Integrative approaches and complementary techniques for complex analysis

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The challenge of myriads of complexes

Protein act as complexes. From analysis of ~ 6200 yeast proteins

30 000 binary interactions (by focused small scale experiments)

Affinity purification of 1732 proteins \rightarrow 232 complexes composed of # 7.5 proteins per complex

9 partners per protein and 3.6 partners per domain (not all direct or at the same time)

Distribution of protein complexes in the PDB (intervals of 250 residues)

Under-representation of structures from large complexes in view of the estimated average of 7.5 protein per complex



Many macromolecules are recalcitrant to main structural biology methods

Structural data on complex systems is often limited to isolated subunits and their domains or to low resolution envelopes by SAXS or EM.

Difficulty to express/reconstitute (incomplete bochemical characterization) and poorly abundant

Conformational heterogenity (prevents cristallisation and high resolution cryo-EM or do not stay intact during analysis (dissocation and/or aggregation on the EM grid)

Integrative determination of macromolecular structures

Aim: combine heterogenous data (information of any source) and propose hybrid models to provide the best possible description of the system (a set of models consitent with available data).





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Atomic, multi-scale and topologic models

Genomic region from 3C data



MOLECULAR STRUCTURE OF
NUCLEIC ACIDSJ. D. WATSONNo. 4356April 25, 1953J. D. WATSON
F. H. C. CRICKJ. D. WATSON
F. H. C. CRICKNATURE



To understand and modulate cellular processes, we need their models. These are best generated by considering all available information.

Integrative approaches

1/ Principles of integrative structure determination

2/ The example of the Nuclear Pore Complex

3/ A few experimental methods for complex analysis

Integrating modelling platforms

Integrative modeling platform (IMP)

Russel D, Lasker K, Webb B, Velazquez-Muriel J, Tjioe E, et al. Putting the pieces together:integrative modeling platform software for structure determination of macromolecular assemblies. PLoS Biol 2012;10, e1001244.

Inferential Structure Determination (ISD) framework

Rieping W, Nilges M, Habeck M. ISD: a software package for Bayesian NMR structure calculation. Bioinformatics 2008;24:1104–5.

HADDOCK

van Zundert GC1, Rodrigues JP1, Trellet M2, Schmitz C3, Kastritis PL4, Karaca E4, Melquiond AS5, van Dijk M6, de Vries SJ7, Bonvin AM1. The HADDOCK2.2 Web Server: User-Friendly Integrative Modeling of Biomolecular Complexes. J Mol Biol. 2016 Feb 22;428(4):720-5.

RNABuilder

Flores SC, Sherman MA, Bruns CM, Eastman P, Altman RB. Fast flexible modeling of RNA structure using internal coordinates. IEEE/ACM Trans Comput Biol Bioinform 2011;8:1247–57.

Integrative Modeling Platform (IMP) http://integrativemodeling.org



The four stages of integrated modelling



Integrative structure modeling of RNA Polymerase II stalk

RNA Pol II is a eukaryotic complex that catalyzes DNA transcription to synthesize mRNA strands Eukaryotic RNA polymerase II contains 12 subunits, Rpb1 to Rpb12

The yeast RNA Pol II dissociates into a 10-subunit core and a Rpb4/Rpb7 heterodimer

Rpb4 and Rpb7 are conserved from yeast to humans, and form a stalk-like protrusion extending from the main body of the RNA Pol II complex

Rpb4/Rpb7



1/ Gathering data

Determine the localization of two subunits of the yeast RNA Polymerase II, Rpb4 and Rpb7 (stalk), hypothesizing that we already know the structure of the remaining 10-subunit complex based on:

2/ Representing and translating data into restraints

- chemical cross-linking coupled with mass spectrometry (CX-MS),
- negative-stain electron microscopy (EM),
- X-ray crystallography data



4/ Analysis and assesment



Integrative structure modeling of RNA Polymerase II stalk



Gathering data Represent and translate data into restraints Sampling good scoring configurations Analysis and assesment

Experimental map of entire complex at 20.9Å resolution

RX-ray structures of the 10-subunit core of RNA Pol II and of parts of Rbp4 and Rbp7

