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1 **Interaction of the N-terminus of ecdysone receptor isoforms with the ligand**
2 **binding domain**

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10 **Figures:** 5

11
12 **Abbreviated title:** N/C interaction of EcR isoforms

13
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26 **Abstract**

27

28 Ecdysone receptor (EcR) isoforms exert different biological functions, although they
29 vary only in their N-terminal domain. Despite identical C-termini, which mediate
30 hormone-induced activity, the influence of ligand is isoform specific, which indicates
31 an N/C-interaction. The position of helix 12 with and without hormone varies among
32 isoforms and modifies N/C–interaction determined by fluorescence resonance-
33 energy transfer (FRET), which depends on the salt bridge between helices 4 and 12
34 of the ligand-binding domain (LBD). Disruption of the salt bridge by mutation of K497
35 (helix 4) had no effect on basal N/C interaction, but prevented the hormone-induced
36 increase, which was partially restored by a salt bridge with reversed polarity. The
37 heterodimerization partner Ultraspiracle (Usp) can compensate for the disruption of
38 the salt bridge. Without ligand the AB-domains of EcR-A and EcR-B1, but not EcR-
39 B2, interact with the LBD via K497 and repress transcriptional activity. This
40 intramolecular cross talk between N- and C-terminus along with the position of helix
41 12 stabilized by K497 regulates transcriptional activity of EcR isoforms.

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44 **Keywords:** Corepressor, *Drosophila*, N/C-interaction, FRET, insect, nuclear receptor.

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Abbreviations used

Aa: amino acid, AF: activation function, AR: androgen receptor, CFP: cyan fluorescent protein, DBD: DNA binding domain, EcR: ecdysone receptor, EcRE: ecdysone response element, ER: estrogen receptor, FRET: fluorescence resonance energy transfer, LBD: ligand binding domain, NTD: N-terminal domain, PPAR: Peroxisome proliferator – activated receptor PR: progesterone receptor, RAR: Retinoic acid receptor, RXR: retinoid X receptor, Usp: Ultraspiracle, VP16: herpes simplex transcriptional activator protein, wt: wild type, YFP: yellow fluorescent protein.

78 **1. Introduction**

79 The functional ecdysone receptor, which mediates the signal transduction of moulting
80 hormones of arthropods, is generally considered as a heterodimer of ecdysteroid
81 receptor and Ultraspiracle, the invertebrate orthologue of RXR (Yao et al., 1993)
82 although there are several indications that EcR (Costantino et al., 2008) and Usp
83 (Jones and Sharp, 1997; Spindler et al., 2009) also exert separate functions in the
84 absence of the heterodimerization partner.

85
86 In *Drosophila melanogaster* three isoforms of EcR are known, which differ in length
87 and sequence of their N-terminal AB - domain (Talbot et al., 1993). The isoforms
88 EcR-A, EcR-B1, and EcR-B2 exert different functions during development and
89 cannot substitute for each other (Bender et al., 1997; Schubiger et al., 2003; Davis et
90 al., 2005). EcR - A is predominantly expressed in cells that proliferate and
91 differentiate during metamorphosis, while EcR-B1 and EcR-B2 are present in larval
92 cells, which are eliminated by apoptosis during metamorphosis. In *Drosophila*
93 *melanogaster*, only one isoform of the heterodimerization partner Usp is found,
94 widely expressed during development and involved in the activation of ecdysone-
95 dependent genes (Hall and Thummel, 1998).

96
97 As expected, basal transcriptional activity of EcR isoforms varies (Dela Cruz et al.,
98 2000; Mouillet et al., 2001; Hu et al., 2003; Beatty et al., 2006; Ruff et al., 2009) due
99 to the different AF-1 sequences present in the AB-domains of EcR isoforms.
100 Although AF-2, located in helix 12 of the ligand- binding domain (Hu et al., 2003), is
101 identical for all EcR isoforms, hormone-induced transcriptional activity also varies
102 considerably among EcR isoforms expressed in vertebrate and in insect cells
103 (Spindler et al., 2009). Therefore, we speculated that the NTD of EcR may interact

104 with AF-2 to regulate the transcriptional capability of the receptor complex as proven
105 previously for several vertebrate nuclear receptors, e.g. AR (Doesburg et al., 1997;
106 Schaufele et al., 2005), ER (Cvoro et al., 2007) and PR (Tetel et al., 1999), either by
107 determination of transcriptional activity (Gianni et al., 2003) or by direct
108 measurements of fluorescence resonance energy transfer (FRET) of receptor
109 proteins tagged with fluorescent receptor fusion proteins (Grant et al., 2001; van
110 Royen et al., 2009).

111
112 The amino acid lysine in helix 4 (K497) of the ligand-binding domain of EcR plays an
113 essential role for the function of the ecdysone receptor and is involved in the
114 formation of the salt bridge between helix 4 and helix 12 (Bourget et al., 2000;
115 Renaud and Moras, 2000) and in ligand binding (Grebe et al., 2003). Since mutation
116 of K497 has more severe effects on transcriptional activity compared with mutation of
117 the partner amino acid of the salt bridge (E648 in helix 12), we assumed that an
118 additional function is associated with lysine 497. We therefore studied the functional
119 role of K497 in more detail. In order to separate the impact of the salt bridge from
120 other receptor functions mediated by K497, we mutated not only K497 but also the
121 partner of the salt bridge located in helix 12 E648. This allowed the formation of a salt
122 bridge, although with opposite polarity, whereas other functions associated with K497
123 were still disrupted.

124
125 In our experiments, we mainly used vertebrate cell lines to study the functionality of
126 the receptor complexes, ensuring that the test system was devoid of endogenous
127 EcR and Usp interfering with the function of heterologously expressed nuclear
128 receptor proteins (Beatty et al., 2006; Henrich et al., 2009). The subclone of CHO
129 cells used in our experiments contained no detectable amounts of RXR (Nieva et

130 al.,2008; Azoitei et al., 2009), which ruled out that this vertebrate receptor may
131 functionally have replaced Usp (Thomas et al., 1993). We used fluorescence
132 resonance energy transfer (FRET) technique, which allows monitoring of
133 intramolecular interactions and conformational changes that are crucial for receptor -
134 mediated signal transduction with non-invasive techniques, while preserving the
135 cellular protein network, compartmentalization and spatial cell arrangement (Eidne et
136 al., 2002).

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156 **2. Material and methods**
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158 **2.1. Plasmids:**

159 Plasmids coding for wild type and mutated isoforms of EcR tagged with CFP at the N
160 - terminus and with YFP at the C - terminus (Fig.1) were obtained by subcloning the
161 sequences coding for EcR isoforms lacking the F - domain (aa 654–878 of EcR-B1)
162 into the pECFP-C1 vector (Clontech, Mountain View, USA).

