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Extension of *Drosophila melanogaster* life span with a GPCR peptide inhibitor

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G protein–coupled receptors (GPCRs) mediate signaling from extracellular ligands to intracellular signal transduction proteins[1]. Methuselah (Mth) is a class B (secretin-like) GPCR, a family typified by their large, ligand-binding, N-terminal extracellular domains[2]. Downregulation of *mth* increases the life span of *Drosophila melanogaster*[3]; inhibitors of Mth signaling should therefore enhance longevity. We used mRNA display selection[4,5] to identify high-affinity (K_d = 15 to 30 nM) peptide ligands that bind to the N-terminal ectodomain of Mth. The selected peptides are potent antagonists of Mth signaling, and structural studies suggest that they perturb the interface between the Mth ecto- and transmembrane domains. Flies constitutively expressing a Mth antagonist peptide have a robust life span extension, which suggests that the peptides inhibit Mth signaling *in vivo*. Our work thus provides new life span–extending ligands for a metazoan and a general approach for the design of modulators of this important class of GPCRs.

Because of their participation in numerous cell processes, GPCRs are the targets of approximately half of marketed drugs, and new GPCR ligands continue to be pursued and developed[6]. Naïve approaches toward GPCR ligand identification typically involve high-throughput screening of a molecular library (10^2 to 10^5 unique members) in functional, cell-based assays[6–8]. A powerful alternative approach for rapidly isolating new ligands is *in vitro* peptide selection using mRNA display, which allows access to very high library complexities ($>10^{13}$) in a robust format[4]. High-affinity peptide ligands for RNA, small-molecule, and protein targets have been identified by mRNA display selection[5].

The crystal structure of the hexahistidine-tagged Mth ectodomain shows that the mature, N-terminal extracellular domain of Mth is a stably folded, glycosylated protein of 195 residues[9]. Given that the ectodomains of other class B GPCRs maintain recognition of their cognate ligands independently of their transmembrane cores[10,11], we targeted the Mth ectodomain for *in vitro* selection to isolate putative modulators of Mth signaling. We expressed and purified a specifically biotinylated construct of the Mth ectodomain[12] to avoid using the weak hexahistidine epitope as an immobilization tag for selection[13]. Hence, the Mth ectodomain was homogeneously presented, perhaps mimicking the juxtaposition of the ectodomain and transmembrane domain in the context of the full-length receptor.

We constructed a random 27-mer peptide mRNA display library. After eight rounds of selection, with the final four rounds including preclearing steps on matrix without target and specific elution with free, nonbiotinylated Mth, we obtained a final eighth-round pool that showed high activity for Mth and negligible nonspecific binding (**Fig. 1**). DNA sequencing of individual clones from the final selection round revealed a highly conserved consensus—[R/P]XXWXXR—which we term the RWR motif (**Table 1**). This motif was not found in the recently identified Mth peptide agonist Stunted[14], and the shortness of the consensus precluded any statistically significant homology to the *D. melanogaster* proteome.

We synthesized several selected peptides for binding analysis by surface plasmon resonance (SPR). Peptides containing the RWR motif demonstrated high affinity (K_d < 30 nM) to the Mth ectodomain (**Table 1**). A scrambled version of the R8-12 synthetic peptide, as well

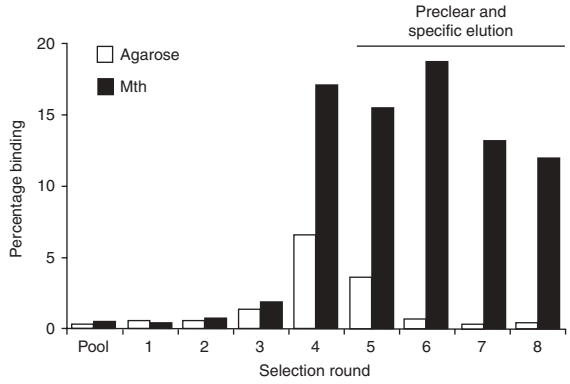

Figure 1 Selection of a 27-mer peptide library against the Mth ectodomain. RNase-treated, ^{35}S-methionine–labeled mRNA displayed peptides from each round of selection were assayed for binding to immobilized Mth (black) or to matrix alone (white). Preclearing and competitive elutions were performed in the fifth through eighth rounds to eliminate nonspecific binding peptides.