Chemical cross-linking coupled with mass spectrometry (CX-MS)





https://integrativemodeling.org/



Experimental map

https://integrativemodeling.org/

Representation:

Scoting function * SEV Define a scoring function, by which the individual structural models will be scored based on the input data

A simple sum of individual restraints

Each restraint maps to one of our input experiments physical/statistical information

> Sequence connectivity restraint: residues that are adjacent in sequence will also be close in space due to the peptide bond

Excluded volume restraint: one protein cannot occupy the same space as another

EM restraints: A density overlap function to compare the GMM approximation of our model (em components) with that of the EM map (target_gmm_file)

No electrostatics or stereochemistry; very different to a typical molecular mechanics simulation

Represent and translate data into restraints

Gathering data



Analysis and assesment



Define a scoring function, by which the individual structural models will be scored based on the input data

A simple sum of individual restraints

Each restraint maps to one of our input experiments or other physical/statistical information

Sequence connectivity restraint: residues that are adjacent in sequence will also be close in space due to the peptide bond

Excluded volume restraint: one protein cannot occupy the same space as another

EM restraints: A density overlap function to compare the GMM approximation of our model (em_components) with that of the EM map (target_gmm_file)

Cross-linking restrains: protein and residue numbers for each of the two linked residues (cross linker length,

Represent and translate data into restraints

Gathering data



Analysis and assesment



Here Monte Carlo is used to sample (not minimize) system (generate many models that satisfy the data)

Need to define a set of movers: rigid_bodies defines the components that will be moved as rigid bodies (in this case, the parts of Rpb4 and Rpb7 for which we have atomic structure). Unstructured regions will move as flexible beads.

Sampling:

Simplex Conjugate Gradients Monte Carlo Brownian Dynamics Molecular Dynamics Replica Exchange Divide-and-conquer enumeration



Srb (super rigid body)

https://integrativemodeling.org/

Cluster (group by similarity) the sampled models to determine high-probability configurations.

- Chose a reference and align (superpose) all structures
- Calculate distances between structures (RMSD)
- Calculate localization densities for selected subunits



https://integrativemodeling.org/

Gathering data

Examples of Recently Determined Integrative Structures



https://integrativemodeling.org/

Topological models from MS-based hybrid analysis



Politis et al. 2015 (Robinson and Sali's labs)

The main limitation: sample preparation

buffer exchange or desalting procedure

Ultra centrifugation

- micro-concentrators :
- Microcon, Centricon, Amicon (Millipore)
- Vivaspin (Vivasciences)



- centrifuge
- 5 to 7 cycles at least
- takes time but very efficient procedure !

Size exclusion chromatography

gel filtration colums :NAP-10 et NAP-5 (GE Healthcare)



- Often 2 runs with a concentration step in between
- takes less time but dilutes the sample

Equilibrium dialysis

- dialysis or mini-dialysis units :
 - Slide-A-Lyzer minidialysis (Pierce)



- dilutes the samples
- very easy to perform (overnight)

Insights into disassembly pathways and composition of sub-complexes



Subunit composition and stoichiometry Dissociation pathways, sub-complexes. -acceleration voltage, pressure-



Hernandez, EMBO Rep 2008



A topological model from MS data

Native-MS data and excluded volume restraints (r = f((MM))) are not sufficient

Additional constrains are needed





Subcomplex	ADCD	ADCDE
Number of subunits	4	5
Subcomplex connectivity restraint and excluded volume restraint		
Fraction of topologies that match	8.95	7.52
the native topology (%)		
*Subcomplex connectivity restraint, binary interactions and excluded v		
Fraction of topologies that match	35.6	43.51
the native topology (%)		

Integrated representation of the eIF3 yeast initiation translation factor



Politis et al. 2015 (Robinson and Sali's labs)

