and downregulated expression of TTC4 and Cdc2 suggested that two cell cycles were covered during the time-course stimulation with FBS.

To gain insight into the cell cycle profile of N2A cells during FBS stimulation, it was necessary to perform FACS analysis. Firstly, the efficiency of the serum starvation method was assessed by looking at what proportion of total cell population was arrested in G0/G1 phase after treatment. FACS analysis revealed that compared to nonsynchronous cells that were cultured in growth medium supplemented with 10% FBS, the majority of serum starved cells were in G0/G1 phase (Fig. 3.15, panel B). Most importantly, the expression of TTC4 was 3-fold higher in nonsynchronous, proliferating cells than in quiescent, G0/G1- arrested cells (Fig. 3.15, panel A). To investigate the expression of TTC4 in specific stages of the cell-cycle, G0/G1 cells were first treated with 10 % serum and 5 ug/ml aphidicolin to arrest the cells in G1/S and then in 10 % FBS without aphidicolin for indicated time periods to obtain cells in S-, G2/M- and M-phase of the cell cycle (for details see Materials and Methods). Subsequent RT-PCR analysis revealed that TTC4 expression increased during cell cycle progression, significantly, the greatest increase in TTC4 expression occurred during the G1/S transition (Fig. 3.16, panels A and B).

Further confirmation of stage-specific progression through the cell cycle was obtained by examining morphology of N2A cells during serum starvation and aphidicolin treatment. Cells arrested in G0/G1 displayed typical differentiated neuronal-like morphology with extended neurites. Before entry into S phase, at G1/S transition, cells became rounded and during the S phase there was an evident increase in cell volume and size. Throughout mitosis, there was an increased proportion of cells undergoing division and at the end of mitosis the divided cells began to separate (Fig. 3.17).

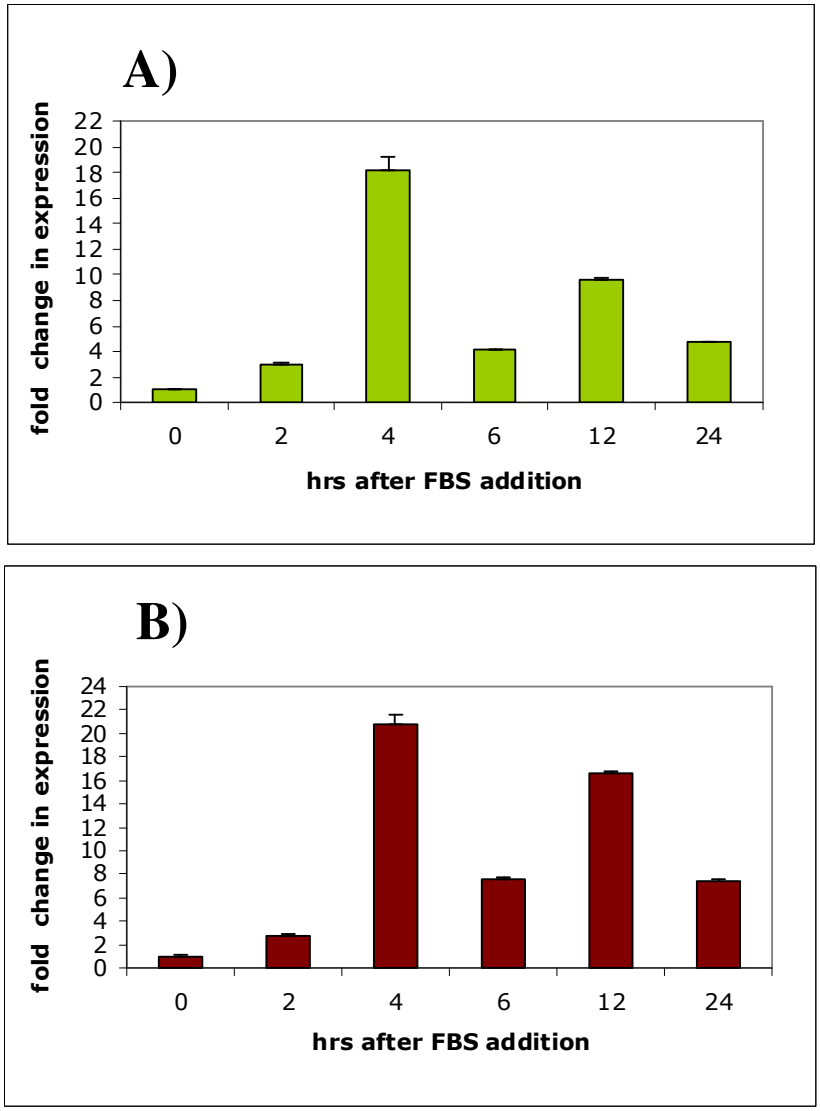

Fig. 3.14. The effect of FBS stimulation on TTC4 and Cdc2 expression in N2A cells – TTC4 and Cdc2 follow a similar pattern of expression in response to FBS. N2A cells were serum starved for 49 hrs and then stimulated with 10 % FBS for indicated time periods. A) RT-PCR analysis of TTC4 expression, B) RT-PCR analysis of Cdc2 expression.

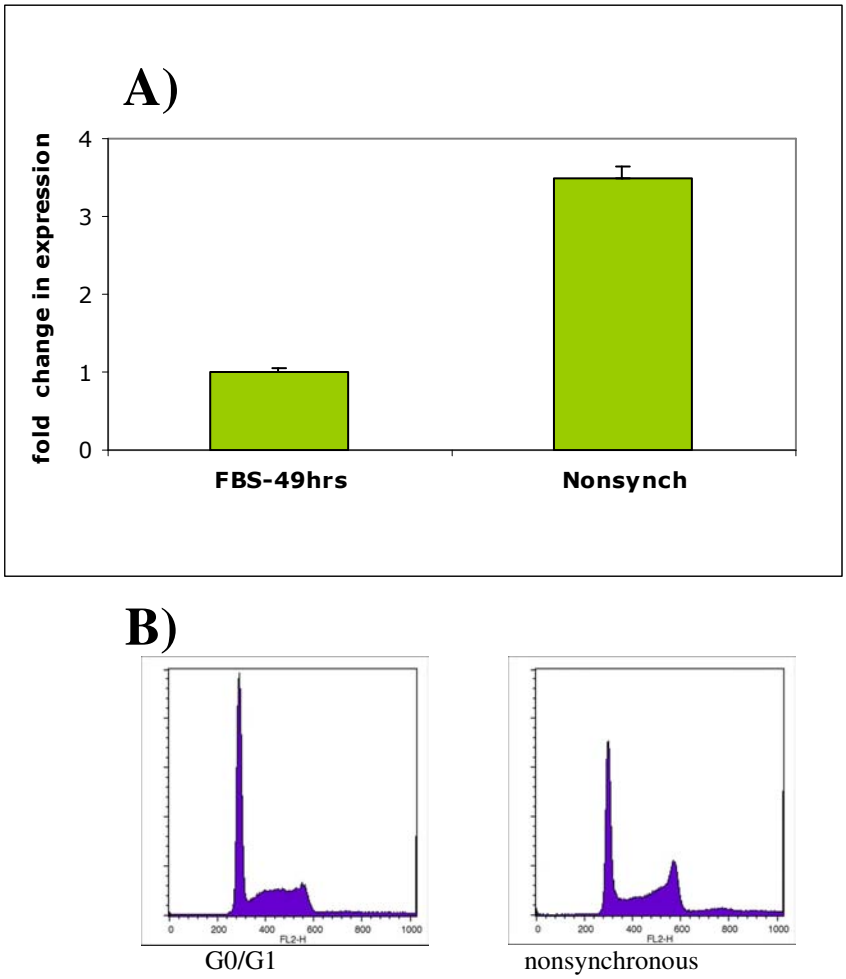

Fig. 3.15. The effect of serum on TTC4 expression in N2A cells – TTC4 is upregulated in FBS-stimulated, nonsynchronous, proliferating cells. N2A cells were either serum starved or cultured in the medium containing 10 % FBS for 49 hrs A) RT-PCR analysis of TTC4 expression, B) FACS analysis of treated N2A cells stained with propidium iodide (see Materials and Methods)

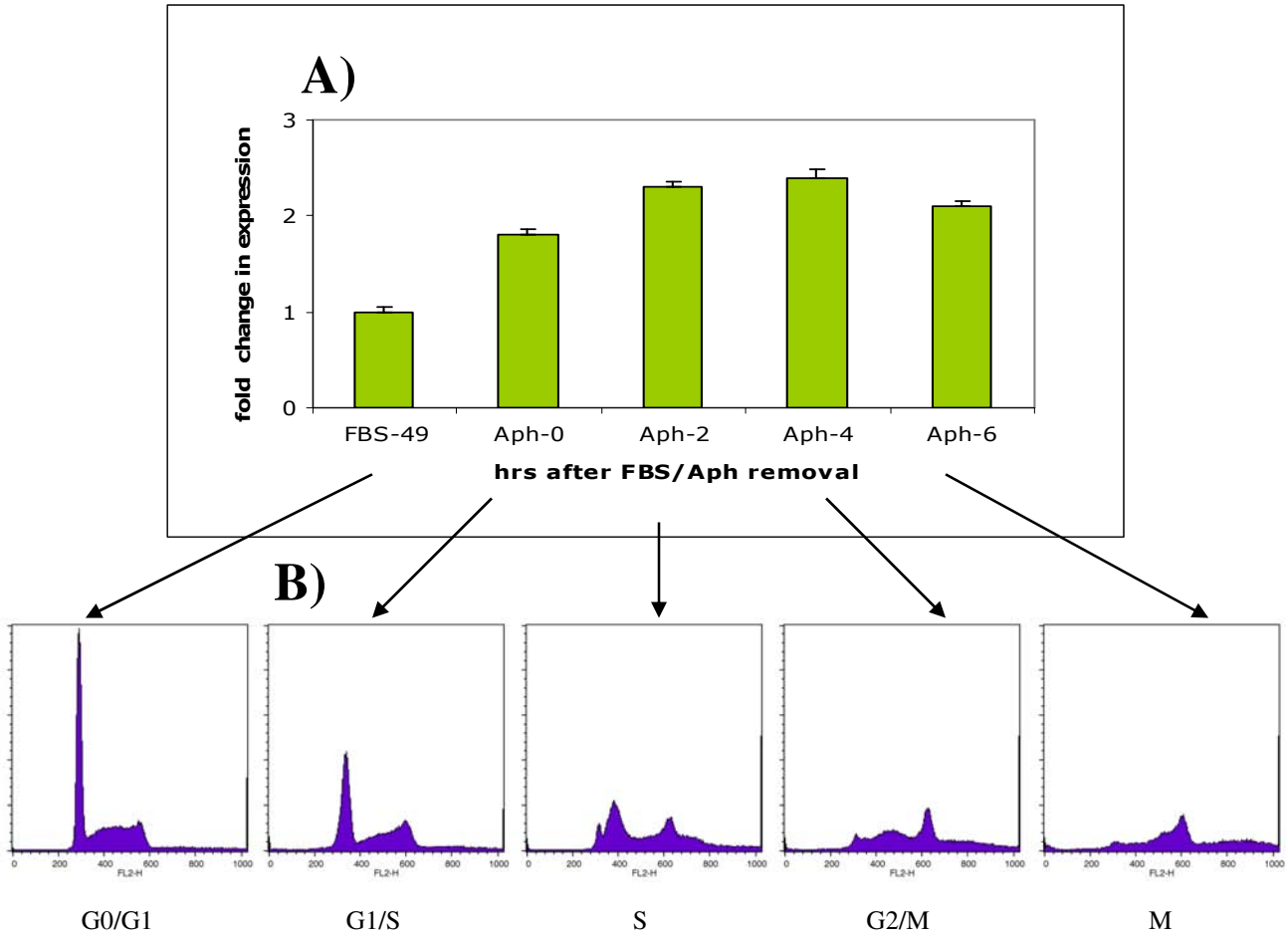

Fig. 3.16. Expression of TTC4 in different stages of the cell cycle in N2A cells – TTC4 is upregulated during the G1/S transition. N2A cells were serum starved for 49 hrs and then treated with 10 % FBS and 5 ug/ml aphidicolin for 24 hrs. Aphidicolin was removed from the serum-containing media at indicated time points. A) RT-PCR analysis of TTC4 expression, B) FACS analysis of treated N2A cells stained with propidium iodide.