163
164 To this end, the open reading frame of DNA sequences coding for the EcR isoforms
165 were amplified by PCR from the plasmids pEYFP-EcR-A, pEYFP-EcR-B1 and
166 pEYFP-EcR-B2. If necessary, restriction sites (*Hind*III, *Bam*HI) were introduced by
167 PCR. The following forward primers were used (*Hind*III restriction site underlined:
168 5'-CGG CAT GGA CGA GCT GTA CAA G-3' (EcR-A, EcR-B1, EcR-B2, CFP-EcR-A);
169 5'-GGA AAGCTT CG ACC TTA CCT AGC-3' (EcR-A Δ 1-14aa); 5'-GGA AAGCTT CT
170 GCA GGC CCA TC-3' (EcR-A Δ 1-29aa); 5'-GAG AAGCTT ATG TTG ACG ACG AGT
171 GG-3' (EcR-A-YFP). Reverse primers (*Bam*HI restriction site underlined): 5'-GAG
172 GGA TCC AAC GTC CCA GAT C-3' (all constructs except CFP-EcR-A); 5'-GAG
173 GGATCC TTA AAC GTC CCA GAT CTC-3' (CFP-EcR-A, termination codon
174 italicized).

175
176 After double digestion, the PCR products were ligated into the *Hind*III and *Bam*HI
177 restriction sites of pECFP-C1 using T4 ligase (Promega, Madison, USA). The double-
178 tagged constructs were obtained by cloning the YFP sequence (taken from the
179 pEYFP-N1 vector; Clontech, Mountain View, USA) into the *Bam*HI and *Xba*I
180 restriction sites. For EcR-A-YFP, the PCR product was cloned into the *Hind*III and
181 *Bam*HI sites of the pEYFP-N1 vector. Additional base pairs between the *Bam*HI

182 restriction site and ATG of the YFP sequence were deleted with QuikChange Site-
183 Directed Mutagenesis Kit (Stratagene, La Jolla, USA) according to the
184 manufacturer's instructions using a forward primer 5'-GGG ACG TT GGATCC ATG
185 GTG AGC AAG GGC-3' and its reverse complement.

186

187 Point mutations K497E and E648K were introduced using QuikChange Site-Directed
188 Mutagenesis Kit according to the manufacturer's instructions. The following primers
189 and their reverse complements were used (nucleotides introducing the mutation are
190 underlined):

191 EcR-K497E: 5'-CAG ATC ACG TTA CTA GAG GCC TGC TCG TCG G-3'

192 EcR-E648K: 5'-G CCC AAG TTC CTC GAG AAG ATC TGG GAC GTT C-3'

193 In addition, the following plasmids were used for transfection studies: wild type Usp,
194 Usp Δ DBD (deletion of amino acids 104–169 encoding DBD of Usp), pVP16-Usp I
195 (encoding aa 98–507 of Usp, which includes the last six amino acids of the N-
196 terminal AB-domain of wild type Usp), and pVP16-Usp III (aa 170–507 of wild type
197 Usp encoding the DE-domains (Beatty et al., 2006). The integrity of all plasmids was
198 confirmed by DNA sequencing (GATC, Konstanz, Germany).

199 Transcriptional activity was evaluated with the reporter plasmid pEcRE-tk-Luc
200 carrying tandemly repeated copies of the hsp27 ecdysone response element
201 attached to the minimal constitutive thymidine kinase promoter and a luciferase
202 reporter gene as described previously in detail (Beatty et al., 2006). A cotransfected
203 pGL4.74 [hRluc/TK] plasmid (Promega, Madison, USA) containing the *Renilla*
204 *luciferase* gene was used for normalization of transcriptional activity on transfection
205 efficiency.

206

207 **2.2. Cell culture and transfection:**

208 CHO-K1 cells were grown in DMEM/F12 medium (Gibco / Invitrogen, Carlsbad, USA)
209 supplemented with 5% of fetal bovine serum (Sigma, Taufkirchen, Germany) under
210 5% CO₂. For luciferase reporter assay, cells were seeded in six-well plates (Nunc,
211 Wiesbaden, Germany) with 4×10^5 cells per well. After 24 hours cells were
212 transfected with nanofectin (PAA, Pasching, Austria) according to the manufacturer's
213 instructions. Each well received 3 µg of plasmid DNA (0.75 µg of pEcRE-tk-Luc, 0.25
214 µg of pGL4.74 [hRluc/TK], and 1 µg of plasmids expressing EcR and Usp each; in
215 case of experiments without Usp 2 µg of the plasmid expressing EcR). After 4 hours
216 of incubation the medium containing the transfection mix was replaced by fresh
217 culture medium. One hour later muristerone A (Sigma, Steinheim, Germany) was
218 added (final concentration 1 µM). 24 hours after transfection cells were lysed by
219 shaking in 1x passive lysis buffer (PLB 5x, Promega, Madison, USA; 100 µl per well).
220 To improve homogenization the suspension was sucked several times through a thin
221 needle (0.4 × 20 mm, Terumo, Leuven, Belgium).

222
223 For ligand binding assay, about 1.5×10^6 cells were seeded in 10-cm cell culture
224 dishes (Greiner Bio-One, Frickenhausen, Germany) and transfected with nanofectin
225 after 24 hours. Each 10-cm plate received 7 µg of total plasmid DNA. 24 hours after
226 transfection, proteins were extracted from the cells as described previously (Azoitei
227 and Spindler-Barth, 2009).

228
229 Human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA, USA) were
230 maintained at 37°C under 5% CO₂ in minimal essential medium with Earle's salts
231 supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml
232 penicillin. Drosophila S2 cells were grown at 25 °C in Schneiders Drosophila medium
233 (Gibco/Invitrogen, Carlsbad, USA) supplemented with 10 % fetal bovine serum. Cells

234 were transiently transfected using Fugene HD transfection reagent (Roche Molecular
235 Biochemicals, Mannheim, Germany) following the manufacturer's instructions. For
236 FRET analysis, cells were seeded on glass coverslips and transfected with 2 µg of
237 total plasmid DNA (1µg of dually tagged EcR CFP/YFP-tagged EcR and 1µg Usp
238 construct, In case of experiments without Usp 2 µg of dually tagged EcR construct).
239 The ratio of DNA coding for CFP-EcR-A and EcR-A-YFP used for transfection was
240 1:3.

241
242 Luciferase reporter activities were assayed with the Dual Luciferase Reporter Assay
243 System (Promega, Madison, USA) according to the manufacturer's instructions to
244 compensate differences in transfection efficiency. Transcriptional activities were
245 additionally normalized on receptor concentration determined by quantification of
246 specific Western blot signals to eliminate variations in receptor protein content of EcR
247 isoforms due to differences in the stability of receptor proteins, which is mainly
248 determined by the AB-domain of nuclear receptors.