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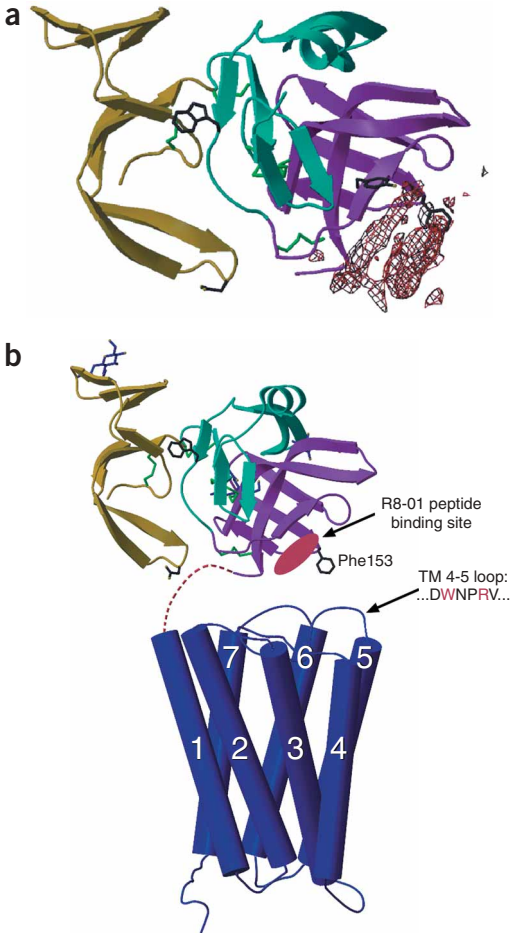

Figure 2 Structure of the Mth ectodomain in complex with the R8-01 15-mer peptide. (**a**) Electron density reveals the putative peptide binding site on the Mth ectodomain (shown as a ribbon diagram) from an averaged 3.5-Å $F_o - F_c$ map contoured at 9 σ. Trp120 (a previously proposed natural ligand binding site[9]), Tyr130 (at the R8-01 binding site), and Asp46 and Phe153 (suggested to interact with the extracellular face of the transmembrane domain[9]) are shown as stick models. (**b**) Scaled model depicting the full-length structure of Mth (adapted from ref. 9). The transmembrane domain is depicted by the structure of rhodopsin[30] as a representative GPCR. Data collection and refinement statistics are available in **Supplementary Table 2** online.

as W7A and R10A mutants of R8-14, showed no measurable binding by SPR at concentrations up to 5 μM. Additionally, a fluorescently labeled analog of R8-12 was shown to bind full-length Mth expressed in cell culture (**Supplementary Fig. 1** online). Hence, despite targeting the Mth ectodomain, selected peptides recognize the full-length GPCR.

It is not clear why arginine and proline should be interchangeable at the first consensus position of the RWR motif. Proline could provide a conformational anchor, whereas arginine makes favorable electrostatic contacts that ultimately result in two peptides with similar binding affinities to Mth. Analysis of the amino acid types at each position in the aligned peptides also reveals several trends that are dependent on whether arginine or proline is in the first position (**Supplementary Fig. 2** and **Supplementary Table 1** online).

In vitro competition binding studies suggest that the selected peptides share the same binding site. Synthetic, unlabeled peptide R8-01 (10 μM) competed with radiolabeled, full-length R8-01 and R8-04 for binding to immobilized Mth ectodomain, which resulted in 96% and 94% reductions in binding, respectively, compared with the amount bound without competitor. N-Stunted, a 30-mer synthetic peptide previously shown to activate the Mth receptor[14], also competed for binding to the Mth ectodomain: at 30 μM, N-Stunted reduced binding of radiolabeled R8-01 by 79% compared with binding without competitor. These results suggest that the natural ligand binding site is an interaction "hot spot"[15] and is at least partially reconstituted by the Mth ectodomain. Alternatively, allosteric competition may occur through Mth conformational changes upon ligand binding.