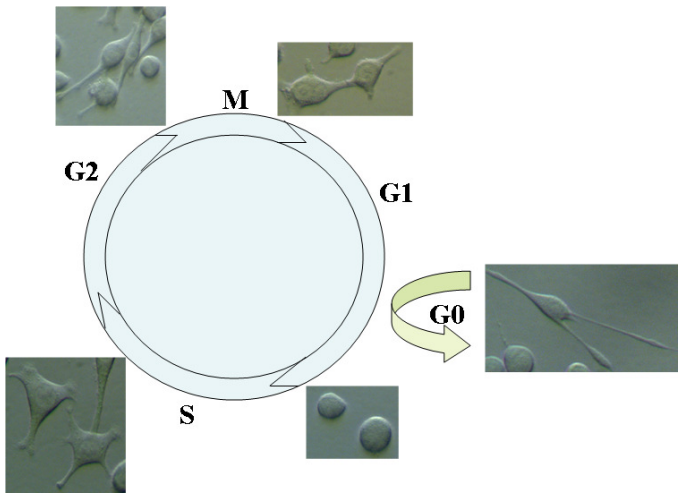

Fig. 3.17. Morphology of N2A cells in different stages of the cell cycle. Cells from the same treatment as in Fig. 3.16.

3.4. DISCUSSION

It is well established that K^+ channel activity positively influences cell proliferation and that it is essential for the G1/S progression of the cell cycle (Ghiani *et al.* 1999, Kahl and Means 2003, Lang *et al.* 2005, MacFarlane and Sontheimer 2000, Pardo 2004, Santella 1998, Wang 2004, Wonderlin and Strobl 1996). Accordingly, many cancers show selective upregulation of K^+ channels (Bianchi *et al.* 1998, Pillozzi *et al.* 2002, Wang *et al.* 2002). This study is the first to show a link between modulation of K^+ channel activity and changes in expression of mouse TTC4 gene. The results indicate that 1) depolarization stimuli and blocking K^+ channel activity downregulates TTC4, 2) K^+ channel opener mallotoxin and mitogen bradykinin attenuate depolarization-induced downregulation of TTC4 in mouse neuroblastoma cells. Furthermore, the results suggest that the expression of TTC4 is cell cycle-dependent, TTC4 is upregulated in actively proliferating cells and the extent of upregulation is the greatest at the G1/S transition of the cell cycle. This strongly indicates that TTC4 may be a cancer-related gene.

The mechanism by which K^+ channel activity regulates the expression of TTC4 is likely to involve Ca^{2+} signalling. Ca^{2+} is a key messenger in many important cellular processes (Berridge *et al.* 1998, Fig. 3.18), and to ensure tight regulation of Ca^{2+} signalling pathways, multiple points of control are required. By having multiple points of control, it is possible to regulate multiple cellular pathways through a single messenger. The complexity of Ca^{2+} signalling occurs at two levels, route of Ca^{2+} entry and mode of Ca^{2+} entry. By combining different routes and modes of Ca^{2+} of entry, different processes are triggered. Such level of complexity of Ca^{2+} signalling is particularly important in the view of K^+ channel activity. K^+ channels regulate both cell proliferation and apoptosis and both of these processes involve Ca^{2+} oscillations (Lang *et al.* 2005, Pardo 2004, Wang 2004). It is essential therefore that cells are able to differentiate between Ca^{2+} signals that would trigger K^+ channel-induced cell proliferation as opposed to K^+ channel-induced apoptosis.

Changes in membrane potential cause oscillations in intracellular Ca^{2+} levels via two main pathways: 1) extracellular influx of Ca^{2+} through voltage-operated Ca^{2+} channels (VCCs), store-operated Ca^{2+} channels (SOCCs) and receptor-operated Ca^{2+} channels (RCCs) and 2) inositol 1,4,5-trisphosphate (IP_3) - dependent Ca^{2+} release from intracellular stores (Berridge *et al.* 1998, Fig. 3.19). It is unlikely that extracellular

Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels or NMDA receptor-operated channels contributes to regulation of TTC4 expression given that L-type channel inhibitor nifedipine and RCC agonist NMDA had no appreciable effect on depolarization-induced downregulation of TTC4 (Fig. 3.5 and Fig. 3.6). On the other hand, attenuation of depolarization-induced downregulation of TTC4 by bradykinin (Fig. 3. 12), suggests that intracellular Ca^{2+} release and the IP_3 pathway may be important in regulating TTC4 expression. In both, non-excitable and excitable cells, stimulation by mitogens leads to activation of the phosphoinositide (PIP) metabolism (Greco *et al.* 2005, Zweifach and Lewis 1993). By activating PIP metabolism, mitogens control differentiation and proliferation of various cell-types. Bradykinin is a mitogen capable of stimulating cell proliferation via MAPK pathway (Greco *et al.* 2005). Stimulation of the B2 bradykinin receptor leads to a signalling cascade that involves phospholipase C (PLC) activation and degradation of phosphoinositides to generate IP_3, activation of IP_3 receptors in the endoplasmatic reticulum (ER) and release of Ca^{2+} from intracellular stores (Fig. 3.19). This ultimately results in transient increases of intracellular Ca^{2+} (Higashida *et al.* 1986, Luo *et al.* 1995, Yano *et al.* 1984). However, the increase in intracellular Ca^{2+} resulting from bradykinin stimulation is unlikely to directly affect TTC4 expression. The reason for this is that the increase of intracellular Ca^{2+} concentration causes transient membrane depolarization (Fanger *et al.* 2001, Imtiaz *et al.* 2007), an effect which, on its own, negatively regulates TTC4 expression. Efflux of K^+ ions is needed to restore the membrane potential towards more hyperpolarized values (Fanger *et al.* 2001). Thus, without counterbalancing K^+ efflux, that is primarily brought upon activation of K^+ channels, the increase of intracellular Ca^{2+} would lead to further cell depolarization. Consequently, any factor that inhibits K^+ efflux would tend to have depolarizing effects on the membrane potential, including raising extracellular K^+ and inhibiting K^+ channel activity. Taken together this neatly explains why a) increasing extracellular K^+ downregulates TTC4, b) blocking K^+ channels exacerbates depolarization-induced downregulation of TTC4 and c) Ca^{2+} downregulates TTC4 even at low K^+ concentrations and exacerbates depolarization-induced downregulation of TTC4. For the later, increasing extracellular Ca^{2+} would also lead to membrane depolarization by causing influx of Ca^{2+} and thereby resulting in increased intracellular Ca^{2+} concentration. The later observations suggests that even though regulation of TTC4 expression may not require L-type Ca^{2+} channels or

NMDA receptor channels, other types of Ca^{2+} channels may be involved in this process. There are two main subgroups of voltage gated Ca^{2+} channels, high threshold-activated channels which require high depolarization stimuli (L,N,P,Q and R-type channels), and low threshold-activated channels which conversely, require small depolarizations for activation (T-type channels, Dolphin 1995, Fox *et al.* 1987, Wolfe *et al.* 2003). TTC4 downregulation in the presence of elevated extracellular Ca^{2+} (2.3 mM), at the lowest extracellular K^+ concentration (10 mM), indicates that T-type channels may be involved in Ca^{2+}-mediated depolarization induced downregulation of TTC4. Also, given that at highest K^+ concentration (85 mM), TTC4 downregulation is exacerbated by 2.3 mM Ca^{2+}, it is possible that high threshold activated Ca^{2+} channels, other than L-type Ca^{2+} channels, also contribute to transcriptional regulation of TTC4. At 30 mM KCl TTC4 expression was not downregulated by 2.3 mM Ca^{2+}. The most plausible explanation for this result is that the addition of 30 mM KCl causes an increase in membrane depolarization that is beyond the threshold of T-type Ca^{2+} channel activation (and hence results in closure of T-type Ca^{2+} channels), but is below the threshold required for activation of L, N, P, Q and R-type Ca^{2+} channels. Of high threshold-activated channels, L-type and N-type channels have been linked to transcriptional events (Fukuchi *et al.* 2005, Gallin and Greenberg 1995, Guerini *et al.* 1999, Rubing et al. 2007, Welman *et al.* 2001), whereas the function of other channels has been linked to other events such as neurosecretion (Wolfe *et al.* 2003). Having excluded L-type channels based on the lack of effect of nifedipine on TTC4 expression, it remains possible that N-type channels may be regulating TTC4 expression. The inability of the Ca^{2+} chellator EGTA to suppress depolarization-induced TTC4 downregulation in the presence of elevated extracellular Ca^{2+} may be due relatively slow chellating properties of EGTA (Isaacson and Murphy 2001).

Another question remains to be resolved. If bradykinin effect on TTC4 expression cannot be attributed to the release of Ca^{2+} from intracellular stores directly via the IP_3 pathway, in which case we would expect downregulation of TTC4, how does bradykinin mediate this effect, that ultimately, results in attenuation of depolarization-induced downregulation of TTC4? The answer to this paradox appears to be rather simple. Bradykinin effect is indirect, it is likely to be mediated through activation of Ca^{2+} activated, voltage-gated K^+ channels which then cause membrane hyperpolarization and increase Ca^{2+} influx through store-operated Ca^{2+} channels. It is

this second wave of intracellular Ca^{2+} increase, resulting from K^+ channel activity, that may directly regulate TTC4 expression. This scenario also elegantly fits the effects of mallotoxin and growth factors on TTC4 expression (Fig. 3.20). Mallotoxin is a K^+ channel opener (Wu *et al.* 2007, Zakharov *et al.* 2005) and growth factors in serum have been shown to activate K^+ channels (refs). Mallotoxin was found to attenuate depolarization-induced downregulation of TTC4 (Fig. 3.9-12) and addition of serum to nonproliferating cells caused upregulation of TTC4 (Fig. 3.14 and Fig. 3.15). It is well known that store-operated Ca^{2+} channels open in response to depletion of intracellular Ca^{2+} stores (Berridge *et al.* 1998, Fanger *et al.* 2001, Wulff *et al*. 2004, Zweifach and Lewis 1993). However, Ca^{2+} influx through SOCCs depends on the electrochemical gradient: the influx of Ca^{2+} is greatly reduced at depolarizing potentials. Activation of K^+ channels thus necessary to provide the driving force for Ca^{2+} entry through SOCCs by establishing and maintaining a hyperpolarized membrane potential (Fanger *et al.* 2001, Hess *et al.* 1993, Wulff *et al*. 2004, Zweifach and Lewis 1993).

The two-step process of intracellular Ca^{2+} increase 1) directly as a result of Ca^{2+} release from intracellular stores and the transient depolarization and 2) indirectly, as a result of Ca^{2+} influx throough SOCCs mediated by K^+ channel activity and membrane hyperpolarization, is an excellent example of how combining distinct routes and modes of Ca^{2+} entry triggers different signalling cascades and hence different cellular processes. The first pathway results in the activation of a PKC-dependent Ras/MEK/ERK pathway and transcription of early/immediate-response genes, transcription factors c-Fos and c-Jun. (Barlow *et al.* 2006, Fanger *et al.* 2001, Juretic *et al*. 2006). c-Fos and c-Jun constitute the AP-1 dimeric transcription complex which regulates the expression of genes involved in an array of important cellular processes, such as cell proliferation, differentiation, apoptosis and stress response (Bossis *et al.* 2005, Shaulian and Karin 2002). AP-1 binds to CREB/AP-1 responsive elements to initiate the transcription of target genes (Juretic *et al*. 2006). The second Ca^{2+} signalling pathway has been shown to activate nuclear factor of activated T cells (NFAT) transcription factor-dependent genes (Barlow *et al.* 2006, Berridge *et al.* 1998, Fanger *et al*. 2001), through activation of Ca^{2+}/calmodulin - dependent serine/threonine phosphatase calcineurin (Carrasco and Hidalgo 2006, Crabtree 2001, Loh *et al.* 1998). Even though other pathways that result in intracellular increase of Ca^{2+} have also been shown to stimulate both CREB and NFAT-dependent

transcription, for example, IP_3 pathway can also induce NFAT-dependent transcription (Barlow *et al.* 2006, Carrasco and Hidalgo 2006), the specificity of the resulting cellular responses is strictly dependent on the route of Ca^{2+} entry and the duration of the Ca^{2+} signal (Barlow *et al.* 2006, Berridge *et al.* 1998). NFAT family consists of four members, NFATc1, NFATc2, NFATc3 and NFATc4. NFAT family of transcription factors have been long known to play key roles in mediating immune responses (Fanger *et al.* 2001). Recently however, it has been shown that NFAT members may have more ubiquitous roles, in fact, they are important in regulating expression of genes involved in development and differentiation. Multiple functional roles of NFAT members in different cell types are linked to differences in Ca^{2+} - mediated NFAT activation (Crabtree 2001). The second pathway of Ca^{2+} influx through SOCCs following Ca^{2+} depletion from intracellular stores has specifically been associated with cell proliferation. The fact that triggering of cell growth and proliferation requires a sustained Ca^{2+} signal explains the dependency of NFAT-mediated proliferation-responses on the second Ca^{2+} pathway (Berridge *et al.* 1998, Fanger *et al*. 2001). Namely, in contrast to activation of the second Ca^{2+} pathway, activation of the IP_3 pathway and the following release of Ca^{2+} from intracellular stores, only results in a transient increase in intracellular Ca^{2+}. That is because intracellular Ca^{2+} stores are finite and further increase of Ca^{2+} to generate a sustained signal, requires activation of SOCCs in the plasma membrane (Berridge *et al.* 1998, Fanger *et al*. 2001).