249

250 **2.3. Western blotting and quantification of receptor content:**

251 Cell extracts (25 µg protein/lane) were separated on SDS gels (Lämmler et al., 1970).
252 Gels were electroblotted on nitrocellulose membranes (BA 85, 45 µm pore size,
253 Schleicher and Schuell, Dassel, Germany). Membranes were stained with Ponceau
254 to check transblotting efficiency. Membranes were soaked in blocking buffer (20 mM
255 Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6, 0.02% Thimerosal) containing 3%
256 milk powder (low fat, < 1 %) and 1 % bovine serum albumine (Sigma, Steinheim,
257 Germany) for 1 hour and then probed over night with a monoclonal first antibody
258 diluted in the same buffer (anti-GFP, Clontech, Heidelberg, Germany, diluted 1:500,
259 or anti-EcR, DDA2.7, diluted 1:1000, respectively). For detection, a peroxidase-

260 conjugated secondary antibody diluted in TBS-T (20 mM Tris-HCl, 137 mM NaCl, 0.1
261 % Tween 20, pH 7.6) was used (anti-rabbit IgG, # A-6667, Sigma, Deisenhofen,
262 Germany, diluted 1:500, or anti-mouse IgG, # A-5906, Sigma, Deisenhofen,
263 Germany, diluted 1:3000, respectively). Signals were visualized with SuperSignal
264 West Dura Extended Duration Substrate (Pierce, Rockford, USA). Specific bands
265 were detected by the Chemi-Smart 5000 photo documentation system (Vilber
266 Lourmat, Eberhardzell, Germany) and quantified relative to a standard probe using
267 software Bio-1D (Vilber Lourmat, Eberhardzell, Germany). The linearity of the
268 intensity of specific receptor bands was confirmed with a calibration curve as
269 described previously (Braun et al., 2009).

270

271 **2.4. Ligand binding assay:**

272 CHO cells were transfected as described above and cell extracts prepared as
273 described previously (Azoitei and Spindler-Barth, 2009). Ligand-binding was
274 determined with $^3\text{[H]}$ -Ponasterone A (specific activity 7.9 TBq/mmol) using a filter
275 assay (Turberg and Spindler, 1992). Briefly, cell extracts were incubated routinely
276 with $^3\text{[H]}$ -Ponasterone for 5 hrs at 4°C. Free ligand was removed by filtration using
277 NC45 nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and
278 washed with 3 x 4 ml ice-cold washing buffer (20 mM HEPES, 20 mM NaCl, 10 %
279 glycerol (v/v), 1 mM EDTA, 1 mM β -mercaptoethanol, pH = 7.9). Bound radioactivity
280 was measured with a liquid scintillation counter (Tri - Carb 1500, Perkin Elmer,
281 Rodgau-Jügesheim, Germany) and the non-specific binding, determined by
282 competition with 1 μM 20-OH-Ecdysone, subtracted. As additional control extracts
283 from non - transfected cells were used (< 100 cpm). The purity of $^3\text{[H]}$ -Ponasterone A
284 was routinely checked by HPLC analysis prior to use.

285

286 **2.5. FRET measurements:**

287

288 HEK 293 cells were grown on glass coverslips for 48 h. Coverslips were then
289 mounted in a custom-made chamber and covered with incubation buffer (138 mM
290 NaCl, 6 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5.5 mM glucose, 2 mg/ml bovine serum
291 albumin, and 10 mM HEPES, pH 7.5). FRET analysis was performed with an inverted
292 microscope Axiovert 100 equipped with a Plan-Apochromat x63/1.4 objective (Carl
293 Zeiss, Göttingen, Germany). CFP and YFP were alternately excited at 410 and
294 515 nm with a monochromator (Polychrome II; TILL Photonics, Gräfelfing, Germany)
295 in combination with a dual reflectivity dichroic mirror (< 460 and 500–520 nm;
296 Chroma Technology, Rockingham, VT, USA). Emitted light was filtered through 460–
297 500 (CFP) or 535–580 nm (YFP) band pass filters changed by a motorized filter
298 wheel (Lambda 10-2; Sutter Instruments, Novato, USA), and detected with a cooled
299 CCD camera (Imago; TILL Photonics, Gräfelfing, Germany). For FRET analysis, CFP
300 (40 ms) and YFP (8 ms) emission without acceptor bleach were recorded during
301 20 cycles. Then, 60 cycles were recorded with an additional 2-s illumination per cycle
302 at 512 nm to bleach YFP. FRET was assessed as recovery of CFP (donor)
303 fluorescence during YFP (acceptor) bleach. . FRET efficiencies were obtained as
304 described by Voigt et al. (2005), which allows to calculate the distance between
305 donor and acceptor according to Förster's theory (Evers et al., 2006; Patterson et
306 al., 2000). In brief, the FRET efficiency E is expressed as $E = 1 - (F_{DA}/F_D)$, where F_{DA}
307 and F_D are intensities of the donor fluorescence in the presence and absence of the
308 FRET acceptor, respectively. The increase in CFP fluorescence during acceptor
309 bleach was plotted against the remaining YFP fluorescence, yielding a regression
310 line that served to extrapolate F_{DA} and F_D
311 The data of each bleaching experiment were averaged over two to six single cells.

312 Means and SD were computed from three to five independent transfections each
313 comprising at least three bleaching experiments.

314

315 **2.6. Statistical evaluation:**

316 Statistical significance of differences among groups was determined on the basis of a
317 two-sided, matched pair Student's t-test. Förster's distances calculated from FRET
318 efficiencies according to Evers et al. (2006) and Patterson et al., (2000) reveal that
319 significant differences in FRET efficiency correspond to a differences in the distance
320 between donor and acceptor of 4.5-14 Å.

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338 **3. Results**

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340 **3.1. Functional properties of EcR isoforms labelled with CFP/YFP**

341 The concentration of tagged receptor proteins is even increased especially for EcR -
342 A and EcR - B2 compared with non-tagged receptors (Fig. 2). No degradation
343 products of EcR isoforms tagged with fluorescent proteins CFP at the N-terminus and
344 YFP at the C - terminus can be detected on Western blots, which rules out the
345 possibility that interaction of receptor proteins with degraded fragments might cause
346 increased FRET signals. The concentration of RXR, which may heterodimerize with
347 EcR in the absence of Usp (Thomas et al., 1993)) in the CHO-K1 subline and HEK
348 cells used for our experiments is below detection limit and does not cause any
349 measurable physiological effect (Nieva et al., 2008). This implies that the proximity of
350 N – and C - terminus of EcR isoforms can be studied in the absence of a
351 heterodimerization partner. Basal transcriptional activity of wild type (Table 1) and
352 mutated EcR isoforms (data not shown) is still observed, although considerably
353 reduced compared with non-tagged receptors, which is in accordance with data
354 reported from vertebrate receptors (Schaufele et al., 2005). Induction of
355 transcriptional activity by muristerone A (Table 1) is in the same range as previously
356 observed for EcR isoforms (Ruff et al., 2009), although the affinity of the ligand
357 ponasterone A is considerably impaired ($K_D = 48.8$ nM for CFP-EcR-A-YFP/wt - Usp
358 compared to $K_D = 7.0$ nM for EcR-A/wt – Usp) (Azoitei and Spindler-Barth., 2009).