Topological models from MS-based hybrid analysis





X-link/MS experiments



MW 572.43; Crosslink Mass 138.07 Spacer Arm 11.4 Å

"Na⁺

"Na

Na⁺0

Linear and circular representation



Rappsilber, 2010, 2012

H/D exchange



Peptides that interact with a partner

Integrative approaches

1/ Principles of integrative structure determination

2/ The example of the Nuclear Pore Complex

3/ A few experimental methods for complex analysis

A founding example: the nuclear pore complex (NPC)

Yeast NPCs are ~50 Mda structures built of multiple copies of some ~30 different proteins (nucleoporins), totalling at least 456 protein molecules

Each NPC is a plastic structure embedded in the nuclear envelope and is composed of eight morphologically similar 'spokes' surrounding a central Tube

Filling this tube and projecting into both the cytoplasmic and nuclear sides are flexible filamentous domains from proteins termed FG (phenylalanine-glycine) repeat nucleoporins; these domains form the docking sites for transport factors that carry macromolecular cargoes through the NPC



Albert et al. 2007, 2008

Integrating spacial restrains from proteomic data



Experimental data





Label free quantitation by MS



MaxQuant iBAQ: Schwanhäusser, B. *et al.* Global quantification of mammalian gene expression control. *Nature* **473**, 337–342 (2011).



Label free quantitation by MS

Label-free quantification approaches aim to correlate the mass spectrometric signal of intact proteolytic peptides or the number of peptide sequencing events with the relative or absolute protein quantity directly.

Relative quantitation strategies compare the levels of individual peptides in a sample to those in an identical, but experimentally modified, sample.

Absolute quantification can be obtained *estimated* from analysis of several mass spectrometric signal (TOP3 where the intensity of the selected peaks is taken into account) or the number of peptide sequencing events (emPAI == exponentially modified Protein Abundance Index).


Quantitation by MS

Label free quantitation (MAxQuand iBAQ) intensity-Based Absolute Quantification

QconCAT MS. (Internal standard = synthetic concatemer of tryptic peptides)

SILAC MS Relative quantification between preparations (Stable Isotope Labelling in Culture)

In vivo Calibrated Imaging Absolute quantification

Lipid Analysis Membrane composition

Native MS Absolute mass of isolated complexes















CryoET and sub-tomogram averaging

- 3D map at approximately 28 Å resolution, 1864 sub-tomograms
- nuclear envelope membrane







Gaussian Mixture Models (GMMs)





SAXS analysis and Ab-initio reconstruction



Localization of proteins by immuno-EM. Immuno-EM montages for Pom152–PrA nuclei and Ndc1–PrA nuclear envelopes with goldlabelled antibodies. (Alber, 2007)





Comparative modelling

- 1/ Search for templates (or 'parents')
- 2/ Align the target sequence with the parent(s)
 - structurally conserved regions
 - structurally variable regions
- 3/ Inherit the SCRs from the parent(s)
- 4/ Build the SVRs
- 5/ Build the sidechains
- 6/ Refine the model
- 7/ Evaluate errors in the model