In the context of this study, the findings that NFAT members influence proliferation of different cell types such as pancreatic, fibroblasts, adypocites, lymphocytes and neurons (Buchholz *et al.* 2006, Caetano *et al.* 2002, Graef *et al.* 2003, Hogan 2003, Neal and Clipstone 2003), and are also important in tumor development (Viola *et al.* 2005), is of particular interest. Most significant are perhaps the findings of Buchholz *et al.* (2006), that NFATc1 is capable of activating c-myc oncogene in pancreatic cancer cells. Considering that c-myc oncogene has been found to occupy the TTC4 promoter in Burkitt lymphoma cells (Li *et al.* 2003), and that TTC4 homologs carry NFAT-binding consensus sequence in the first 2000 bp upstream of the starting ATG codon (Fig. 3.13), it is very tempting to speculate that NFAT transcription factors may be the ultimate link between K^+ channel activity, Ca^{2+} signalling and the function of the cancer-related gene TTC4. This hypothesis however, awaits further validation. Thus far, it may be concluded that transcriptional activation of TTC4 requires

hyperpolarization and K^+ channel activity and it is likely to be a part of a second cascade of Ca^{2+} signalling possibly involving NFAT transcription factors.

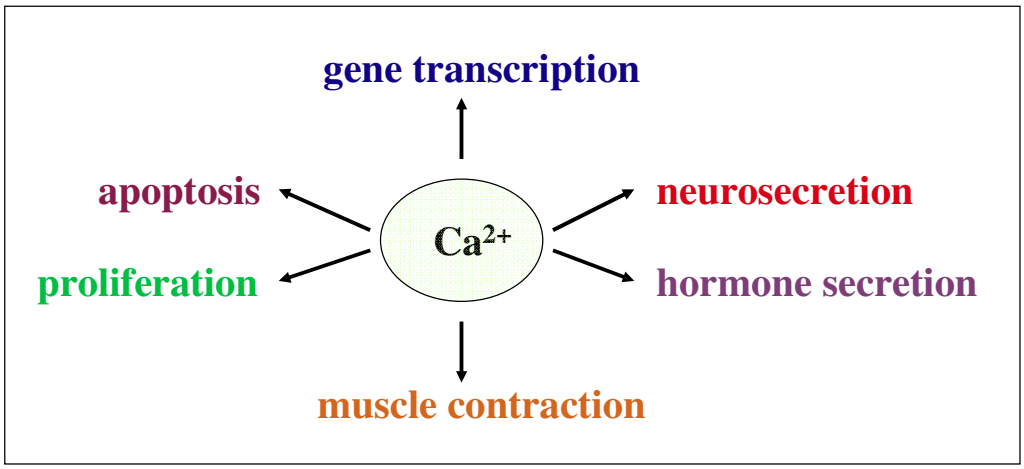

Fig. 3.18. Multiple roles of Ca^{2+} signalling.

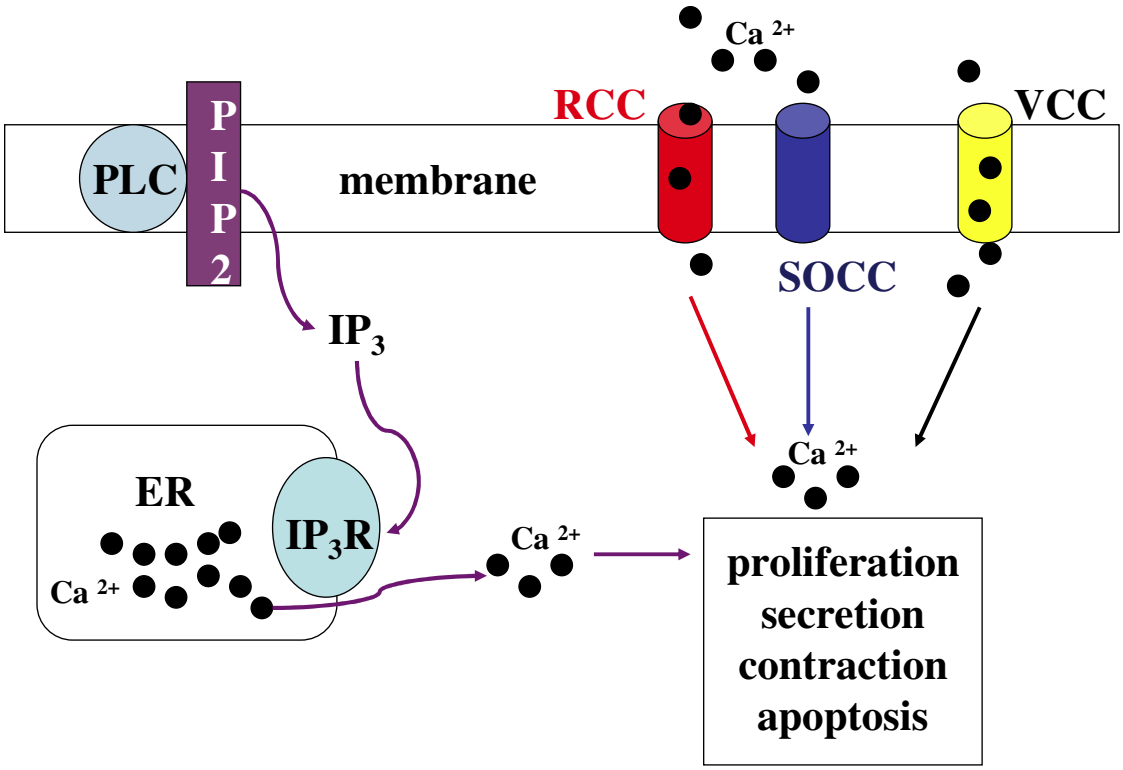

Fig. 3.19. Regulation of intracellular Ca^{2+} increase by the IP_3 pathway and Ca^{2+}-channel operated extracellular pathway. PLC-phospholipase C, PIP2-phosphatidylinositol 4,5-bisphosphate, IP_3-inositol 1,4,5-trisphosphate, IP_3R- IP_3 receptor, ER-endoplasmatic reticulum, RCCs-receptor-operated Ca^{2+} channels, SOCCs- store-operated Ca^{2+} channels, VCCs-voltage-operated Ca^{2+} channels.

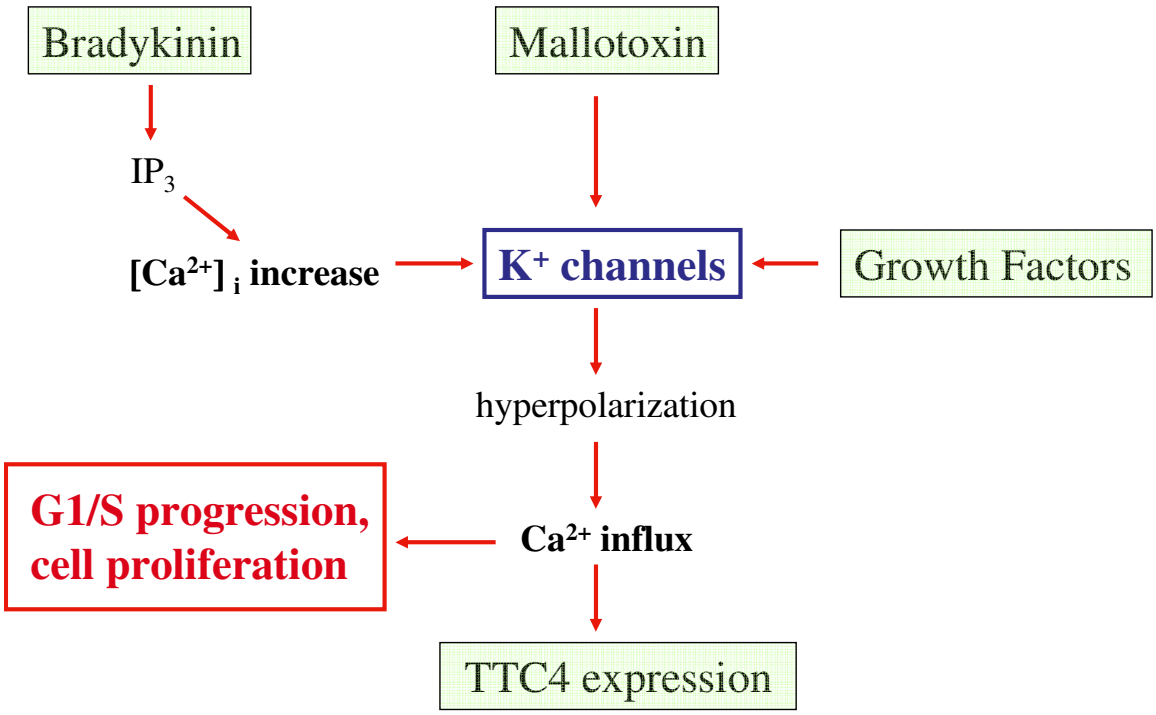

Fig. 3.20. Pathways of bradykinin, mallotoxin and growth factor-induced regulation of TTC4 expression – convergence point: activation of K^+ channels and K^+ channel-induced Ca^{2+} influx.

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To suppress or not to suppress: cell proliferation, development, cancer and the AmTPR/Dpit47/TTC4 TPR family saga

4.1. INTRODUCTION

Cancer is a progressive disease which stems from genetic alterations that perturb the delicate balance between cell proliferation and differentiation (Loop *et al.* 2004, Okubo and Hogan 2004, Willert *et al.* 2002). These two processes are regulated at a highly orchestrated level and are critical for proper development of multicellular organisms (Okubo and Hogan 2004, Willert *et al.* 2002, Zechner *et al.* 2003). Early development features extensive cell proliferation that ceases once maturity is reached. In adult organisms most cells are differentiated and quiescent and proliferation is restricted to specific pools of cells in tissues which require constant turn-over (for example, intestinal epithelia, Muncan *et al.* 2006, Pinto *et al.* 2003, Pinto and Clevers 2005). Such proliferative cell compartments are kept in check by numerous mechanisms of regulation. Not only is there a large number of regulatory genes, but also a network of signalling cascades that prevent cells from entering a state of aberrant proliferation (Hahn and Weinberg 2002, Hanahan and Weinberg 2000, Kearsey and Cotterill 2003, Sivaprasad *et al.* 2007, Stoeber *et al.* 2001). Consequently, cancer is rarely caused by a mutation in a single gene, rather it requires an accumulation of mutations affecting multiple genes involved in cell growth (Dean 1998, Hahn and Weinberg 2002, Hanahan and Weinberg 2000, Loop *et al.* 2004, Okubo and Hogan 2004, Willert *et al.* 2002). Despite the genetic complexity, most cancers develop from two classes of mutations: 1) activation of oncogenes and 2) inactivation of tumor suppressor genes. Notably, both oncogenes and tumor suppressors play fundamental roles in development (Dean 1998). For example, oncogenes E2F, Myb, Myc and Ras are important regulators of cell proliferation, growth and differentiation. Their mutational activation correlates with a significant proportion of human tumours (Classon and Harlow 2002, Dick *et al.* 2000, Karim and Rubin 1998, Li *et al.* 2003, Loop *et al.* 2004, Moodie and Woolfman 1994). On the other hand, tumor suppressor gene p53, a critical mediator of DNA-damage induced cell-cycle progression, is inactivated in 40-50% of all human cancers (Ashur-Fabian *et al.* 2004, Hollstein *et al.* 1991).