We determined the crystal structure of the Mth ectodomain in complex with an RWR motif peptide to identify the binding site. Electron density putatively corresponding to the R8-01 15-mer peptide (**Table 1**) places the binding site near the C terminus of the ectodomain (**Fig. 2a**). In the context of the full-length receptor, this suggests that the peptides bind at an interface between the Mth ectodomain and extracellular loops (**Fig. 2b**). These results contradict the hypothesis that the single exposed tryptophan residue in the Mth ectodomain is the binding site for the natural ligand[9]. Further fluorescence studies with Mth, R8-01 and R8-04 confirmed that Trp120 is not required for peptide binding to Mth (**Supplementary Fig. 3** online).

The second extracellular loop (EL2) of Mth contains a WXXR peptide sequence (a partial RWR motif) that may interact with the ectodomain in a similar fashion as the RWR motif peptides and form a distinct surface for agonist binding (**Fig. 2b**). However, at concentrations of up to 150 μM, a synthetic EL2 peptide did not compete with radiolabeled R8-01. This may not preclude an *in vivo* interaction, because the affinity may be enhanced in the full-length receptor, in which the EL2 sequence and the ectodomain are colocalized. Additionally, a high-affinity, optimized interaction may be undesirable

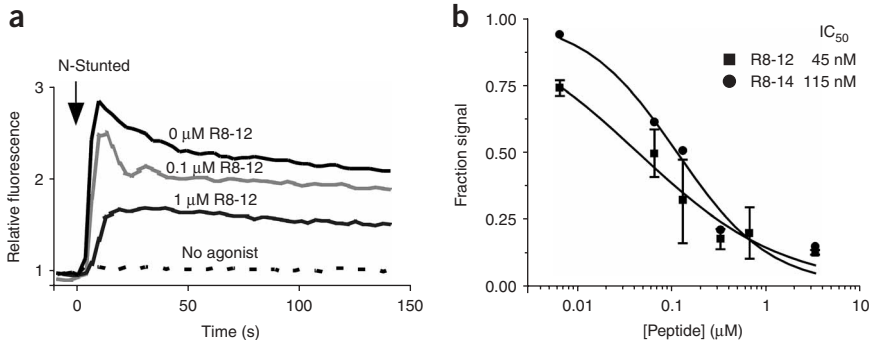

Figure 3 RWR motif peptides are antagonists of Mth signaling. (**a**) Mth activation by Stunted results in intracellular calcium mobilization and enhanced fluorescence. The N-Stunted agonist peptide (20 μM final) was added to HEK-Mth cells preincubated with and without the indicated concentration of R8-12 peptide. The dashed line indicates a control in which only buffer (without Stunted agonist) was added. (**b**) Concentration dependence of the inhibition of Mth signaling by the R8-12 and R8-14 peptides. The fluorescence values at a time point ~13 s after the addition of N-Stunted agonist (10 μM final) are expressed as a fraction of the fluorescence observed in the absence of antagonists (± s.d. when more than one trial was performed).

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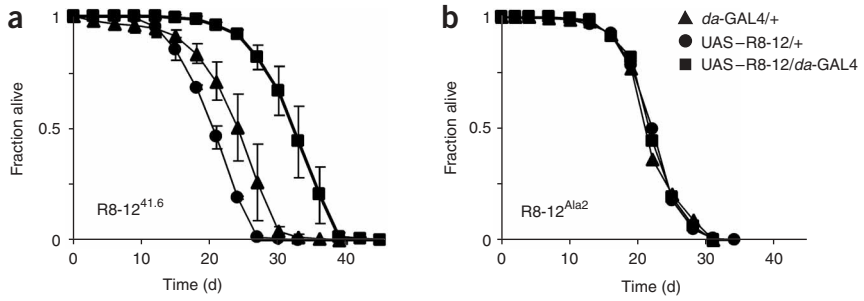