Homology modeling

1 (ou several) 3D structure(s)
 1 multiple sequence alignment



htrß 1	209	PTDEEWELIKTVTEAHVATNAOGSHWKOKRKFLPEDIGOAPIVNAPEGGKVD	260
hPPARa	199	ETADLKSLAKRIYEAYLKNFNMNKVKARVILSGKASNNPFFVIHDMETLCMAEKTLVAKLVANGIONKEVE	269
hROR1	269	SMAELEHLAONISKSHLETCOYLREELOOITWOTFLOEEIENYONKOR	316
hVDR	124	LSEEOORIIAILLDAHHKTYDPTYSDFCOFRPPVRVNDGGGSHPSRPNSRH+++LSEEDSDDPSVTLELSO	223
dE75A	339	ELDDOPRLLAAVLRAHLETCEFTKEKVSAMBORARDCPSYSMPTLLACPLNPAPE	403
rNGFI-B	322	PDASPTNLLTSLIRAHLDSGPNTAKLDYSKFOELVLPRFGKED	364
hRARy	182	LSPOLEELITKVSKAHOETFPSLCOLGKYTTNSSAD	223
	· ·		
hRXRα	224	SSANEDMPVERILEAELAVEPKTETYVEANMGLNPSSP	261
rHNF-4	135	YEDSSLPSINALLOAEVLSOOTTSPISGINGDIRAKR	171
dusp	232	NSVSRDFSIERIIEAEORAETOCGDRALTFLRVGPYSTVOPDY	274
hCOUP-TFI	181	GHCYLSGYTSLLLRAEPYPTSRYGSOCMOPNN	212
		AF2-a	
hER	308	ISLTADOMYSALLDAEPPTLYSEYDPTRPFSE	339
hPR	680	DIOLIPPLINLLMSIEPDVIYAGHDNTKPDTS	711
hAR	666		697
hGR	525		556
hMR	731		762
	/31		/01
		H1 (H2)	
		signature	
		h hh ϕ_{AK} hp F I. DO II. h hh	
Ի	261	LEARSHEWET TYDA TYDUNDER KILDWECELDOT TLLKGCOMETMSLEADUR YNDES	320
hppapo	270	UP THE COORDENS THE TREE AT A BASIN IN CARE THE COMPANY AND A COMMUNICATION AND A COMU	320
hPOP1	317	VALUE OCCUPY THE A CONTRACT AND ADDRESS OF THE ADDR	376
hIDD	31/		3/0
IVDR	224		203
CLE/SA	404	LOSEQEF SQRFARVIRGVIDFAGAIFGFQLLTQDDKFTLLKAGLFDALFVRLICAFDSSI	403
rNGFI-B	365	AGDVQQFYDLLSGSLDVIRKWAEKIPGFIELSPGDQDLLLESAFLELFILRLAYRSKPGE	424
hrary	224	LGLWDKFSELATKC <mark>IIKIVEFAKRLPGFTGLSIADQ</mark> ITLLKAACLD <mark>ILML</mark> RICTRYTPEQ	283
nRXRO	262	NDPVTNICQAADKQLFTLVEWAKRIPHFSELPLDDQVILLRAGWNELLIASFSHRSIAVK	321
rHNF-4	172	IASITDVCESMKEQLUVLVEWAKYIPAFCELULDDQVALLRAHAGEHLLLGATKRSMVFK	231
dusp	275	KGAVSALCQVVNKQ <mark>L</mark> FQ <mark>MVEYARMMPHF</mark> AQ <mark>V</mark> FLD DQ VI <mark>LL</mark> KAAWIELLIANVAWCSIVSLDDG+++QP	361
hCOUP-TFI	213	IMGIENIÇELAARL <mark>L</mark> FS <mark>AVEWAR</mark> NIPFFPDLQITDQVŞLLRLTWSELFVLNAAQCSMPLHV	273
hER	340	ÅSMMGLLTNLÅDRELVHMINWAKRVPGFVDLTLHDOVHLLECAWLEILMIGLVWRSMEHP	399
hPR	712	SSLLTSLNQLGERQLLS <mark>VVKWSKSLPGF</mark> RNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSG	773
hAR	698	AALLSSLNELGEROLVHVVKWAKALPGFRNLHVDDOMAVIOYSWMGLMVFAMGWRSFTNVNS	759
hGR	557	WRIMTTLNMLGGROVIAAVKWAKAIPGFRNLHLDDOMTLLOYSWMFLMAFALGWRSYROSSA	618
hMR	763	ENLLSTLNRLAGKONIOVYKWAKYLPGFKNLPLEDOITLIOYSWMCLSSFALSWBSYKHTNS	824
	100		0
		H3 H4 H5	
		whith h	
hmp@1	221		202
hTRβ1	321	E h h Lh h	383