Genome-wide genetic screens and microarray expression studies have revealed a large number of genes involved in cancer development. However, the molecular mechanisms by which these genes contribute to tumorigenesis are largely unknown (Li *et al.* 2003, Loop *et al.* 2004). Due to the intimate link between carcinogenesis and malfunctioning of developmental programs, investigation of developmental gene function is necessary to improve our understanding of cancer pathology. In that context, the present study aimed to elucidate the molecular and cellular function of AmTPR1/Dpit47/TTC4, a family of genes that encode TPR proteins implicated in cell proliferation and development.

4.2. The human TTC4, tumor suppressor or oncogene ?

The human TTC4 locus maps to a region on chromosome 1 (1p31) that has been associated with a number of malignancies (Schwab *et al.* 1996, Su *et al.* 1999). Su *et al.* (1999) mapped the TTC4 gene to a 15-Mb region within 1p31 overlapping with a region frequently displaying loss of heterozygosity (LOH) in breast cancer. They assigned the 28-kb TTC4 gene to that region, implying a possible function of TTC4 as a tumour suppressor gene. However, another study (Hey *et al.* 2000) implies that TTC4 may map to a different interval on 1p31 lying outside the region displaying LOH and this point remains controversial. Clinical data from Poetch *et al.* (2000) showed point mutations in TTC4 in 6 of 25 metastases, 2 of 17 nodular melanomas and 2 of 17 superficial spreading melanomas. Of particular interest is to note that the most prevalent of all melanoma mutations found by Poetch *et al.* (2000), was A→C change affecting codon 77 and resulting in a substitution of a polar glutamate residue (E) to hydrophobic alanine (A). Even though codon 77 lies outside of the TPR motifs, it is positioned in the close vicinity of the first TPR. Thus the E→A substitution could possibly affect the overall conformation of the TTC4-superhelix structure and consequently the function of the protein. In contrast to Poetch *et al.* (2000), subsequent investigations (Irwin *et al.* 2002) found no mutations in the TTC4 coding region in 40 melanoma cell lines, derived from primary cutaneous melanoma or metastases. However, Irwin *et al.* (2002) did not investigate the possibility of transcriptional silencing of the TTC4 gene and since cell lines rather than clinical material were used, direct comparisons between their results and those of Poetch *et al.* (2000) cannot be made. Accordingly, a role of TTC4 in melanoma cannot be ruled out

on the basis of Irwin *et al.* (2002) results. Further data suggests that TTC4 may be an oncogene rather than being a tumor suppressor. TTC4 expression appears to be positively regulated by the c-Myc oncogene in Burkitt lymphoma cells (Li *et al.* 2003). Furthermore, investigation of genomic alterations in non-small cell lung cancers conducted by Kim *et al.* (2005), lead to the identification of minimally altered regions of chromosomal gains and losses (MAR-Gs and MAR-Ls), that were associated with clinicopathological features of lung cancer. Significantly, a number of known tumor suppressors were identified in MAR-Ls, for example IRF1, CDKL3, RAD50 and PTEN (Heikkinen *et al.* 2006, Vazquez *et al.* 2000, Xie *et al.* 2003). On the other hand, well known oncogenes PIK3CA, ECT2, FGR, LCK and MYCL1 (Eguch *et al.* 2007**,** Karakas *et al.* 2006, Kim *et al.* 2006, Link and Zutter 1995, Marth *et al.* 1988), were associated with MAR-Gs. In contrast to its assumed role as a tumor suppressor, TTC4 was also found in a MAR-G and cautiously denoted by Kim *et al.* (2005), as a "cancer-related" gene. It is of interest to note that the Kim *et al.*'s (2005) study illustrates recurring themes in the molecular basis of carcinogenesis: 1) cancer is not caused by one but by a number of mutations, 2) tumor suppressors and oncogenes have opposing roles in cancer development.

4.3. Comparative genomics: introducing the TTC4 gene family

In order to clarify the existing discrepancies on TTC4 role in tumorigenesis the present study made use of comparative genomics approach, based on the fact that TTC4 homologs have been identified in species other than mammals. The *Acropora* AmTPR1, *Nematostella* NvTPR1 (XP_001637204), *Drosophila* Dpit47 (NP_525106.2), *Caenorhabditis* C17G10 (NP_495087.1), *Danio* (NP_001002122.1) and *Mus* TTC4 (NP_082485.1), proteins share more than 50% amino acid similarity with the human TTC4. It is well established that important developmental genes show high degree of conservation among different metazoan phyla (Metazoa represent animals with true tissues, Technau *et al.* 2005). In that respect, the scleractinian coral *Acropora*, a member of Anthozoa, is exceptionally informative. Despite being simple animals with only one body axis and two germ layers, Anthozoa are strikingly complex on a genetic level (Ball *et al.* 2002, Ball *et al.* 2004, Kortschak *et al.* 2003, Miller *et al.* 2000, Miller and Ball 2000, Technau *et al.* 2005). Most intriguingly, a surprisingly large number of developmental genes thought to be vertebrate-specific are also present in corals but absent from more complex invertebrates such as

Drosophila and *Caenorhabditis* (Ball *et al.* 2004, Kortschak *et al.* 2003, Technau *et al.* 2005). Anthozoans as model organisms offer further significant advantages over complex invertebrates. Being the most basal class within the Cnidaria and also the simplest animals with true tissue level of organization, anthozoans are critically important in understanding ancestral function of metazoan genes (Ball *et al.* 2002, Ball *et al.* 2004, Kortschak *et al.* 2003, Miller *et al.* 2000, Miller and Ball 2000, Technau *et al.* 2005). Nevertheless, *Drosophila* as a model organism is particularly applicable for studying cancer-related gene function since more than two thirds of known human cancer genes have homologs in *Drosophila* and their misregulation leads to development of neoplastic phenotypes that are strikingly similar to malignant changes that characterize cancer in humans (Loop *et al.* 2004).

4.4. Invertebrate AmTPR1/Dpit47 and the Hsp90 co-chaperone machinery in DNA replication in eukaryotes

What lessons are to be learnt from the coral and the fly TTC4-like proteins ? First, both *Acropora* AmTPR1 and *Drosophila* Dpit47 are Hsp90 co-chaperones that also interact with DNA polymerase α, an enzyme central to the eukaryotic DNA replication complex (Crevel *et al.* 2001, Chapter 2, Fig. 2.13.). Apart from DNA polymerase α, Dpit47 presumably interacts with three other proteins involved in initiation of DNA replication: cdc6, orc2 and orc5 (Cotterill, unpublished). Dpit47 is also found in association with Hsp70 and thus belongs to the Hsp70/Hsp90 multichaperone complex (Crevel *et al.* 2001). Based on Crevel *et al.*'s study (2001), DNA polymerase activity is inhibited when complexed with Dpit47, suggesting a regulatory role of the fly TPR co-chaperone in DNA replication. Consistent with this hypothesis, Dpit47/DNA polymerase α interaction is only observed in proliferating cells (Crevel *et al.* 2001). Furthermore, consistent with its chaperone role, it is possible that Hsp90 forms a ternary complex with Dpit47/DNA polymerase α in order to relieve the inhibitory effect of Dpit47 and to establish a replication-permissive conformation of the polymerase. *In vivo*, activity of Hsp90 is critically dependent on its intrinsic ATPase activity (Grenert *et al.* 1999, Panaretou *et al.* 1998, Prodromou *et al.* 1999, Pearl and Prodromou 2000, Richter *et al.* 2002). It was shown that geldanamycin, a specific inhibitor of Hsp90 ATP-ase, stabilises the interaction between Dpit47 and DNA polymerase α and that the addition of ATP reverses the

stabilising effect of geldanamycin on Dpit47/DNA polymerase α complex (Crevel *et al.* 2001). These results not only support the hypothesis of Hsp90-mediated activation of DNA polymerase α but also strengthen the link between Dpit47 function and regulation of DNA replication.

The involvement of Hsp90 chaperone complex in cell cycle regulation is not unprecedented. Initiation of DNA replication is a remarkably ordered process, involving a plethora of protein complexes and accessory proteins (Kearsey and Cotterill 2003, Kelly and Brown, 2000, Sivaprasad *et al.* 2007, Stoeber *et al.* 2001). It is therefore not surprising that one of the most abundant cellular proteins, the Hsp90 chaperone, is implicated in its control (Helmbrecht *et al.* 2000). In eukaryotes, which possess large genomes, multiple origins of replication are required. Subsequently, processes which ensure simultaneous activation and also inhibition of reactivation of these points prior to completion of chromosomal synthesis, have to be tightly co-ordinated so that each daughter cell receives only one and complete copy of the genome (Fitzpatrick *et al.* 2002, Sivaprasad *et al.* 2007). This level of organisation features multiple points of regulation which effectively buffer against error. Notably, a significant number of proteins involved in cell cycle control are well known Hsp90/Hsp70 clients, for example: G1-Cdks, cyclins, transcription factors p53, pRb and E2F (Fig. 4.1, Helmbrecht *et al.* 2000). In the context of the present discussion, the G1-Cdks are relevant given that they mediate E2F-dependent transcriptional activation of G1/S and S phase (DNA replication associated) genes (Johnson and Schneider-Broussard 1998, Muller and Helin 2000). It is important to note that the association of Hsp90 and Dpit47 may be an additional and unique point of control in terms of cell cycle regulation, since the vast majority of Hsp90 targets are signalling molecules and transcription factors (Fig. 4.1). The latter are thus components of the upstream regulatory mechanism, being associated with transcriptional events, whereas Dpit47, with its direct inhibitory effect on the polymerase α, represents a downstream component (Fig. 4.1). Therefore, in the Hsp90 chaperone machinery, there are seemingly two kinds of fail-safe mechanisms that ensure proper progression of cell cycle events.

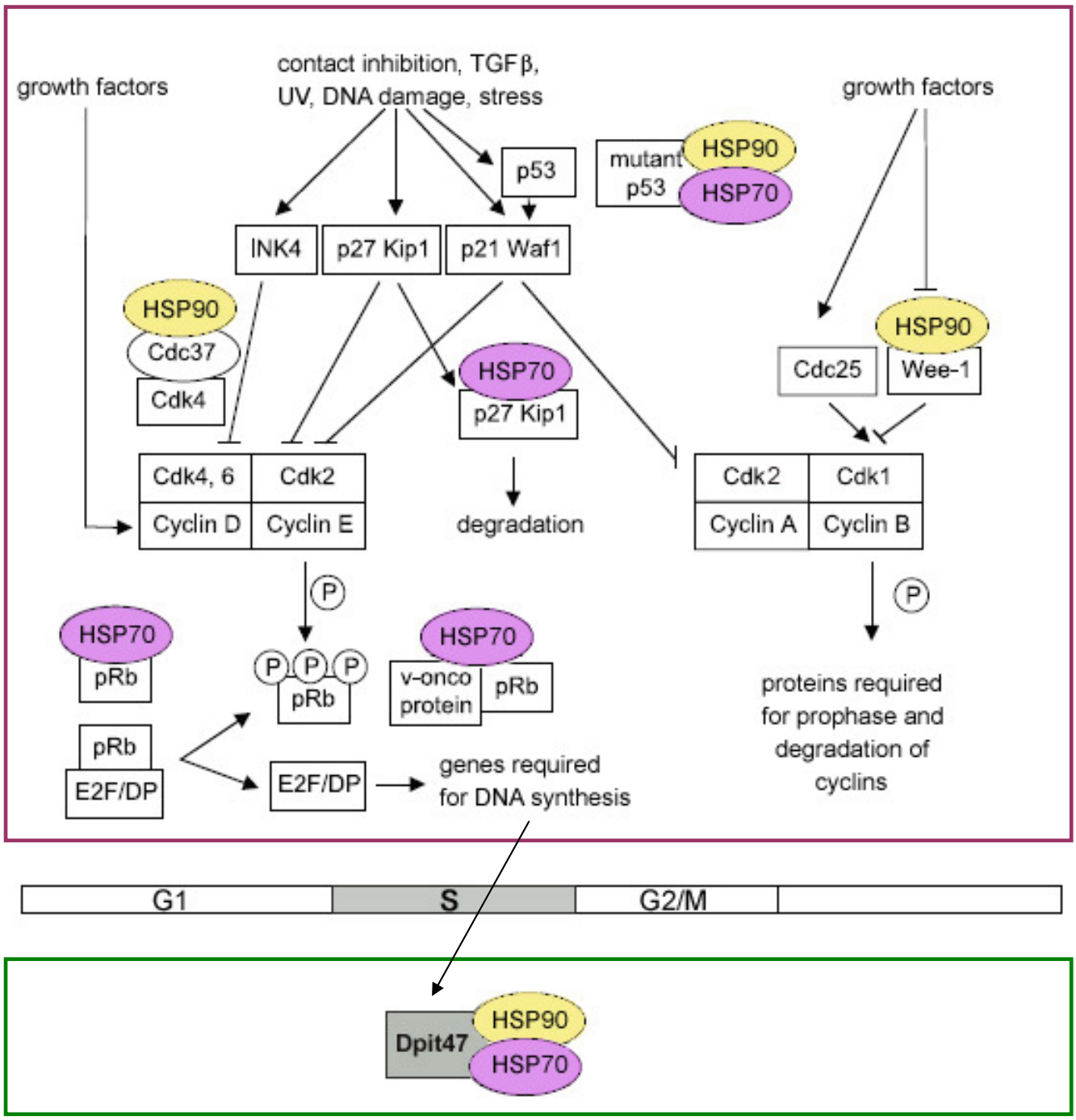

Fig. 4.1. Cell cycle regulation by the Hsp90/Hsp70 chaperone network. Boxed in purple is the upstream regulatory component of the Hsp90/Hsp70 network constituting of signaling molecules: cyclins, cyclin-dependent kinases (Cdk), Cdk inhibitors (INK4, p27KIP1 and Waf), cell division cycle proteins (Cdc) and transcription factors (p53, EF2 and pRb). Boxed in green is Dpit47/Hsp90/Hsp70 complex, the downstream component of the network (adapted from Helmbrecht *et al.* 2000).