359

360 **3.2. FRET efficiency of dually tagged EcR varies for different isoforms.**

361 FRET efficiencies of dually tagged EcR isoforms vary depending on the AB-domain
362 of EcR (Table 2). Basal FRET efficiency is low for EcR-A and EcR-B1. In the
363 presence of the ligand muristerone A FRET signalling increases with EcR - A, but not

364 EcR – B1. The hormone - induced increase depends on the presence of aa 15-29 of
365 EcR - A, a peptide with the sequence STLPSHILLQQQLAA, as was shown by
366 deletion experiments. In the presence of the hormone, FRET signals do not increase
367 following deletion of aa 1-29 (Table 2) or truncation of the complete isoform- specific
368 AB-domain of EcR-A (data not shown), whereas deletion of aa 1-14 results in FRET
369 signals comparable to full length EcR- A. In contrast, FRET efficiency of EcR-B2 is
370 already high in the absence of the hormone and is not further stimulated in the
371 presence of muristerone A.

372
373 Control experiments with cotransfected CFP-EcR-A and EcR-A-YFP resulted in
374 rather low FRET signals (< 2 %), indicating that the FRET signals in dually tagged
375 EcR isoforms predominantly represent intramolecular and not intermolecular
376 interaction of CFP- and YFP-tagged receptor proteins. Time course experiments
377 revealed that heterodimerization and ligand binding take about 10-15 min and
378 maximal FRET signals can be observed after this incubation time (data not shown).

379

380 **3.3. Influence of Usp on FRET efficiencies of EcR isoforms**

381 The heterodimerization partner Usp slightly increases basal and significantly
382 stimulates hormone-induced FRET efficiency of EcR-A (Table 3). Enhanced
383 constitutive FRET efficiency is observed only with wild type Usp. Deletion of the
384 DNA-binding domain (DBD) of Usp diminishes the hormone-induced increase in
385 FRET efficiency compared with the wild type heterodimer, but does not alter basal
386 FRET. In contrast to EcR - A, FRET efficiencies of both EcR - B isoforms are not
387 increased by Usp (EcR - B1/wt - Usp: basal FRET efficiency = 5.4 % ± 1.2 %, with
388 hormone = 6.1 % ± 1.7 %; EcR - B2/wt - Usp: basal = 9.0 % ± 0.4 %, with hormone =
389 6.8 % ± 0.4 %).

390

391 To rule out, that cell- type specific conditions may influence FRET efficiencies of
392 EcR, FRET studies were also performed with transfected *Drosophila* S2 cells, which
393 abundantly express Usp, but no EcR (Spindler et al., 2009). Neither in the absence
394 nor in the presence of muristerone A were significant differences in FRET
395 efficiencies for EcR-A/wt-Usp detected between vertebrate cells (Table 3) and insect
396 S2 cells (basal FRET efficiency: 7.0 % \pm 1.2 %, with hormone: 11.1 % \pm 1.5 %).

397

398 **3.4. Influence of the salt bridge in the ligand-binding domain of EcR on FRET** 399 **efficiency**

400

401 Ligand binding of nuclear receptors is often accompanied by a conformational
402 change of the ligand-binding domain (Bourget et al., 2000; Renaud and Moras,
403 2000). The altered position of helix 12 in the holo - receptor is stabilized by formation
404 of a salt bridge between helix 4 and helix 12 (Fig. 3). If the salt bridge is disrupted
405 either by mutation of lysine (K) at position 497 in helix 4 to glutamic acid (E) or E at
406 position 648 in helix 12 to K, basal FRET efficiency is not altered in EcR - A as
407 expected, but the hormone -induced increase is abolished (Table 4). The affinity of
408 the ligand itself is not reduced by disruption of the salt bridge (EcR - A^{K497E}/wt - Usp:
409 $K_D = 23.4$ nM; EcR - A^{E648K}/wt - Usp: $K_D = 19.5$ nM) compared with the non-mutated
410 construct ($K_D = 48.8$ nM, see above).

411

412 Heterodimers of EcR - A^{K497E} and wt - Usp exhibit increased FRET signals in the
413 presence of the hormone, comparable with the corresponding wild type receptor
414 complex (Table 4). In case of EcR^{E648K}, Usp is unable to compensate the influence of
415 the mutation in helix 12. Formation of a salt bridge with reversed polarity using

416 liganded EcR^{K497E/E648K}/wt - Usp (Table 4) only slightly increased FRET efficiency.
417 With both EcR-B isoforms, neither point mutation showed an effect on FRET
418 efficiency (data not shown).

419

420 **3.5. Mutation of lysine in helix 4 enhances transcriptional activity of EcR - A**
421 **and EcR-B1.**

422 Besides participation in the salt bridge between helix 4 and helix 12 the amino acid,
423 which corresponds to K497 in EcR is known to be a conserved corepressor binding
424 site of nuclear receptors (Lempiäinen et al., 2005). In fact, basal transcriptional
425 activity of EcR-A^{K497E} (36-fold) and EcR-B1^{K497E} (4-fold) is enhanced compared with
426 the corresponding wild type EcR isoforms, whereas no further increase is observed
427 with EcR-B2^{K497E} (Fig. 4). As shown for mutated EcR-A, Usp modulates the extent of
428 basal transcriptional activity, but does not prevent the hormone-induced increase in
429 transcriptional activity of the mutated receptor complexes (Fig. 5). No gain-of-function
430 is observed in the presence of the hormone (data not shown). The AB - domain of
431 Usp enhances basal activity of receptor complexes with wild type EcR-A, but reduces
432 the increase of transcriptional activity caused by the exchange of K497 to E (5-fold).
433 Exchange of the original AB - domain of Usp by the activation domain of VP16
434 (Usp I) increases basal activity in wild type and mutated receptor complexes (7-fold),
435 whereas deletion of the DBD (Usp III) impairs basal transcriptional activity already in
436 receptor complexes with wild type EcR-A, but has no major influence on fold increase
437 (5-fold) of constitutive transcriptional activity of the mutated EcR.