hTR β 1 hPPAR α	321 330	E h h Lh h ETLTLNG-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLINSSDRPGLAC MLVAYGNGFITHEFLKSLKK-FFCDIMEEKFDFAMKFNA-LELDDSDISLFVAAI <mark>IC</mark> GGDRPGLLN	383 393
hTRβ1 hPPARα hROR1	321 330 377	E h h Lh h ETLTLNG-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLLMSSDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMEPKFDFAMKFNA-LELDDSDISLFVAAIICCGDRPGLLN NTVYFDN-GKYASPDVFKSL-GCEDFISFVFEFGKSLCS-MHLTEDEIALFSAFVLMSADRSVLQE	383 393 438
hTRβ1 hPPARα hROR1 hVDR	321 330 377 284	E h h Lh h ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLIMSSDRPGLAC MLVAYGNGFITREFLKSLRK-PFCDIMEPKFDFAMKFNA-LELDDBDISLFVAAIGCGDRPGLLN NTVYFDN-GKXASDPUFKSL-GCEDFISFVFEGKSLCS-MHLTEDEILAFSAFVLMSADRSMLQE MSWTCGNQDYKYRVSDVTKA-GHSLELIEPLIKFQVGLKKLNLHEEEHVLLMAICIVSPDRPGVQD	383 393 438 338
hTRβ1 hPPARα hROR1 hVDR dE75A	321 330 377 284 464	E h h Lh h ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLIMSSDRPGLAC MLVAYGNGFITREFLKSLRK-PFCDIMEPKFDFAMKFNA-LELDDSDISLFVAAIIC MTVJFDN-GKYASPDVFKSL-GCEDFISFVFEFGKSLCS-MHLTEDEIALFSAFVIKSADRSWLQE MSWTCGNOPKYRVSDVTKA-GHSLELIEFLIKFQVGLKKLNHEEEHVLIMAICIVSPDRPGVQD NSIICLN-GQVMRRDAIQNG-ANARFLVDSTFNFAERMNSMNLTDAEIGLFCAIVLITPDRPGLRN	383 393 438 338 527
hTRβ1 hPPARα hROR1 hVDR dE75A rNGFI-B	321 330 377 284 464 425	E h h Lh h ETLTLNG-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLUNSSDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMEPKFDFAMKFNA-LELDDBDISLFVAAIICGDRPGLLN NTVYFDA.GKXASPDVFKSL-GCEDFISFVFEGKSLCS-MLHITEDEIALFSAFVIMSADRSMLQE MSWTCGNQDYKKNVSDVTKA-GHSLELIEPLIKFQVGLKKLNLHEEHVLLMAICIYSPDRPGVQD NSIICLN-GQVMRRDAIQNG-ANARFLVDSTFNFAERMNSMNLTDAEIGLFCAIVLITPDRPGLRN GKLIFCS-GLVLHRLQCRRGFGWIDNILAFSRSLHG-LGVDVPAFACLSALVLIT	383 393 438 338 527 486
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hTRβ1 hPPARα hROR1 hVDR dE75A rNGFI-B hRARγ	321 330 377 284 464 425 284	E h h Lh h ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLLMSSDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMERKFDFAMKFNA-LELDDBDISLFVAAIICGDRPGLLN NTVYFDN-GKXASPDVFKSL-GCEDFISFVFFEGKSLCS-MHLTEDEIALFSAFVIMSADRSMLQE MSWTCGNQDYKKYVSDVTKA-GHSLELIEPLIKFQVGLKKLNLHEEHVLLMAICIYSPDRPGVQD NSITCLN-GQVMRRDAING-AMARFLVDSTFMFARMNSMILTDAEIGLFCAIVLTPDRPGLRN GKLIFCS-GLVLHRLQCARGFGDMIDMILAFSRSLHS-LGVDVPAFACLSALVLTTDRHGLQD DTMTFSD-GLTLNRTQMHA-GFGPLTDLVFAFAGQLLF-LEMDDTETGLSAICLICGDRMLEE	383 393 438 338 527 486 346