4.5. AmTPR1/Dpit47/TTC4 - developmental genes and proto-oncogenes ?

Interaction with Hsp90 may only be circumstantial evidence linking AmTPR1 and Dpit47 function with the cell cycle. However, in a broader perspective, the expression characteristics of both of these invertebrate TTC4 homologs are consistent with important developmental roles as specific regulators of cell proliferation. A discernable characteristic of genes involved in cell proliferation is their high expression in early development, when most tissues are mitotically active (Katzen and Bishop 1996). Consistently, Dpit47 is most prominently expressed during early embryogenesis (Crevel *et al.* 2001). Furthermore, in the case of *Acropora* AmTPR1, in situ hybridisation shows that, after gastrulation AmTPR1 transcript localizes to a specific subpopulation of trans-ectodermal cells (Chapter 2, Fig. 2.7 and 2.8). The trans-ectodermal cells in anthozoans cross the ectoderm-endoderm boundary, thus they are likely to be analogous to the interstitial cells of Hydra (Bode 1996). Noteworthy, the interstitium of Hydra is a unique cellular compartment with stem cell-like properties (Bode 1996). It is well established that pathways that regulate stem cell renewal are also involved in abnormal cell proliferation associated with pathogenesis of cancer (Pardal *et al.* 2005). Taken together, these observations also support a role of AmTPR in cell proliferation and cancer. In addition, AmTPR1 expression is markedly increased in embryos treated with the glycogen synthase kinase-3 (GSK-3) - specific inhibitor alsterpaullone, implicating the involvement of canonical Wnt/β-catenin pathway in regulating AmTPR1 expression (Chapter 2, Fig. 2.9, panels A and B). In agreement with this hypothesis is also the presence of several putative TCF/LEF binding sites within the AmTPR1 promoter (Chapter 2, Fig. 2.9, panel C). The members of the Wnt/β-catenin family are highly conserved regulators of developmental processes in multicellular organisms (Moon *et al.* 1997, Moon *et al.* 2002, Okubo and Hogan 2004, Sato *et al.* 2004, Willert *et al.* 2002, Zechner *et al.* 2003). Significantly, by the virtue of activating genes that 1) positively influence proliferation, stem cell-like properties and 2) inhibit differentiation, hyperactive Wnt/β-catenin signalling is found as a common predisposing mechanism to tumor formation in distinct histological backgrounds. Colorectal cancer, melanoma, medulloblastoma, gastric and prostate cancer, hepatocellular, lung and embryonic carcinomas have all been linked with inappropriate activation of Wnt/β-catenin pathway (Liu *et al.* 2002, Morin *et al.* 1997, Okubo and Hogan 2004, Rubinfeld *et al.* 1997, Tetsu and McCormick 1999, Willert *et al.* 2002). An important conclusion

stems from these observations, the localization of AmTPR1 mRNA to a stem cell-like compartment and the apparent positive regulation of AmTPR1 by Wnt/β-catenin are more consistent with a pro-proliferative and oncogenic role of AmTPR1 than a tumor suppressor role. In the view of functional conservation between AmTPR1 and the human TTC4 this hypothesis is in concordance with Kim *et al.*'s (2005) study and therein implied oncogenic function of TTC4.

Like AmTPR1, Dpit47 is expressed in a manner consistent with its proposed role in cell proliferation and development. However, from that point, the characteristics of the coral and the fly gene are seemingly divergent. At a protein level, *Drosophila* Dpit47 is most prominently expressed in early embryos and pupae and expressed at much lower or undetectable levels in late embryos and larvae (Crevel *et al.* 2001). Interestingly, the expression levels of Dpit47 mRNA and protein appear to be markedly different during *Drosophila* development**.** As shown by in situ hybridisation, early in embryogenesis (stages 6-11), Dpit47 transcript was not detectable above background while later in development (stages 13-16), the expression was strongly associated with the central nervous system (Chapter 2, Fig. 2.10). These results imply a complex regulation of Dpit47 activity that occurs both, at the level of protein and mRNA, a feature that is indicative of genes important for development (Welch and Wang 1992). Consistently, analysis of Dpit47 promoter indicates that Dpit47 expression may be mediated by E2F and Myb (Chapter 2, Fig. 2.11). Both transcription factors and protooncogenes, E2F and Myb are crucial regulators of cell growth and proliferation and thus fundamentally important for normal development of many organisms (Duronio *et al.* 1995, Golay *et al.* 1994, Hao *et al.* 1995, Ito 2005, Johnson and Schneider-Broussard 1998, Katzen and Bishop 1996, Lewis *et al.* 2004, Li *et al.* 2003a, Li *et al.* 2003b, Muller *et al.* 2001, Sitzmann *et al.* 1995, Toscani *et al.* 1997, Trauth *et al.* 1994, Turque *et al.* 1997, Vara *et al.* 2003). E2Fs regulate differentiation of numerous cell lineages including adipocytes, myocytes, myoblasts, pancreatic exocrine cells, hematopoietic progenitors, lymphocytes (Hlaing *et al.* 2004, Li *et al.* 2003a, Li *et al.* 2003b, Muller *et al.* 2001, Vara *et al.* 2003, Zhu *et al.* 2001). Similar to E2F, the developmental functions of Myb are remarkably extensive. In *Drosophila,* Myb is essential for proper embryonic and imaginal development. Accordingly, suppression of Myb function correlates with decreased embryonic viability, impairments in fertility, wing development (Beall *et al.* 2004, Katzen and Bishop 1996, Lewis *et al.* 2004). In vertebrates, prominent Myb-

directed processes include embryogenesis, spermatogenesis, hematopoiesis, CNS and breast tissue development (Kauraniemi *et al.* 2000, Mucenski *et al.* 1991, Sitzmann *et al.* 1995, Thomas *et al.* 2005, Toscani *et al.* 1997, Trauth *et al.* 1994, Turque *et al.* 1997). In conclusion, the presence of both E2F and Myb sites in Dpit47 promoter implies that Dpit47 function is required for development in *Drosophila*.

Moreover, in close proximity to E2F and Myb, Dpit47 promoter contains two DREF (DNA replication element-binding factor), binding consensus sequences (Chapter 2, Fig. 2.11). In *Drosophila*, DREF exclusively regulates expression of genes involved in cell cycle and cell proliferation such as cyclin A, cyclin E, PCNA and orc2 (Hochheimer *et al.* 2002, Hyun *et al.* 2005, Matsukage *et al.* 1995, Ohno *et al.* 1996, Seto *et al.* 2006). Genes implicated in Dpit47 function, DNA polymerase α, E2F and Myb are also targets of DREF (Hyun *et al.* 2005). Interestingly, E2F and DREF act in synergy to promote DNA replication and cell proliferation in *Drosophila* (Fitzpatrick *et al.* 2002, Hochheimer *et al.* 2002, Seto *et al.* 2006). Accordingly, many of the DREF-responsive genes are also subjected to E2F-mediated transcriptional regulation: cyclins A and E, PCNA, Orc2 and Myb (Hochheimer *et al.* 2002, Muller *et al.* 2001, Royzman *et al.* 1999, Seto *et al.* 2006, Thacker *et al.* 2003, Zhu *et al.* 2004). In the case of PCNA, E2F-mediated transcription itself is DREF-dependent given the inability of E2F to induce activation of a PCNA reporter from a construct lacking the DREF site (Hochheimer *et al.* 2002).

While DREF appears to be specifically required for the induction of G1/S and S phase associated genes (Hyun *et al.* 2005), the temporal regulation of gene expression exerted by E2F and Myb is far more complex. In mammals, *Drosophila* and plants, evidence has accumulated that suggests an indispensable role of E2F and Myb in regulating G2/M transition of the cell cycle (Fitzpatrick *et al.* 2002, Ito 2005, Katzen *et al.* 1998, Zhu *et al.* 2004). As an example the expression of mitotic genes cyc B and cdc2 is mediated by both E2F and Myb (Zhu *et al.* 2004). While E2F directly interacts with the cdc2 promoter, Myb-orchestrated cdc2 expression is dependent on the presence of a functional E2F binding site within the Myb promoter. Since Myb is an E2F target expressed at G1/S, the regulation of cdc2 expression by Myb and E2F represents an example of G1/S-controlled transcription of a G2/M specific gene (Zhu *et al.* 2004). The presence of DREF sites in Dpit47 promoter as well as its interaction with G1/S phase- specific genes DNA polymerase α and possibly Cdc6, Orc2 and

Orc5 (Crevel *et al.* 2001, Chapter 2, Fig. 2.11), suggests that it may function as a G1/S rather than G2/M-specific E2F/Myb target.

The role of Myb in Dpit47 function is particularly intriguing considering its unique functional properties in *Drosophila* development. Vertebrate Mybs are a family of three closely related genes: C-Myb, A-Myb and B-Myb (Nomura *et al.* 1988, Turque *et al.* 1997). In contrast, *Drosophila* possesses a single Myb gene which is most closely related to the vertebrate C-Myb (Fitzpatrick *et al.* 2002, Katzen and Bishop 1996). Furthermore, *Drosophila* Myb (*Dm* Myb) is also able to induce vertebrate Myb-specific reporter construct activation (Fitzpatrick *et al.* 2002). Despite being highly related to vertebrate family of Myb genes *Dm* Myb evolved specific functions to match the highly specialized requirements in *Drosophila* development. Namely, *Dm* Myb is expressed in all mitotically active tissues but absent from larval polyploid tissues that undergo endoreplication (eg. salivary glands, Fitzpatrick *et al.* 2002, Katzen and Bishop 1996). Consistent with this, ectopic expression of *Dm* Myb in polyploid endocycling cells, suppresses endoreplication through a mechanism that inhibits S phase-dependent DNA synthesis (Fitzpatrick *et al.* 2002). In contrast, in mitotic diploid cells, *Dm* Myb in concert with E2F and DREF, induces proliferation by promoting S phase progression (Fitzpatrick *et al.* 2002). Several conclusions can be made from these observations, first, *Dm* Myb itself is not required for DNA replication in *Drosophila*, given that polyploid tissues undergo DNA replication in its absence. Instead of *Dm* Myb, G1/S progression of endocycling cells is promoted by E2F and DREF (Fitzpatrick *et al.* 2002, Hirose *et al.* 1999). Second, in mitotic cells, the ability of *Dm* Myb to suppress endoreplication is utilized to ensure DNA replication occurs only once per cell cycle and thus, represents a mechanism of maintaining genomic stability (Fitzpatrick *et al.* 2002). Finally, the synergistic aspect of regulation of *Dm* Myb/E2F/DREF in mitotic cells and the presence of their respective binding sites in Dpit47 promoter provides a strong link between Dpit47 function and G1/S progression.