438

439

440

441

442 **4. Discussion**

443

444 ***4.1. The AB-domain of EcR modulates constitutive and hormone - induced N/C***
445 ***interaction in an isoform - specific manner.***

446

447 Full transcriptional activation by steroid hormone receptors requires functional
448 synergy between the ligand-independent transcriptional activation domain AF-1
449 located in the amino-terminal region and the ligand-dependent transcriptional
450 activation domain AF-2 at the C - terminus. As a mechanism for achieving this
451 functional synergy, physical intramolecular association between N- and C-terminal
452 domains of the receptor has been demonstrated in vertebrate nuclear receptors
453 (Tetel et al., 1999).

454

455 According to analysis of the crystal structure, ligand binding induces a conformational
456 change of the ligand-binding domain upon hormone binding (Renaud and Moras,
457 2000). The most obvious change is the repositioning of the C - terminal helix 12,
458 which protrudes from the LBD core in the apo - receptor and folds back towards the
459 LBD in the liganded holo - receptor. This conformation is stabilized by the formation
460 of a salt bridge between helix 4 and helix 12 (Renaud et al., 1995), which is of
461 functional relevance for transcriptional activity of EcR as shown by mutational
462 analysis (Grebe et al., 2003; Bergman et al., 2004; Grebe et al., 2004).

463

464 The movement of helix 12 results in an increased FRET signal of the liganded holo -
465 receptor, indicating a stabilized interaction of N - and C - terminus as described for
466 vertebrate nuclear receptors like AR (Schaufele et al., 2005) and ER (Cvoro et al.,
467 2007; Paruthiyil et al., 2009). This hormone - dependent increase in N/C interaction

468 was functionally confirmed by mammalian two - hybrid assays and by direct protein-
469 protein interaction assays *in vitro* (Tetel et al., 1999).

470

471 The results obtained with EcR-A are in agreement with these previous reports and
472 demonstrate that the repositioning of helix 12 in the liganded EcR-A also occurs
473 without Usp, despite the considerably reduced hormone-binding capability in the
474 absence of a heterodimerization partner (Azoitei and Spindler-Barth, 2009).

475 However, in case of EcR-B1, FRET signals remained low even in the presence of the
476 ligand. We cannot exclude that in EcR-B1 the distance between N- and C-terminus
477 and/or the 3D-conformation may prevent an increase in FRET coupling efficiency
478 (Koterba and Rowan, 2006; Paruthiyil et al., 2009). It is also possible that the
479 enhanced intramolecular FRET efficiency of EcR - B2, which is already observed in
480 the absence of the ligand may simply reflect the reduced distance between the
481 shorter NTD and the LBD of EcR-B2 compared with EcR-A and EcR-B1. Conversely,
482 these different FRET levels may represent a distinct conformation of the apo -
483 receptor, where the position of helix 12 is more similar to the liganded holo - receptor
484 as described for RAR β and RAR γ in contrast to RAR α (Farboud et al., 2003). We
485 have to keep in mind that there exists a considerable degree of dynamic variability in
486 the position of helix 12 among receptor isoforms. Receptor activation may not
487 necessarily require repositioning of helix 12 as shown for PPAR γ (Uppenberg et al.,
488 1998). Tanenbaum et al. (1998) report that helix 12, which is not essential for ligand
489 binding and dimerization of ER α , is still in the apo-position even in the liganded holo -
490 receptor.

491

492 Isoform-specific variations of interactions between the LBD and the NTD contribute
493 to altered functionality of EcR, as reported previously for vertebrate receptors, e.g. for

494 PR (Tetel et al., 1999). Different basal transcriptional activities of EcR isoforms were
495 reported repeatedly (Dela Cruz et al., 2000; Hu et al., 2003, Mouillet et al., 2001,
496 Beatty et al., 2006; Ruff et al., 2009). The low basal transcriptional activity of EcR-A
497 into transfected in mammalian cells (Mouillet et al., 2001) and insect cells (Hu et al.,
498 2003) was consequently attributed to a repressive function in the AB domain of EcR-
499 A in contrast to the activating functions of EcR-B isoforms, although the molecular
500 mechanism for the selective repression by EcR-A was still unknown.

501

502 ***4.2. FRET efficiency and transcriptional activity are not correlated***

503 Transcriptional activity and N/C interaction of EcR isoforms as determined by FRET
504 do not change in parallel as was described previously for several vertebrate
505 receptors, e.g. AR (Schaufele et al., 2005). This is most obvious for EcR - A, which
506 exhibits rather low constitutive and hormone-induced transcriptional activity. In
507 contrast, basal transcriptional activity of EcR-B1 is considerably higher (Ruff et al.,
508 2009) despite low FRET efficiency, which remains low even in the presence of the
509 ligand. In addition to the conformational change of helix 12 associated with ligand
510 binding (Bourget et al., 2000), N/C interaction and transcriptional activities of nuclear
511 receptors are modulated by many factors e.g. by heterodimerization and interaction
512 with comodulators. Apart from direct association of N- and C-terminus, N/C
513 interaction also can be mediated indirectly by comodulator interaction as suggested
514 previously (Ikonen et al., 1997; Need et al., 2009). Isoform - specific comodulator
515 recruitment is reported for vertebrate receptors like ER indicating that comodulator
516 binding is modified by the AB - domain of nuclear receptors (Bai and Giguère, 2003).
517 Other receptor functions like DNA binding may be also not correlated with
518 transcriptional activity (Beatty et al., 2006; Braun et al., 2009) underlining the
519 complex interplay of various factors regulating transcriptional activity.

520

521 **4.3. Influence of the heterodimerization partner on FRET efficiency and**
522 **transcriptional activity**

523 The heterodimerization partner Usp modifies FRET efficiency and transcriptional
524 activity. Whereas FRET signalling is mainly influenced by the ligand-binding domain
525 of Usp, transcriptional activity of the heterodimer is modulated not only by the AB-
526 domain of EcR, as outlined already by Dela Cruz et al., (2000), but also by the N-
527 terminus of Usp (Ruff et al., 2009). Cross-talk of nuclear receptors mediated by the
528 N-terminal AF-1 domain has been reported for vertebrate receptors like ER α and
529 RAR β ; e.g., the N-terminal region of ER α modulates the transcriptional activity of
530 RAR β . In addition, the AB - domain of the heterodimerization partner RAR modifies
531 N/C interaction of ER (Rousseau et al., 2003). N- and C-terminus of both
532 dimerization partners may also be involved in comodulator binding. According to
533 Zamir et al. (1997) receptor dimers interact with comodulators and the C - termini of
534 both receptors are required; e.g. heterodimerization with RXR provides the
535 interaction surface necessary for corepressor recruitment of RAR/RXR heterodimers
536 in the non-liganded state.