Figure 4 Expression of the Mth antagonist R8-12 peptide extends fly life span. (**a**) Male life span for the UAS–R8-12 transgene insertion line (41.6). ▲, heterozygous control for the *daughterless*-GAL4 driver (*da*-GAL4/+). ●, heterozygous control for the UAS–R8-12 peptide construct in the absence of the driver (UAS–R8-12/+). ■, the combination in which the *da*-GAL4 driver, by activating UAS promoter elements, drives expression of the R8-12 peptide (UAS–R8-12/*da*-GAL4). Data are mean ± s.d. (**b**) Male life span for a mutant UAS–R8-12 transgene (Ala2). Flies were maintained at 29 °C, and approximately 90 to 120 flies were used for each trial.

for the modulation of natural signaling. Indeed, the bias of *in vitro* selection for high-affinity ligands may favor the recovery of antagonists rather than agonists, which are evolved for function rather than binding.

The Stunted peptide was previously identified as an agonist for Mth using a cell-based fluorescence reporter system for calcium mobilization, which is a common consequence of GPCR activation[14]. We used the same technique here to determine whether the selected peptides antagonize Mth signaling. R8-12 and R8-14 were strong antagonists of N-Stunted–induced Mth signaling, with 50% inhibitory concentration (IC_{50}) values of 45 ± 10 and 115 ± 25 nM, respectively (**Fig. 3**). The W7A and R10A mutants of R8-14, as well as the R8-12 scrambled peptide, failed to antagonize Mth at concentrations up to 10 μM. No signaling was induced by any of the peptides in nontransfected control human embryonic kidney (HEK) cells. RWR motif peptides likely antagonize Mth by blocking interaction with agonist. However, it is also possible that the peptides desensitize the receptor (for example, by triggering internalization) or block GPCR oligomerization, which may be required for proper signaling.

Given that downregulation of *mth* increases life span[3], we speculated that the *in vivo* application of a Mth antagonist would affect *D. melanogaster* longevity. We generated transgenic flies that constitutively express the R8-12 peptide using the UAS/GAL4 system[16]. Expression of the R8-12 peptide extended the mean and maximal adult life spans at 29 °C by 38% and 26%, respectively (**Fig. 4a**). Two other independent UAS–R8-12 insertion lines showed similar life span increases (**Supplementary Fig. 4a** online). F1 heterozygous lines, each carrying one of the two P elements (*da*-GAL4 or UAS–R8-12), showed life spans similar to those of parental controls, which suggests that the longevity seen with peptide expression is not due to heterosis (**Supplementary Fig. 4b**). Additionally, expression of a mutant R8-12 peptide in which critical residues of the RWR motif were mutated to alanine resulted in no extension of life span (**Fig. 4b**). Male survival curves at 25 °C also show life span extension with R8-12 expression (**Supplementary Fig. 4c**). These results suggest that the expressed R8-12 peptide antagonizes Mth *in vivo*. However, because the role of other Mth-like receptors in longevity is unknown, and because other Mth-like extracellular domains have significant sequence similarity to the Mth ectodomain (up to 60% identity), the action of the R8-12 peptide may be mediated by other proteins. Studying the effects of R8-12 expression on other phenotypes observed in the *mth* mutant may reveal the relationships between aging, synaptic transmission and fertility[3,17–19].

In vitro selection has emerged as a powerful alternative to screening approaches for the identification of modulators of G protein signaling[20,21]. Our work provides a clear strategy for designing functional ligands for class B GPCRs, a protein family associated with a number of human diseases[2]. New expression and presentation platforms for transmembrane proteins are needed to broaden the utility of mRNA display selection for developing novel ligands. Selections targeting full-length GPCRs expressed in cells remain an unattractive option owing to the large background of cell-surface proteins. The recent successes in assembling pure GPCRs in paramagnetic proteoliposomes[22] and in a reconstituted bilayer on a biosensor surface[23,24] may be favorable alternatives for presenting a transmembrane protein for selection. The method we describe for the identification of GPCR effectors may provide new tools that are applicable to aging and other fields.