Genomic instability resulting from deregulation of the cell cycle is a significant contributing factor in cancer development (Classon and Harlow 2002, Fung *et al.* 2003, Loop *et al.* 2004, Manak *et al.* 2002, Shepard *et al.* 2005, Sivaprasad *et al.* 2007). Not surprisingly, mutations affecting genes that participate in DNA replication, origin assembly and signalling cascades related to cell cycle progression have often been linked with molecular genesis of tumors (Loop *et al.* 2004, Kim *et al.* 2005,

Shepard *et al.* 2005, Sivaprasad *et al.* 2007). Extensive investigations based on correlating specific tumor phenotypes with altered gene expression profiles revealed a remarkably high number of genes potentially involved in tumorigenesis (Loop *et al.* 2004, Kim *et al.* 2005). In that context, the results of a study on transcriptional profiling of brat^{k06028} - associated brain tumor in *Drosophila*, extend the role of Dpit47 as a *Dm* Myb target with a potentially oncogenic role in *Drosophila* development. Brat is a tumor suppressor involved in translation repression, ribosomal biogenesis and regulation of cell growth. It is a member of a highly conserved family of proteins that have been implicated in tumorigenesis both in flies and humans (Arama *et al.* 2000, Jensen *et al.* 2001, Loop *et al.* 2004, Torok and Etkin 2001). Suppression of brat function in flies causes neoplastic overgrowth, metastasis and lethality in *Drosophila* larvae and pupae (Arama *et al.* 2000, Loop *et al.* 2004). Two independent extensive microarray analysis that compared the transcriptome of adult wildtype flies with flies harbouring a homozygous brat^{k06028} allele, identified 321 genes with significantly altered expression profiles associated with brat^{k06028} mutation. Markedly, one fifth of these genes were highly homologous to mammalian cancer-related genes (Loop *et al.* 2004). Not only were *Dm* Myb and Dpit47 identified in the brat^{k06028} transcriptome but also, both genes were upregulated to a strikingly similar extent between the two independent microarray analysis (3.03/2.27-fold vs 3.57/5.92-fold upregulation, Dpit47/ *Dm* Myb, 1st and 2nd experiment respectively). Similar to Kim *et al.*'s (2005), work, Loop *et al.*'s (2004), study provides invaluable data that is not only consistent with the requirement for oncogene activation and tumor supressor inactivation to cancer development but also points to the seemingly unexpected role of TTC4/Dpit47 in oncogenesis. While TTC4 activation may be involved in human lung cancers, Dpit47 upregulation in brat^{k06028} mutant flies implies a CNS-specific oncogenic function of Dpit47 in *Drosophila*. In further support of this hypothesis, Dpit47 mRNA was shown to be restricted to the CNS in stages 13-16 of *Drosophila* development (Chapter 2, Fig. 2.10) when CNS is the predominant proliferative tissue in *Drosophila* (Hao *et al.* 1995). Moreover, in these later stages, *Dm* Myb mRNA is also absent from most tissues with the exception of the CNS where *Dm* Myb is highly expressed (Katzen and Bishop 1996). Interestingly, of the cell cycle-related genes that were aberrantly expressed in brat^{k06028} tumor, many show CNS-specific expression (Fig. 4.2), and are consequently involved in pathological and non-pathological neuronal development (Loop *et al.* 2004, Ohnuma and Harris 2003). Thus, both *Dm*

Myb and Dpit47 may be added to the growing mosaic of genes that link neurogenesis with cell cycle control (Ohnuma and Harris 2003).

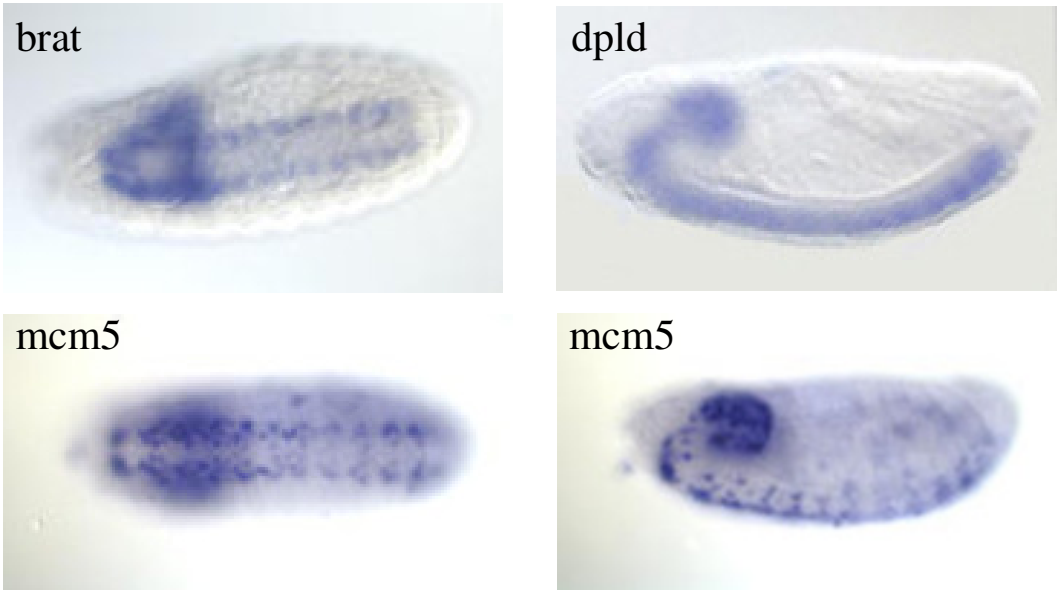

Fig. 4.2. CNS expression of genes involved in cell proliferation and cell cycle in *Drosophila* embryos. Images were retrieved from Berkeley Drosophila Genome Project (www.fruitfly.org/cgi-bin/ex/insitu.pl): brat (CG10719), dpld (CD1624) and mcm5 (CG4082). Stage 13-16 embryos are shown. In all cases, transcript expression is detected in the ventral nerve cord and embryonic brain.

Even though the key developmental factors and cell cycle genes that govern neurogenesis appear to be known, their interactions are complex and poorly understood (Ohnuma and Harris 2003). In that respect, the precise role of *Dm* Myb in *Drosophila* CNS is currently unclear (Katzen and Bishop 1996, Loop *et al.* 2004). Nevertheless, the present study has revealed that Dpit47 may function as a *Dm* Myb-transcriptional target during *Drosophila* CNS development and that consequently, aberrant *Dm* Myb/Dpit47 expression is likely to be a contributing factor in the pathogenesis of brat^{k06028} brain tumor.

Conclusively, both AmTPR1 and Dpit47 appear to be developmental genes and putative proto-oncogenes. Intriguingly, the regulatory aspect of gene expression seems to differ between the two invertebrate TTC4 homologs. While AmTPR1 expression may be linked to the canonical Wnt/β-catenin pathway, Dpit47 expression

may depend on synergistic actions of *Dm* Myb, E2F and DREF. The possible reason for this divergence may be in the fact that, even though highly conserved, mechanisms of cell cycle regulation have unique components related to tissue specificity. An example of this was already mentioned in the context of *Dm* Myb function in *Drosophila* endocycling and mitotic cells. Another interesting example is the cell-type dependent regulation of cyclin D1 expression by the Wnt pathway in mammalian cells. In HCT116 colon cancer cells Pitx2 (a bicoid-related homeodomain factor), in concert with LEF, represses the cyclin D1 promoter while in C2C12 myoblasts, cyclin D1 promoter repression is mediated only by Pitx2. In both cell lines, the repressive actions of Pitx2/LEF and Pitx2 respectively, are relieved by activation of β-catenin (Baek *et al.* 2003).

Taking into consideration the above given examples and the morphological distance between flies and corals, it seems plausible that AmTPR1 and Dpit47, in addition to having common roles, have also evolved lineage-specific roles and are therefore subjected to different regulatory mechanisms. This principle may extend to the human TTC4 gene due to its functional association with lung cancer and melanoma, as opposed to Dpit47 association with brat^{k06028} brain tumor. Accordingly, the mechanisms that regulate TTC4 expression may have mammalian-specific components.

A role for Wnt/β-catenin pathway in regulating Dpit47 expression cannot be precluded based on the lack of TCF/LEF binding sites in Dpit47 promoter, given that β-catenin can associate with other transcription factors to control gene expression. For example, β-catenin interacts with E2F-4 and the associated p130/HDAC1 co-repressor complex to regulate the expression of c-Myc in C2C12 cells (Baek *et al.* 2003). It is further suggested by Baek *et al.* (2003), that a subset of G1 phase-related genes, including c-Myc, cyclin D1 and cyclin D2 is synergistically repressed by E2F-4, Pitx2 and LEF and that this repression is relieved in response to Wnt/β-catenin activation. These findings raise the question whether a similar β-catenin/E2F - dependent mechanism of cell cycle gene regulation exists in *Drosophila*. In that respect, it would be of special interest to investigate whether Dpit47 expression can be affected by the *Drosophila* Wnt (Wingless) pathway.

Another open question that is implied from Baek *et al.*'s study (2003), concerns the complex nature of expressional regulation by the members of the E2F family and thus, the precise role of E2F in Dpit47 regulation. In mammals, six members of the

E2F family have been identified thus far: E2F1, E2F2 ,E2F3, E2F4, E2F5 and E2F6 (Baek *et al.* 2003, Muller and Helin 2000, Muller *et al.* 2001, Sawado *et al.* 1998). Based on their structure, affinity for the pocket proteins (pRb, p107 and p130), and functional properties, E2Fs can be divided in three groups. First group includes E2F1-E2F3, which exclusively associate with pRb and act as transcriptional activators that regulate the expression of genes involved in G1/S progression and DNA replication. Consistently, E2F1-E2F3 are expressed in proliferating cells and not in quiescent cells (Muller and Helin 2000, Muller *et al.* 2001, Sawado *et al.* 1998, Zhu *et al.* 2004). In contrast, members of the second E2F group, E2F4 and E2F5, even though expressed throughout the cell cycle, are not capable of inducing S-phase genes, instead, these E2Fs act as transcriptional repressors in quiescent cells (Baek *et al.* 2003, Muller and Helin 2000, Muller *et al.* 2001). The repressive actions of E2F4 and E2F5 are mediated by their specific association with pocket proteins p107 and p130 (Baek *et al.* 2003, Muller and Helin 2000, Muller *et al.* 2001, Sawado *et al.* 1998). The function of the third E2F group member E2F6 is less clear at present. E2F6 does not associate with pocket proteins, however, like E2F4 and E2F5, it appears to act as a repressor of transcription (Muller and Helin 2000, Muller *et al.* 2001, Sawado *et al.* 1998). In *Drosophila*, only two E2F-like proteins have been identified to date: dE2F-1 and dE2F-2. While dE2F-1 represents the fly counterpart of the first group of mammalian E2Fs, dE2F-2 is more similar to the second group (Beall *et al.* 2004, Hao *et al.* 1995, Lewis *et al.* 2004, Ohtani and Nevins 1994, Sawado *et al.* 1998). Thus, the two *Drosophila* E2Fs have opposing functions, one is a transcriptional activator (dE2F-1), and the other is a transcriptional repressor (dE2F-2). Both *Drosophila* E2Fs bind to a sequence that is closely homologous to the mammalian E2F binding consensus (Ohtani and Nevins 1994). In *Drosophila* PCNA promoter, there are three putative E2F recognition sites (I, II and III). Distinctly, there is an overlap in site binding specificity between the two *Drosophila* E2Fs so that site I even though preferentially occupied by E2F-1 also binds E2F-2. Site II seems to be exclusively bound by E2F-2 and in addition to site I, it is required for E2F-2 – mediated repression of the PCNA promoter (Sawado *et al.* 1998). Considering that 1) the two functionally distinct *Drosophila* E2Fs bind to the same site in the PCNA promoter, 2) both *Drosophila* E2F-1 and E2F-2 are expressed in proliferating cells and 3) like in mammals *Drosophila* E2F-1 is not expressed in quiescent cells as opposed to E2F-2 (Hao *et al.* 1995, Sawado *et al.* 1998), indicates that the lack of PCNA expression in quiescent

cells is mainly brought by the repressive action of E2F-2, while in proliferating cells, E2F-1 outcompetes E2F-2 at site I, thus activating PCNA transcription. Analogous to PCNA regulation, Dpit47 may be regulated at the E2F-1/E2F-2 level from a single E2F binding site so that it becomes expressed in proliferating cells in response to E2F-1 whilst being absent from quiescent cells due to E2F-2 – mediated repression. Following observations agree with this hypothesis: 1) Dpit47 interacting partner DNA polymerase α is itself an E2F-target gene (Ohtani and Nevins 1994), 2) both genes show proliferation-restricted expression pattern (Crevel *et al.* 2001, Hirose *et al.* 1991), and consistently, the interaction of Dpit47/DNA polymerase α is only observed in proliferating cells (Crevel *et al.* 2001).