537

538 **4.4. Impact of the salt bridge between helix 4 and helix 12 on FRET efficiency**

539 No significant change in basal N/C interaction was observed for EcR-A^{K497E}. This was
540 expected as the formation of the salt bridge between helix 4 (K497) and helix 12
541 (E648) is associated with ligand binding. The hormone-induced increase in FRET
542 efficiency is abolished thereby confirming that the conformational change
543 associated with formation of the salt bridge between helix 4 and helix 12 is
544 responsible for the increased FRET signal of wild type EcR-A. In contrast, FRET
545 coupling efficiency of EcR-A^{K497E} in the presence of Usp is comparable with wild type

546 receptor complexes, and confirms the hypothesis of Grebe et al. (2003) that the
547 ligand-binding domain of the heterodimerization partner can compensate for the
548 disruption of the salt bridge. According to (Uppenberg et al., (1998), hydrophobic
549 interactions between helix 12 and the remaining part of the ligand-binding pocket
550 additionally contribute to the stabilization of helix 12. As expected, Usp has no
551 influence on EcR^{E648K} mutated in helix 12, which protrudes away from the ligand-
552 binding domain as a result of the mutation. Formation of a salt bridge with opposite
553 polarity restores N/C interaction at least partially, although no increase is obtained in
554 transcriptional activity, which affords the integrity of AF-2 in helix 12.

555

556 ***4.5. The AB - domains of EcR - A and EcR - B1 inhibit basal transcriptional***
557 ***activity by interaction with K497 in helix 4 of the ligand binding domain.***

558 Basal, but not hormone-induced transcriptional activity is considerably enhanced , if
559 lysine at position of 497 is mutated to glutamic acid as previously reported (Beatty
560 et al., 2006). K497 may be involved in two different receptor functions. Besides
561 formation of a salt bridge between helix 4 and helix 12 in the liganded holoreceptor,
562 K497 is part of a conserved corepressor binding site of nuclear receptors
563 (Lempiäinen et al., 2005). Therefore, it is reasonable to assume that mutation of
564 K497 activates transcription by disruption of corepressor interaction with the non -
565 liganded receptor.

566

567 This interpretation is further supported by experiments with N-terminally truncated
568 EcR-A variants. According to deletion experiments, the inhibitory action of the NTD of
569 EcR-A depends on aa 15-29. The sequence LPSHI(LLQQQL) within this peptide
570 shows high similarity with the consensus sequence of the nuclear receptor interaction
571 motif CoRNR box known from vertebrate corepressors SMRT and N-Cor: I/L-X-X-

572 I/V-I (Hu and Lazar, 1999; Cohen et al., 2001) and L-X-X-I/H-I-X-X-X-I/L (Perissi et
573 al., 1999). Therefore, the simplest explanation for the gain-of-function in basal
574 transcriptional activity observed after exchange of K at position 497 to E is an isoform
575 - specific direct intermolecular interaction of the AB-domain of EcR-A and a weaker
576 interaction of the AB-domain of EcR-B1, which lacks the typical CoRNR motif, with
577 the ligand-binding domain via K497 resulting in reduced transcriptional activity.

578
579 The data presented here demonstrate that basal and hormone-induced
580 transcriptional activities of EcR are regulated in an isoform-specific manner by
581 interaction of the NTD with the ligand-binding domain and by variation of the position
582 of helix 12. It is also shown that K497 in helix 4 is involved in two different functions:
583 interaction with the AB- domain in the absence of the ligand and formation of the salt
584 bridge between helix 4 and 12 in the liganded state depending on the isoform.
585 This means that K497 acts as a molecular switch between basal and hormone-
586 induced activity.

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851 **Tables**

852

853 Table 1: Influence of fluorescent tags on transcriptional activity of heterodimers of

854 EcR isoforms and wild type Usp.

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	basal transcriptional activity	fold induction by
	[% of non-tagged receptors]	1 μM muristerone A
CFP-EcR-A-YFP	20.7 % \pm 8.6 %	1.53 \pm 0.34
CFP-EcR-B1-YFP	44.4 % \pm 15.2 %	1.36 \pm 0.38
CFP-EcR-B2-YFP	42.4 % \pm 18.3 %	1.13 \pm 0.38

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860 Table 2: FRET efficiencies of EcR isoforms. Means \pm SD of at least three

861 independent transfection experiments are shown. The differences in FRET

862 efficiencies as indicated by *, ° and + are significant ($p < 0.01$).

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	constitutive	hormone-induced
	FRET efficiency [%]	FRET efficiency [%]
EcR-A	5.0 \pm 1.0 ^{*,°}	8.7 \pm 0.8 ^{°,+}
EcR-B1	4.7 \pm 1.0	5.9 \pm 1.0
EcR-B2	9.9 \pm 1.0 [*]	10.4 \pm 1.0
EcR-A Δ aa1-14	5.8 \pm 1.6	9.8 \pm 1.3
EcR-A Δ aa1-29	5.7 \pm 0.4	5.6 \pm 0.8 ⁺
EcR Δ AB-domain	7.7 \pm 1.3	7.3 \pm 1.7

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867 Table 3: Influence of Usp on FRET efficiency of EcR - A. Means \pm SD of at least
 868 three independent transfection experiments are shown. The differences in FRET
 869 efficiencies as indicated by $^{\circ}$ ($p < 0.03$) and $*$ ($p < 0.05$) and the differences between
 870 constitutive and hormone-induced FRET efficiencies of all receptor complexes
 871 ($p < 0.01$) are significant.

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	constitutive	hormone-induced
	FRET efficiency [%]	FRET efficiency [%]
EcR-A	5.0 \pm 1.0	8.7 \pm 0.8 $^{\circ}$
EcR-A / wt-Usp	6.8 \pm 1.1	12.4 \pm 1.3 $^{\circ,*}$
EcR-A / Usp Δ DBD	4.8 \pm 0.1	9.5 \pm 0.5 $*$

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877 Table 4: Influence of point mutations of EcR - A, which affect the salt bridge between
 878 helices 4 and 12, on FRET efficiencies. Means \pm SD of at least three independent
 879 transfection experiments are shown. The differences in FRET efficiencies indicated
 880 by $*$ ($p < 0.02$) and $^{\circ}$ ($p < 0.05$) are significant.