Table 1 Peptide ligands for the Mth ectodomain

Peptide	Sequence	k_a M^{-1} s^{-1} ($\times 10^5$)	k_d s^{-1} ($\times 10^{-2}$)	K_d nM	χ^2
R8-12	MRLVWIVRSRHFGPRLRMALLGSDRKMW	4.1	0.72	18	1.3
R8-14	MAPRAVWIQRAIQAMFRLASRQESKAFN	7.0	1.2	18	1.7
R8-09b*	MRYVWYLRTKHRRSLRLRSACARGSSA				
R8-03	MGDDMYRIREFLANYRPIWVMRSNLAQL				
R8-01	MNVSWGSFPSSWLQRYYLAKRREADVTL	6.3	1.9	31	1.5
		9.5	5.4	57	0.46
R8-07	MLKYPDTWLARSLSVFYLRKSARQGKSV				
R8-13	MELGQFQRLSLPYQWYLRTISYVSLRTA				
R8-07b*	MSTAGSRARSTSWGTRSPWTWPTPARTG				
R8-04	MVRIGYTSKPGGMNPGNSYTMSIIRMLI	6.1	19.9	326	0.46
R8-08b*	MSSLSPPWPASWSPSRPSAPRAAPSTPT				

RWR motif residues are in bold. The C-terminal constant region (TSGGLRASAI), which was frameshifted or mutated in marked sequences (*), is not shown. The sequences used to make the synthetic peptides are underlined (two peptides, a 22-mer and a 15-mer, were synthesized for R8-01). K_d values were calculated (k_d/k_a) from the kinetic parameters obtained from SPR experiments.

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METHODS

mRNA display library preparation. The initial DNA pool was generated by PCR (0.1 M initial template, 5 total cycles of PCR amplification using Herculase DNA polymerase (Stratagene)) of the 142.1 template(5′-TTAAA TAGCGGATGCACGCAGACCGCCACTAGT(SNN)$_{27}$CATTGTAATTGTAAAT AGTAATTGTCCC-3′; where N is A, C, G, or T, and S is C or G) with the primers 47T7FP (5′-GGATTCTAATACGACTCACTATAGGGACAATTACTATT TACAATTAC-3′) and 21.2 (5′-TTAAATAGCGGATGCACGCAG-3′). This library encoded a T7 promoter for transcription, a 5′ untranslated region sequence, and an open reading frame for the peptide MX$_{27}$TSGGLRASAI. The template DNA was transcribed and purified as described previously[25]. Cross-linking of a puromycin-psoralen linker to the mRNA was performed as described using oligo 28A.1 (5′-(Ps)UAGCGGAUGC(dA)$_{16}$(S9)$_2$(dCdC)Pu-3′; where unlabeled bases are 2′-OMe RNA, Ps is psoralen C6, S9 is spacer phosphoramidite 9, and Pu is puromycin-CPG; Glen Research)[26].

In vitro translation with the mRNA-28A.1 library was performed in Red Nova Lysate (Novagen) according to the manufacturer's instructions with optimized conditions (100 mM KOAc, 0.5 mM MgOAc, 0.4 μM mRNA-28A.1, and ~25 μM overall L-methionine; 10 ml total reaction volume) and ^{35}S-methionine (0.5 mCi ml^{-1} final). RNA-peptide fusions were salt-treated and purified as described previously[25]. Fusions were reverse-transcribed (oligo 21.2) and desalted into Mth buffer (50 mM HEPES-KOH at pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) BSA, 1 μg ml^{-1} yeast tRNA and 0.05% Tween 20) by gel filtration (NAP-25, GE Healthcare). Based on the ^{35}S-methionine incorporated into the RNA-peptide fusions and an average of 1.3 methionine residues per peptide, the initial complexity of the library was approximately 1.5 × 10^{13}.

