

# **Molecular Mechanism of mTOR downstream signaling**

Im Fachbereich Biologie, Chemie, Pharmazie  
der Freien Universitaet Berlin eingereichte  
Dissertation

vorgelegt von  
Stefanie Schalm

Boston, 2003

**Gutachter:**

**Prof. Dr. Ferdinand Hucho**

Freie Universitaet Berlin  
Fachbereich Biologie, Chemie, Pharmazie  
Institut fuer Chemie / Biochemie  
Thieleallee 63, 14195 Berlin

**Prof. Dr. John Blenis**

Harvard Medical School  
Department of Cell Biology  
240 Longwood Avenue  
BLDG: LHRRB  
Boston, 02115 MA  
USA

Datum der Disputation: 17.09.2003

<b>CONTENTS</b>	<b>III</b>
<b>ABSTRACT</b>	<b>VIII</b>
<b>ZUSAMMENFASSUNG</b>	<b>X</b>

<b>1</b>	<b>INTRODUCTION AND AIMS</b>	
<b>1.1</b>	<b>Cancer and Cell growth</b>	<b>1</b>
<b>1.2</b>	<b>Identification of TOR</b>	<b>1</b>
<b>1.3</b>	<b>Regulation of cell growth by TOR signaling</b>	<b>3</b>
<b>1.4</b>	<b>Regulation of mTOR</b>	<b>4</b>
1.4.1	mTOR is a conserved nutrient sensor	4
1.4.2	mTOR senses the energy status	6
1.4.3	Mitogen-dependent mTOR regulation	6
<b>1.5</b>	<b>m TOR downstream signaling</b>	<b>7</b>
1.5.1	Regulation of Cap-dependent translation by 4E-BPs	7
1.5.2	S6K1 signaling	9
1.5.3	Other actions of mTOR signaling	10
<b>1.6</b>	<b>Coordination of mTOR and PI3K-dependent signaling</b>	<b>11</b>
1.6.1	PI3K signaling	11
1.6.2	Regulation of mTOR signaling by the Tsc1/Tsc2 complex	12
1.6.3	Mechanisms of S6K1 and 4E-BP1 regulation	14
1.6.3.1	Regulation of 4E-BPs	14
1.6.3.2	Regulation of S6K1	17
1.6.3.3	Regulation of S6K2	21
<b>1.7</b>	<b>TOR regulation of phosphatases</b>	<b>22</b>
<b>1.8</b>	<b>Limitations in the study of TOR signaling</b>	<b>24</b>
<b>1.9</b>	<b>Identification of TOR binding proteins</b>	<b>25</b>
<b>1.10</b>	<b>Aims of current work</b>	<b>29</b>
<b>2</b>	<b>RESULTS</b>	
<b>2.1</b>	<b>Identification of the TOS motif in S6K1</b>	<b>30</b>

2.1.1	Identification of a conserved region in the N-terminus of S6K1 that is essential for its activation	30
2.1.2	The F5A mutation inhibits phosphorylation of S6K1 at Thr389 and Thr229	32
2.1.3	The F5A point mutation mimics deletion of N-terminus	34
2.1.4	Mutation of phosphorylation sites in S6K1 to acidic residues partially rescues the activity of F5A mutant	36
2.1.5	Deletion of the C-terminus and Thr389Glu substitution rescues F5A activity completely	38
2.1.6	4E-BP1 phosphorylation is inhibited by overexpression of S6K1 with an intact TOS motif	40
2.1.7	The S6K1 F5A mutation prevents amino acid signaling via mTOR towards S6K1	41
2.1.8	Characterization of the C-terminal inhibitory effect of S6K1	43
2.1.9	Ser411 phosphorylation does not affect rapamycin sensitivity	45
2.1.10	The inhibitory effect of the F5A mutation requires the RSPRR motif	46
2.1.11	RSPRR motif is not required for interaction with common mTOR activator	47
<b>2.2</b>	<b>Identification of a TOS motif in 4E-BP1</b>	
2.2.1	The TOS motif in 4E-BP1 is required for its phosphorylation	50
2.2.2	The TOS motif is required for 4E-BP1-raptor complex formation	53
2.2.3	TOS motif is required for efficient <i>in vitro</i> phosphorylation of 4E-BP1 by mTOR	56
2.2.4	Mutation of the TOS motif strengthens the binding of 4E-BP1 to eIF4E	59

2.2.5 Overexpression of 4E-BP1-F114A reduces cell size	60
<b>2.3 Regulation of S6K2</b>	
2.3.1 N- and C-terminus of S6K2 inhibit its activity	63
2.3.2 N-and C-terminal regulation of S6K1	64
<b>3 DISCUSSION</b>	
<b>3.1 Identification of the TOS motif</b>	<b>68</b>
3.1.1 Regulation of S6K1 phosphorylation by the TOS motif	69
3.1.2 Regulation of 4E-BP1 by the TOS motif	72
3.1.3 Conclusions	77
<b>3.2 Regulation of the mTOR target S6K2</b>	<b>78</b>
<b>4 MATERIALS AND METHODS</b>	
<b>4.1 Materials</b>	<b>80</b>
4.1.1 Chemicals	80
4.1.2 Antibodies	81
4.1.3 Primers	81
4.1.3.1 Primers for S6K1 mutants	
4.1.3.2 Primers for 4E-BP1 mutants	
4.1.3.3 Primers for S6K1/S6K2 chimeras	
4.1.4 Buffers and Solutions	84
4.1.5 Enzymes	90
4.1.6 Plasmids	90
4.1.7 Protein A Sepharose	90
4.1.8 Cell lines	91
<b>4.2 Methods</b>	<b>91</b>
<b>4.2.1 Molecular Biology</b>	<b>91</b>
4.2.1.1 DNA Preparation	91
4.2.1.2 DNA Electrophoresis	91

4.2.1.3	Generation of Competent Bacteria DH $\alpha$ 5 and BL21	91
4.2.1.4	Heat Shock Transformation	92
4.2.1.5	Site-directed PCR mutagenesis	92
4.2.1.6	Generation of S6K1 mutants	93
4.2.1.7	Generation of 4E-BP1 mutants	93
4.2.1.8	Generating of S6K1/S6K2 chimera	93
4.2.1.9	Sequencing of DNA	94
<b>4.2.2</b>	<b>Cell Culture and Biochemical Methods</b>	<b>94</b>
4.2.2.1	Calcium phosphate transfection	94
4.2.2.2	Fugene transfection	95
4.2.2.3	Freezing Cells	95
4.2.2.4	Thawing Cells	96
4.2.2.5	Cell Stimulation and Lysis	96
4.2.2.6	Amino Acid withdrawal	96
4.2.2.7	Flow cytometry	96
4.2.2.8	Immunoblots	97
4.2.2.9	Co-immunoprecipitation	98
4.2.2.10	Immune-complex kinase assays	98
4.2.2.11	m <sup>7</sup> GTP Cap-binding assays	99
<b>5</b>	<b>ABBREVIATIONS</b>	<b>101</b>
<b>6</b>	<b>REFERENCES</b>	<b>103</b>
<b>7</b>	<b>APPENDIX</b>	
<b>Acknowledgements</b>		<b>114</b>
<b>Curriculum vitae</b>		<b>115</b>
<b>List of Publications</b>		<b>117</b>