4.6. What lies between K^+ channel activation and cell cycle regulation: mouse TTC4 gene ?

The role of K^+ channels in regulating cell proliferation is a well documented area of research. It is well known that K^+ channel activity positively influences cell proliferation via a mechanism that involves Ca^{2+} signalling and changes in membrane potential. Specifically, activation of K^+ channels causes hyperpolarization of the membrane potential and increases the driving force for Ca^{2+} entry, a condition required for G1/S progression (Ghiani *et al.* 1999, Kahl and Means 2003, Lang *et al.* 2005, MacFarlane and Sontheimer 2000, Santella 1998, Wang 2004, Wonderlin and Strobl 1996). Consistent with this, K^+ channel openers cause hyperpolarization of the membrane potential and promote cell proliferation whereas K^+ channel blockers depolarize the membrane potential and attenuate cell proliferation in both, tumor and non-tumor derived cells (Basrai *et al.* 2002, Coiret *et al.* 2007, Ghiani *et al.* 1999, Huang and Rane 1994, Jensen *et al.* 1999, Pardo 2004, Wonderlin and Strobl 1996). Accordingly, proliferation-inducing stimuli exerted by growth factors, serum and mitogens strongly correlate with activation of K^+ channels (Guo *et al.* 2005, Huang and Rane 1994, Kodal *et al.* 2000, Lang *et al.* 2005, Xu *et al.* 1999). The mitogenic properties of K^+ channels are of particular relevance in terms of understanding both non-pathological and pathological cell proliferation. The latter aspect is an area of extensive research given that in different tumors selective upregulation of K^+ channels is observed as a common mechanism by which abnormal cell proliferation is achieved and maintained (Bianchi *et al.* 1998, Pillozzi *et al.* 2002, Wang *et al.* 2002). K^+

channels are perhaps the most functionally diverse of all ion channels (Pardo 2004), and even though some K^+ currents are known to be associated with more than one cancer, others currents are restricted to a specific type of cancer (Table 4.1). An example of the former is that of the human EAG-related family K^+ currents, which are specifically expressed in a large number of tumor-derived cells, including myeloid leukemia, neuroblastoma and breast cancers but not in their non-tumor derived counterparts (Bianchi *et al.* 1998, Pillozzi *et al.* 2002, Wang *et al.* 2002). In contrast, the inward rectifier K^+ current I_{Kir} has been specifically linked to melanoma (Lepple-Wienhues 1996).

Table 4.1. Distinct K^+ currents involved in regulating tumor cell proliferation. Adapted from Wang 2004.

Type of K^+ current	*Symbol*	*Tumor type*
Ca^{2+} - activated	**$I_{K,Ca}$**	***Glioma, pituitary***
Voltage-gated (Shaker type)	**I_K**	***Neuroblastoma, breast carcinoma, small lung cell carcinoma, prostate cancer, colon cancer, melanoma, lymphoma, hepatocarcinoma***
EAG family of K^+ currents	**I_{EAG}**	***Cervix carcinoma (HeLa),***
neuroblastoma		***(SH-SY5Y), mammary gland carcinoma***
	I_{HERG}	***Myeloid leukaemia, neuroblastoma,***
atrial		***tumor (HL-1), breast cancer (SK-BR-3)***
Inward rectifier	**I_{Kir}**	***Melanoma (SK-MEL-28)***
ATP-sensitive	**$I_{K,ATP}$**	***Cancerous liver epithelia (HepG2,***
HuH-7		***and HFL)***

The inherent complexity of the processes that lie between K^+ channel activation and cell proliferation and the functional diversity of K^+ channels (Pardo 2004, Wang 2004), emphasize the necessity for elucidating the components downstream of K^+ channel activation, so the mechanisms by which K^+ currents contribute to tumor development can be better understood. Noteworthy, factors that positively regulate cell proliferation-dependent K^+ channel activity, have also been shown to positively regulate the cell proliferation-related Hsp70/Hsp90 chaperone activity. Although not necessarily causal, the link between the Hsp70/Hsp90 pathway and K^+ channel-mediated control of cell proliferation has substantial data in support: as with K^+ channels, 1) the expression of Hsp70 and Hsp90 is markedly increased upon growth factor, serum and mitogen stimulation (Hansen *et al.* 1991, Helmbrecht and Rensing 1999, Wu and Morimoto 1985), 2) levels of Hsps are higher in proliferating than in differentiated or quiescent cells (Helmbrecht and Rensing 1999, Hensold and Houseman 1988), 3) tumor cells often show higher expression of Hsp70 and Hsp90 than non-tumor cells (Ferrarini *et al.* 1992, Isomoto *et al.* 2003), 4) Hsp90 expression is particularly high during the G1/S transition of the cell cycle (Jerome *et al.* 1993). Furthermore, in tumor cells, Hsp90 and Hsp70 expression can be directly affected by K^+ channel blockers and the synthesis of both heat shock proteins is Ca^{2+} - dependent. Specifically, treatment of human cervix carcinoma cell line HeLa and the ovarian carcinoma cells A2780 with tetraethylammonium (TEA) or tetrandrine (Tet), selective blockers of large-conductance Ca^{2+}-activated K^+ channels (BK channels), inhibits proliferation and increases cell apoptosis (Han *et al.* 2007). These changes are accompanied with an increased expression of pro-apoptotic proteins (p53, p21 and Bax), and a decreased expression of Hsp70 and Hsp90 (Han *et al.* 2007). Furthermore, in human breast cancer MDA-MB-231 cells, the levels of both Hsp70 and Hsp90 are attenuated in response to removal of either extracellular or intracellular Ca^{2+} by chelating agents EGTA and BAPTA respectively (Kiang *et al.* 2000). In particular, lack of Ca^{2+} negatively affected the translocation of the heat shock transcription factor 1 (HSF1) to the nucleus, thereby reducing its activating potential on Hsp transcription (Kiang *et al.* 2000).

A link between K^+ channels and Hsp70/Hsp90 function in relation to cell proliferation seems improbable in the absence of a downstream effector gene. However, there is a gene that seems to fit the required profile remarkably well: the mouse TTC4, a putative Hsp70/Hsp90 interactor and a gene whose expression not only positively

correlates with activation of potassium channels but also with cell proliferation. Specifically, 1) the expression of the mouse TTC4 gene in neuroblastoma N2A cell line is downregulated in response to depolarizing stimuli and treatment with K^+ channel blocker TEA (Chapter 3, Fig. 3.2 and 3.7), an effect which can be attenuated by the K^+ channel opener mallotoxin and mitogen bradykinin (Chapter 3, Fig. 3.9-3.12), 2) the mechanism by which K^+ channel activity regulates the expression of TTC4 is dependent on Ca^{2+} (Chapter 3, Fig. 3.4), 3) mouse TTC4 expression is cell-cycle dependent and higher in proliferating cells that in quiescent N2A cells (Chapter 3, Fig. 3.15), 4) the greatest extent of TTC4 upregulation is observed at the G1/S transition of the cell cycle (Chapter 3, Fig. 3.16).

Thus far, Hsp90 interaction with the human TTC4 hasn't been verified. However, it is likely that TTC4 interacts with Hsp90 given that its two invertebrate counterparts AmTPR1 and Dpit47 are Hsp90 interactors and that the TPR domains of all three homologs feature a 100% match to the Hsp90 binding consensus (Chapter 2, Fig. 2.15). Furthermore, the implied involvement of the mouse TTC4 gene in cell proliferation suggests that the vertebrate and the non-vertebrate TTC4-like genes are functionally similar. This raises the following question: are there any themes in common to the regulation of expression of the mouse TTC4 and AmTPR1 or Dpit47 ? It was shown that in mouse neuroblastoma N2A cells, treatment with the GSK- 3 inhibitors kenpaullone or LiCl had no effect on TTC4 expression (Chapter 3, Fig. 3.1). Thus, unlike AmTPR1, the expression of the mouse TTC4 appears to be independent of the canonical Wnt/β-catenin pathway, instead it may depend on K^+ channel-associated Ca^{2+} signalling. Changes in membrane potential brought upon K^+ channel activation cause oscillations in intracellular Ca^{2+} levels. Ca^{2+} can signal through numerous cascades to trigger gene transcription, however, the specificity of the transcriptional responses is strictly dependent on the route of Ca^{2+} entry and the duration of the Ca^{2+} signal (Barlow *et al.* 2006, Berridge *et al.* 1998). In that view, two main mediators of Ca^{2+} – dependent transcription are 1) Ras/MEK/ERK-directed transcription factors c-Fos and c-Jun, and 2) nuclear factor of activated T cells (NFAT) transcription factor. Both c-Fos/c-Jun and NFAT pathways function ubiquitously in development (Bossis *et al.* 2005, Buchholz *et al.* 2006, Caetano *et al.* 2002, Graef *et al.* 2003, Hogan *et al.* 2003, Neal and Clipstone 2003, Shaulian and Karin 2002). NFAT members in particular are involved in cell cycle regulation, cell differentiation, cell migration, apoptosis and angiogenesis (Caetano *et al.* 2002,

Horsley and Pavlath 2002, Pu *et al.* 2003, Wang *et al.* 2006, Yao *et al.* 2007, Zaichuk *et al.* 2004). In line with this, NFAT-mediated pathways have been implicated in malignant cell transformation, tumor progression and metastasis (Haitian and Chuanshu 2007, Jauliac *et al.* 2002, Neal and Clipstone 2003, Viola *et al.* 2005, Yiu and Toker 2006). Moreover, given the extensive role of NFAT in tumorigensis, recent efforts are focused towards NFAT-targeted chemoprevention (Haitian and Chuanshu 2007).

Analysis of a 2 kb region upstream of the ATG codon in the mouse and human TTC4 promoter revealed several putative NFAT binding sites (Fig. 4.3). Taken together, the dependence of mouse TTC4 gene expression on K^+ channel activity and Ca^{2+} signalling and the presence of Ca^{2+} - dependent NFAT transcription factor binding sites in the mouse TTC4 promoter, indicates that NFAT may be a an important transcriptional regulator of the mouse TTC4 gene. Conclusively, considering the involvement of NFAT in tumorigenesis, this finding also supports a putative oncogenic role of TTC4 .