***In vitro* selection.** Fusions of peptides with RNA bound to complementary DNA were incubated with ~0.1 ml of Mth-agarose at 4 °C for 1 h, then filtered and washed with 4 × 1 ml Mth buffer followed by 2 × 1 ml Mth buffer without BSA or tRNA. Bound fusions were eluted with 2 × 100 μl of 0.15% SDS. After removal of the SDS (SDS-OUT, Pierce), fusions were isopropanol-precipitated (50 μg ml^{-1} linear acrylamide, 1/40 volume of 3 M NaOAc at pH 5.2, and 1 volume of isopropanol). The reduced salt used for isopropanol precipitation was necessary to prevent inhibition of subsequent PCR, owing to the high salt introduced by the SDS-OUT reagent. Precipitated cDNA was PCR-amplified to produce a new double-stranded DNA pool.

Further rounds of selection were performed as described for the initial round except that *in vitro* translation reactions were smaller (~0.3 ml), less immobilized Mth was used for the selective step (~20 μl), and in rounds 5 through 8, bound fusions were eluted by competition with nonbiotinylated Mth (0.5 mg ml^{-1}) in Mth buffer without BSA or tRNA. Additionally, rounds 5 through 8 included a preclearing step in which the precipitated RNA-cDNA–peptide fusions were passed through columns containing NeutrAvidin-agarose and/or protein G-sepharose to remove peptides with high nonspecific binding for the immobilization matrix.

Kinetics determination by SPR. SPR measurements were performed at 25 °C on a BIAcore 2000 instrument equipped with research-grade streptavidin sensor chips. Biotinylated Mth was immobilized to a surface density of 450 to 700 response units. HBS-EP (10 mM HEPES at pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20 (Tween 20)) was used as the running buffer for all experiments. To collect kinetics data, a concentration series of each peptide was injected for at least 60 s at a flow rate of >45 μl min^{-1}. Raw data was processed with Scrubber (http://www.cores.utah.edu/interaction/scrubber.html) and globally fit with CLAMP using a 1:1 bimolecular interaction model[27]. Sensorgrams for the R8-04 peptide are shown in **Supplementary Figure 5** online.

Cell-based GPCR signaling assay. Calcium response assays were performed with HEK 293 cells stably expressing Mth essentially as described[14]. We used the Mth-B splice variant, which has a longer and different C-terminal cytoplasmic tail than Mth-A. Fluorescence spectra were divided by a baseline average, calculated from the region of data before the addition of N-Stunted. Further details are provided in the **Supplementary Methods** online.

Life span assays. P-element transformants with the full-length R8-12 peptide transgene were generated in a *w*^{1118} background by standard techniques[28]. R8-12^{Ala2} is a W5A and R8A mutant of the full-length R8-12 sequence. UAS-controlled R8-12 lines (UAS–R8-12) were crossed with *da*-GAL4, which had previously been outcrossed with *w*^{1118}. F1 heterozygous adults (0–4 d old) were transferred to new bottles and aged for 2 d, and males were separated under CO_2 anesthesia (30 males per vial). After allowing the flies to recover overnight at 25 °C, flies (3–7 d old) were incubated in a light- and humidity-controlled environment at the indicated temperature. The UAS–R8-12 and *da*-GAL4 lines were each crossed with *w*^{1118} to produce F1 heterozygous animals for controls. Flies were transferred to fresh food vials ((0.45% agar, 5% dextrose, 2.5% sucrose, 8.3% corn meal and 1.5% dried yeast, all (w/v)) with phosphoric and propionic acids supplemented to prevent mold, as previously described[29]) every 3–4 d and scored for survival.

Other methods. Additional methods are provided in the **Supplementary Methods** online.

Accession codes. Protein Data Bank: The atomic coordinates for the structure of the Mth ectodomain with a peptide antagonist have been deposited under PDB ID code 2PZX.

Note: Supplementary information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

W.W.J., A.P.W., Jr. and S.L.D. performed research; all authors designed research and discussed results; W.W.J., A.P.W., Jr., S.B. and R.W.R. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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