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	constitutive	hormone-induced
	FRET efficiency [%]	FRET efficiency [%]
EcR-A ^{K497E}	4.9 \pm 0.1	4.4 \pm 0.4
EcR-A ^{K497E} / wt-Usp	5.5 \pm 0.2 $*$	16.2 \pm 2.3 $*$
EcR-A ^{E648K}	4.8 \pm 0.8	4.6 \pm 0.4
EcR-A ^{E648K} / wt-Usp	5.3 \pm 0.6	5.3 \pm 1.0
EcR-A ^{K497E/E648K}	5.5 \pm 0.6	6.2 \pm 0.4
EcR-A ^{K497E/E648K} / wt-Usp	6.5 \pm 0.7 $^{\circ}$	8.4 \pm 0.2 $^{\circ}$

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Legends

Fig. 1: EcR fusions with fluorescent proteins CFP and YFP used for FRET analyses.

Fig. 2: Specific Western blot signals showing the influence of fluorescent tags on expression of EcR isoforms. Experiments were performed with extracts from transfected HEK293 cells. 40 μ g of protein were loaded per lane.

Fig. 3: Schematic view of the ligand binding domain of EcR with helix 12 (H12) in the open (A) or closed (B) conformation. The closed conformation of helix 12 is often observed in ligand-bound nuclear receptors and is stabilized by a salt bridge between helix 4 and helix 12. In case of EcR, amino acids K497 (helix 4) and E648 (helix 12) are involved in formation of the salt bridge. Scheme modified according to Vivat et al., 1997.

Fig. 4: Comparison of basal transcriptional activity of receptor complexes with wild type EcR and EcR^{K497E}. White bars: wild type EcR - A; black bars: EcR - A^{K497E}. Means + SD of three independent transfection experiments are shown.

Fig. 5: Influence of Usp on basal transcriptional activity of wild type EcR - A and EcR - A^{K497E}. White bars: wild type EcR - A; black bars: EcR - A^{K497E}. Usp I: VP16 - Usp_{CDE}; Usp III: VP16-Usp_{DE}. Means + SD of three independent transfection experiments are shown.