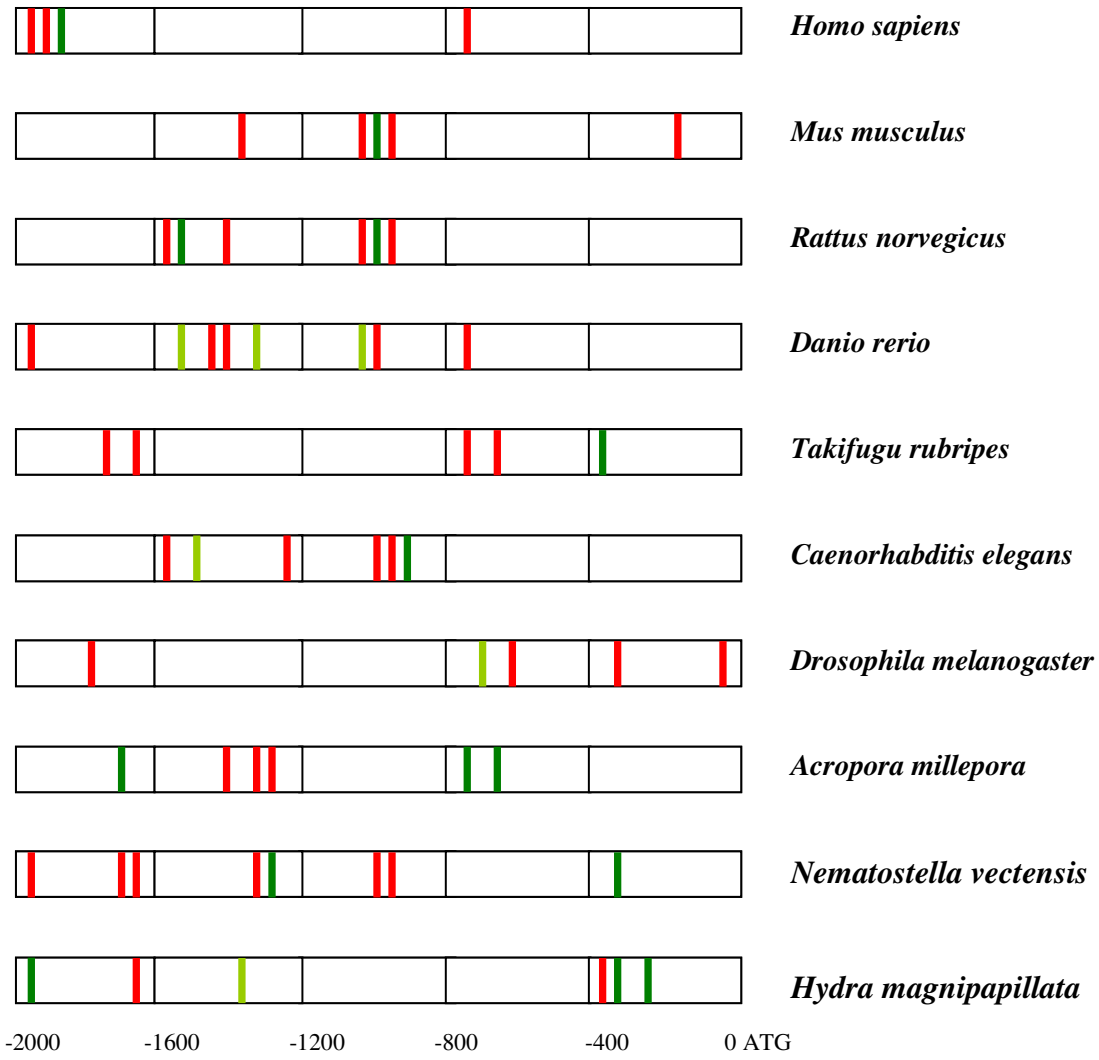

Fig. 4.3. Location of NFAT and TCF/LEF binding sites in the 2000 bp sequence upstream of the ATG codon in TTC4 homologs. NFAT binding sites (GGAAA) are indicated in red and TCF/LEF sites in dark green (sequences matching 100 % the core TCF/LEF-binding consensus A/T A/T CAAAG and CTTTG A/T A/T), and light green (sequences diverging in the terminal A/T nucleotide from the consensus). The promoter sequences were retrieved from the ENSEMBL genome browser database (www.ensembl.org): *H.sapiens* (ENSG00000184313), *M.musculus* (OTTMUSG00000008164), *R.norvegicus* (ENSRNOG00000022624), *D.rerio* (ENSDARG00000044405), *T.rubripes* (SINFRUG00000147013), *C.elegans* C17G10.2 (C17G10.2) and *D.melanogaster* Dpit47 (CG3189); JGI genome database (http://genome.jgi-psf.org/Nemve1/Nemve1.home.html): *N. vectensis* (CL3824); Metazome Hydra genome browser database (http://hydrazome.metazome.net/cgi-bin/gbrowse/hydra/): *H.magnipapillata* (Contig 37986). NFAT and TCF/LEF binding sites were identified by manual inspection.

If TTC4 does indeed function as a proto-oncogene, can this be reconciled with the tumor suppressor role implied by previous studies ? Is there any evidence against TTC4 being an oncogene rather than a tumor suppressor ? As already mentioned, loss of heterozygosity (LOH) on 1p31.1 has been associated with breast cancer pathogenesis (Su *et al.* 1999, Hey *et al.* 2000). However, it is unlikely that loss of TTC4 function plays a role in breast cancer as suggested by Su *et al.* (1999), given that TTC4 locus was subsequently reassigned to a different region on 1p31 (Hey *et al.* 2000). Furthermore, TTC4 exclusion from an interval at 1p31.1 is also implied from Kim *et al.*'s (2005) study. Therein, TTC4 was identified in a region of minimal chromosomal gain (MAR-G) on 1p32.2 (Kim *et al.* 2005). Another study seemingly supporting the tumor suppressor role of TTC4 was conducted by Poetch *et al.* (2000), who found point mutations in TTC4 gene in melanoma.. Nevertheless, their data could be interpreted differently, since mutation per se does not imply loss of function. For example, a serine to phenylalanine substitution at codon 37 of β-catenin (S37F), renders the protein resistant to GSK-3 – mediated degradation and therefore, results in a constitutively active form of β-catenin (Liu *et al.* 2002, Patel *et al.* 2004, Rubinfeld *et al.* 1997). In the case of TTC4, the most frequently occurring mutation in melanoma identified by Poetch *et al.* (2000), was a substitution of a polar glutamate residue (E) to hydrophobic alanine (A), affecting codon 77 in the neighbourhood of the first TPR motif. Poetch *et al.* (2000), interpreted this as a mutation that would result in a "reduction or loss of protein function". However, since there is no functional data to support such a hypothesis, equally it could be concluded that E → A may be a gain of function-like mutation that ultimately results in inappropriate activation of the TTC4.

Moreover, implying the proto-oncogenic function of TTC4 is also the possible involvement of c-Myc in regulating human TTC4 gene expression, as suggested by Li *et al.* (2003). c-Myc is one of the most fundamental regulators of cell proliferation, differentiation and malignant transformation associated with carcinogenesis. Consistently, overexpression of c-Myc has been associated with as much as 30 % of all human cancers (Levens 2003, Loop *et al.* 2004). Notably, in pancreatic cancer cells, the expression of c-Myc is positively regulated by NFAT (Buchholz *et al.* 2006), while in colorectal cancer cells, c-Myc acts a central downstream component of the Wnt/β-catenin pathway in maintaining the proliferative state of the cells (Muncan *et al.* 2006). Thus, depending on the histological background, c-Myc activity

can be regulated by different signalling pathways and transcription factors. Surprisingly, a search for the c-Myc binding consensus CACGTG (Levens 2007, Li *et al.* 2003), in a 2 kb region of both human and mouse TTC4 promoters revealed no putative c-Myc binding sites. However, a single CACGTG site was found in the first intron of the human TTC4 gene (see Appendix D). Since DNA microarrays used in Li *et al.*'s (2003) experiment covered genomic regions from 650 bp upstream to 250 bp downstream of the transcription start, it is possible that this c-Myc intronic site corresponds to the one identified to be bound by c-Myc by Li *et al.* (2003), given that its location in the first intron falls within the described DNA microarray interval. The absence of c-Myc binding sites from the mouse TTC4 promoter sequence implies that there are differences in expressional regulation between the two, at the protein level 98 % homologous mouse and human TTC4 genes. While the expression of the human TTC4 gene may depend on the synergistic action of either NFAT and c-Myc or Wnt/β-catenin and c-Myc, the expression of the mouse TTC4 may solely depend on NFAT.

Conclusively, like its invertebrate counterparts AmTPR1 and Dpit47, the mouse TTC4 appears to be a developmental gene, important for cell proliferation. It is likely to function as a proto-oncogene and it is characterized by its own unique mode of transcriptional regulation. Nevertheless, the promoters of the human, mouse, rat, zebrafish, pufferfish, worm, fly and coral TTC4-like genes do have some common characteristics. Not only do all of these promoters contain multiple NFAT binding sites but also a subset of these have TCF/LEF binding sites in close proximity to the NFAT sites (Fig. 4.3). What is the significance of these observations ? First, Wnt signalling can directly influence NFAT activity through a Ca^{2+} - dependent mechanism (Dejmek *et al.* 2006, Seneyoshi *et al.* 2002, Veeman *et al.* 2003). This pathway, known as a Wnt-5a/Ca^{2+} pathway, plays a role in the pathogenesis of breast cancer and is also involved in dorsoventral patterning (Dejmek *et al.* 2006, Seneyoshi *et al.* 2002). Second, Wnt-5a/Ca^{2+} is different from the canonical Wnt/β-catenin pathway. In particular, Wnt-5a/Ca^{2+} is not subjected to GSK -3 regulation and it does not operate through the β-catenin - mediated TCF/LEF transcription (Kuhl *et al.* 2000, Veeman *et al.* 2003). However, Wnt-5a/Ca^{2+} can inhibit the canonical Wnt/β-catenin signalling and the antagonistic cross-talk between the two pathways controls a number developmental processes (Topol *et al.* 2003, Westfall *et al.* 2003). This

antagonism and the known involvement of hyperactive Wnt/β-catenin signalling in cancer development, suggests that Wnt-5a/Ca^{2+} may normally counter the tumor promoting activities of Wnt/β-catenin (Olson *et al.* 1998, Topol *et al.* 2003). However, there are also instances where Wnt-5a/Ca^{2+} appears to promote the severity of the tumor. For example, the invasive behaviour of metastatic melanoma has been linked with Wnt-5a/Ca^{2+} (Weeraratna *et al.* 2002). Furthermore, Wnt-5a/Ca^{2+} activation of NFAT has also been linked with breast cancer metastasis. Interestingly, in this case the tumor promoting activity of Wnt-5a/Ca^{2+}/NFAT can be simultaneously counteracted by another noncanonical Wnt pathway, the Wnt-5a/Yes-Cdc42-casein kinase I pathway (Dejmek *et al.* 2006)

4.7. Concluding remarks

The regulation of developmental pathways is intricate, typically featuring large numbers of components organized in discrete signalling networks. If there is a cross talk between two developmental pathways the level of complexity increases and so does the level of regulation. In respect of transcriptional regulation of TTC4-like genes, additional levels of complexity may have been achieved in the cases of the fly Dpit47, mouse and human TTC4 genes beyond that seen in the coral AmTPR1 gene, given the more "ancestral" nature of the coral. While AmTPR1 expression appears to be directly regulated by the canonical Wnt/β-catenin pathway, it is possible that the expression of Dpit47 and TTC4 depends on putative downstream effectors of Wnt signalling, E2F and NFAT respectively. These latter may differ in that Dpit47 regulation may be mediated by the canonical Wnt/β-catenin pathway, whereas mouse TTC4 expression may be controlled by the non-canonical Wnt-5a/Ca^{2+} pathway. In the case of the human TTC4 gene, the complexity may have been increased even further by integration of the two opposing Wnt signalling pathways, the Wnt/β-catenin/c-Myc and the Wnt-5a/Ca^{2+}/NFAT pathway, in controlling gene expression. While the two pathways may be acting in concert to regulate the expression of the human TTC4 gene, they may also act in dependence of specific developmental backgrounds.

4.8. REFERENCES

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APPENDIX A

PROPERTIES OF *S. CEREVISIAE* STRAIN EGY48(pSH18-34) AND PLASMID VECTORS

***S. cerevisiae* strain**	**Description**	**Reporters**	**Application**	**Manufacturer**
EGY48 (pSH18-34)	MATα, trp1, his1, ura3, leu2::6LexAop-LEU2	LacZ, LEU2	host for two-hybrid vectors	OriGene Technologies Inc.

***** In EGY48 strain the LEU2 promoter is integrated into the genome, while the LacZ reporter resides on the pSH18-34 vector (see table below)

***S. cerevisiae* plasmid vectors**	**Description**	**Application**	**Manufacturer**
pEG202 (10.2kb)	LexA-DBD, HIS3, amp^R	Yeast two-hybrid bait vector	OriGene Technologies Inc.
pJG4-5 (6.5kb)	B42-AD, TRP1, amp^R	Yeast two-hybrid prey vector	OriGene Technologies Inc.
pSH18-34* (1.1kb)	8 ops.-lacZ, URA3, amp^R	Yeast two-hybrid LacZ reporter vector	OriGene Technologies Inc

***** high sensitivity LacZ reporter plasmid

Note that, in addition to amp^R, all yeast plasmid vectors contain and *E. coli* origin of replication, thereby allowing propagation and selection in a suitable *E. coli* strain (DupLex-A^{TM} Yeast Two-Hybrid System User Manual 1998). This procedure was carried out to obtain a sufficient amount of plasmid template required either for construct preparation, or subsequent yeast transformations of selected constructs (Chapter 2, Materials and Methods).

SELECTION OF EGY48(pSH18-34) TRANSFORMANTS ON SD DROPOUT MEDIUM

***S. cerevisiae* strain**	**Plasmid vectors**	**Selection on SD dropout**
EGY48 $Trp^-His^-Leu^-Ura^-$	pEG202 His+ pJG4-5 Trp+ pSH18-34 Ura+	His, Trp, Ura

APPENDIX B

TRANSCRIPTION FACTOR BINDING SITES WITHNIN PROMOTER SEQUENCES OF AmTPR1 AND RELATED GENES

DATA APPENDICES HAVE BEEN REMOVED

APPENDIX C

TFSEARCH SOFTWARE RESULTS OF DPIT47 PROMOTER

DATA APPENDICES HAVE BEEN REMOVED

APPENDIX D

ENSEMBL EXON REPORT- TRANSCRIPT HUMAN TTC-001

DATA APPENDICES HAVE BEEN REMOVED