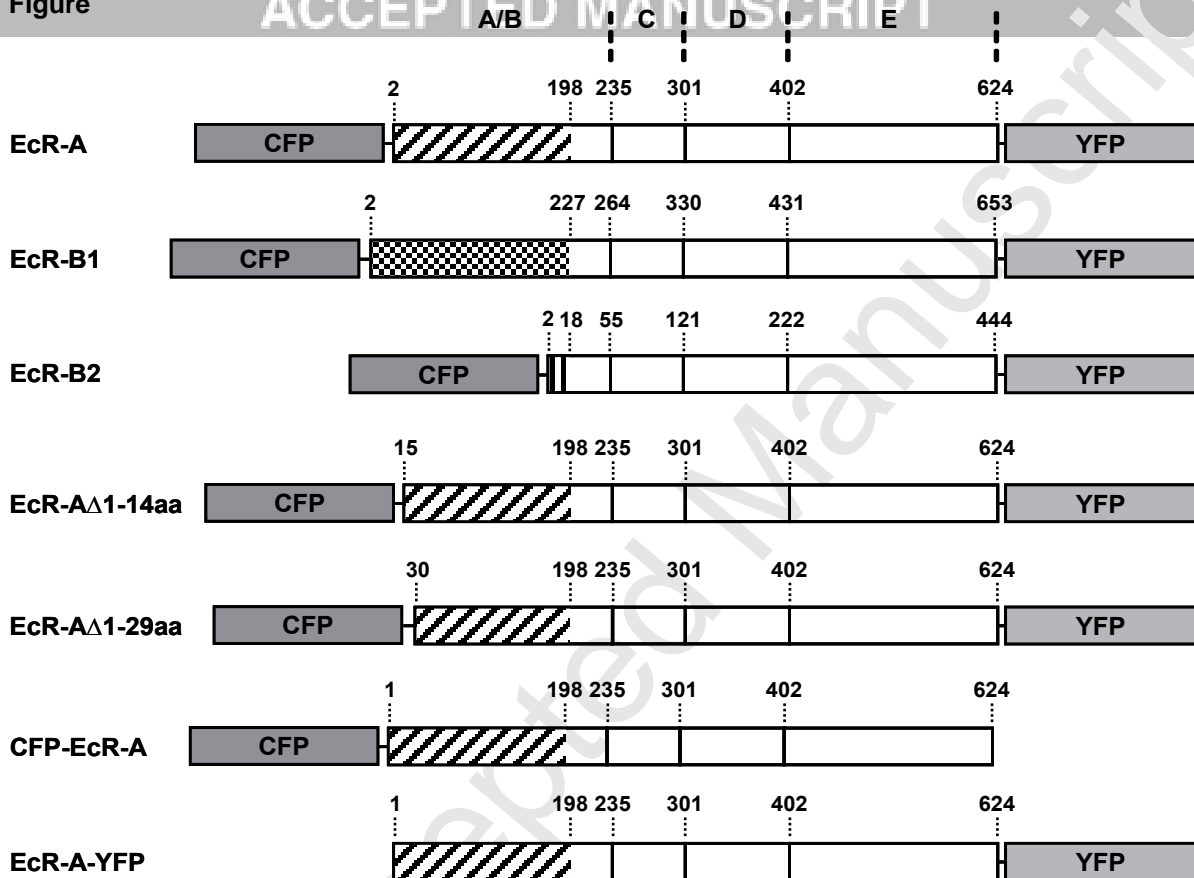


Fig. 1

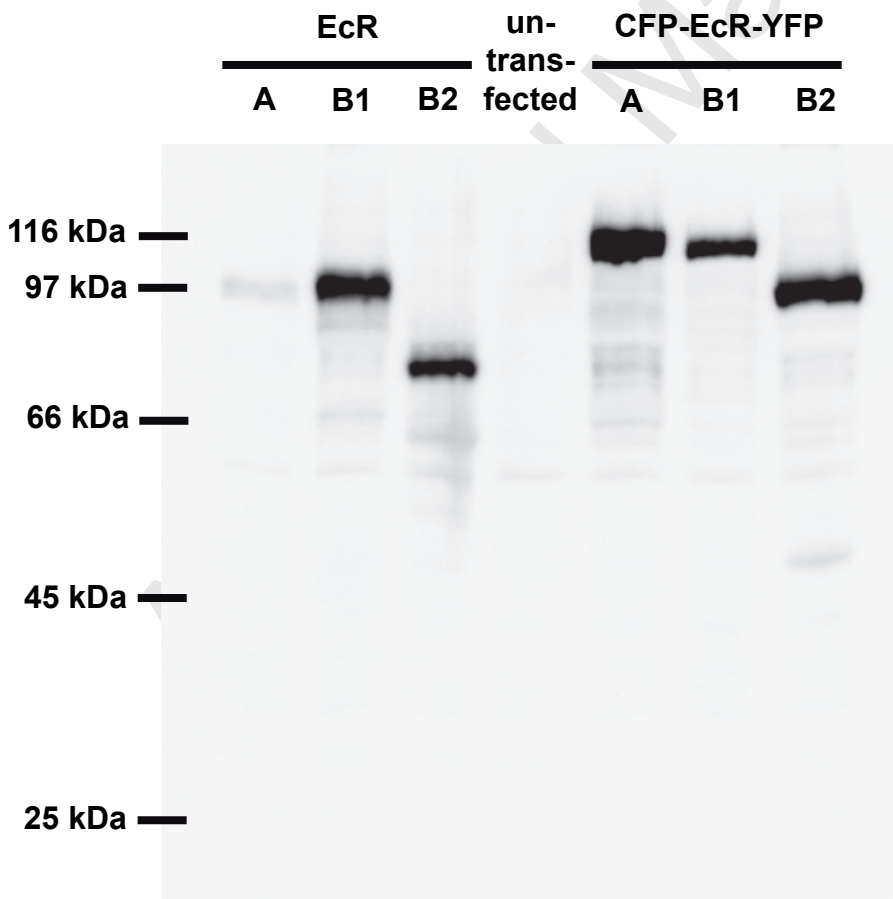
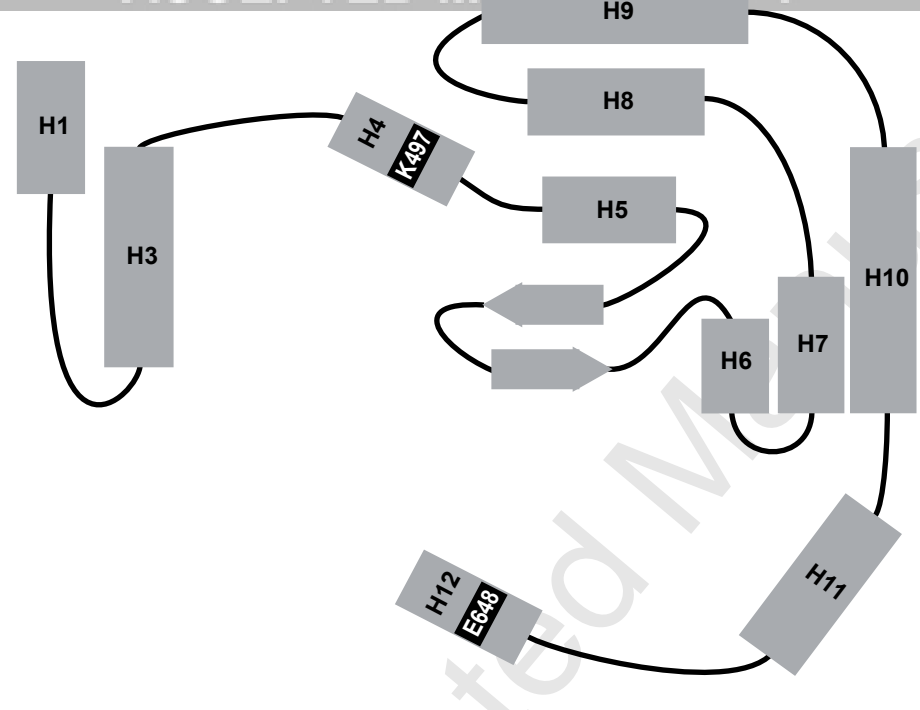


Fig. 2



closed conformation

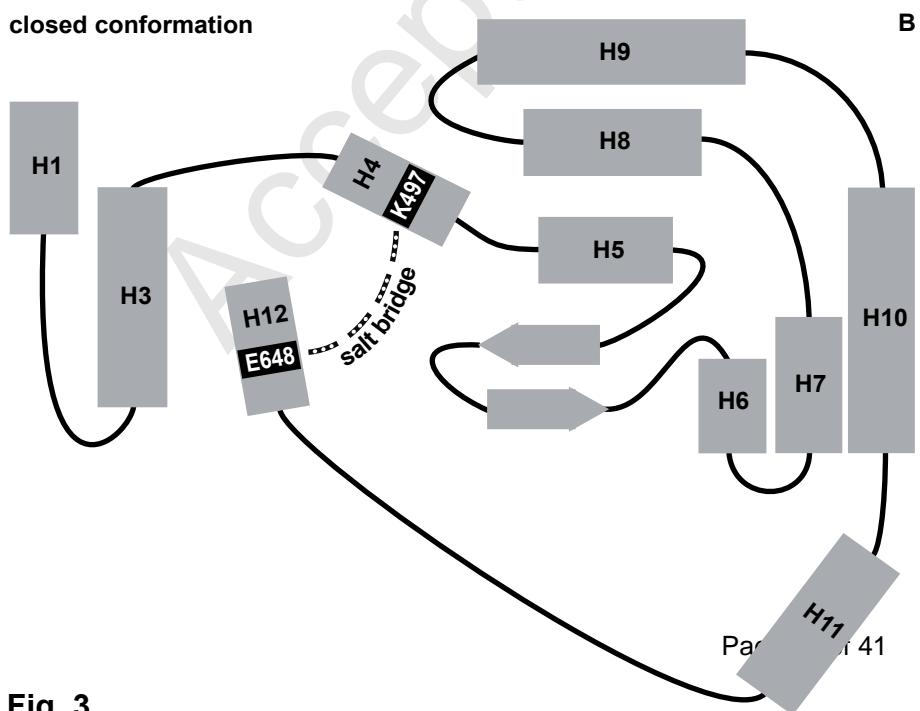


Fig. 3

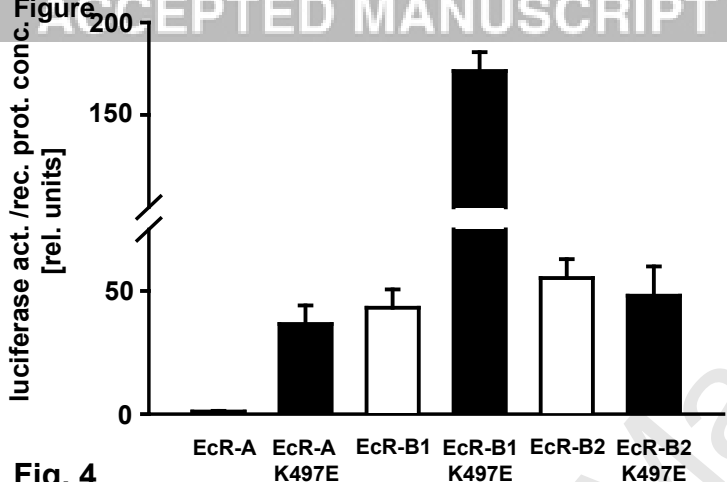


Fig. 4