hTRβ1 hPPARα hROR1 hVDR dE75A rNGFI-B hRARγ hRXRα	321 330 377 284 464 425 284 322	E h h Lh h ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLÄMSSDRPGLAC MLVAYGNGFITREFLKSLRK-PFCDIMEPKFDFAMKFNA-LELDDBDISLFVAATICGDRPGLLN MTVYFDN-GKXASDPUFKSL-GCEDFISVFFEGKSLC3-MHLTEDEIALFSAFVLMSADRSMLQE MSWTCGNQDYKYRVSDVTKA-GHSLELIEPLIKFQVGLKKLNLHEEEHVLLMAICIVSPDRPGVQD NSIICLN-GQVMRRDAIQNG-ANARFLVDSTRNFARSMNSMNLTDAEIGLFCAIVLTPDRPGLRN GKLIFCS-GUVLRLQCARGFGDVIDNILFSRSLH4-LGVUPVAFACLSALVIITDRPGLRD DGILLAT-GLHVHRQMHAA-GFGPLTDLVFAFAGQLLF-LEMIDTETGLSAICLICGDRMDLEE DGILLAT-GLHVHRNSAHSÅ-GVGAIFDRVLTELVSKMRDMQMDKTELGCLRAIVLFNPDSKGLSN	383 393 438 338 527 486 346 385
hTRβ1 hPPARα hROR1 hVDR dE75A rNGFI-B hRARγ hRXRα rHNF-4	321 330 377 284 464 425 284 322 232	E b b Lh b h ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLIMSSDRPGLAC MLVAYGNGFITREFLKSLRK-PFCDIMERKFDFAMKFNA-LELDBDISLFVAATICGDRFGLLN MTVYFDN-GKYASPDVFKSL-GCEDFISFVFERGKSLCS-MHLTEDEIALFSAFVINSADRSMLQE MSWTCGNQDYKRVSDVTKA-GHSELLIEPLIKFQVGLKKLNLHEEHVLLMAICIYSPDRFGVQD MSITCLN-GQVMRRDAIQG-ANAFLVDSTFMFARMNSMNLTDAEIGLFCATVLTPDRFGLRN GKLIFCS-GLVLHRLQCARGFGDMIDMILAFSRSLHS-LGVDVFAFACLSALVLT	383 393 438 338 527 486 346 346 385 297
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$\begin{array}{l} hTR\beta 1\\ hPPAR\alpha\\ hROR1\\ hVDR\\ dE75A\\ rNGFI-B\\ hRAR\gamma\\ hRXR\alpha\\ rHNF-4\\ dUSP\\ hCOUP-TFI\\ \end{array}$	321 330 377 284 464 425 284 322 232 362 274	E h h Lh h ETLITING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLIDEVALLQAVLIMSSDRPGLAC MLVAYGNGFITREFLKSLRK-PFCDIMEPKFPFAMKFNA-LELDDSDISLFVAAICCGDRPGLN MTVYFDN-GKYASPDVFKSL-GCEDFISFVFFGKSLCS-MHLTEDEILFSAFVINSADRSWLQE MSWTCGNQDYKYNSDVTKA-GHSELLIEPLIKFQVGLKKLNLHEEHVLLMAICTVSPDRPGVQD MSITCLN-GQVMRRDAINGG-ANAFLVDSTFNFARMNSMNLTDAEIGFCATVLTPDRPGLRN GKLIFCS-GLVLHRLQCARGFGDWIDNILFSRSLHS-LGVDYPAFACLSALVITDRGLQD DTMTFSD-GLTLNRTQMHAA-GFGPLTDLVFAFAQQLL-LGVDYPAFACLSALVIT	383 393 438 338 527 486 346 385 297 427 337
hTRβ1 hPPARα hROR1 hVDR dE75A rNOFI-B hRARγ hRARγ hRXRα rHNF-4 dUSP hCOUP-TFI	321 330 377 284 464 425 284 322 232 362 274	E h h Lh h ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLIDTEVALLQAVLLMSSDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMERKFDFAMKFNA-LELDDBDISLFVAAIICGDRPGLAC MIVYFDN-GKXASPDVFKSL-GCEDFISVVFFGKSLC3MLTEDEIALFSAFVLMSADRSWLQE MSWTCGNQDYKYNVSDVFKA-GHSLELIEPLIKFQVGLKKLNLHEEHVLLMAICTVSPDRPGVQD NSIICLN-GQVMRFNDIQNG-ANARFLVDSTFMFARMNSMNLITDAREIGFCAIVLITSDRPGLRN GKLIFCS-GLVLHRLQCARGFGDVIDINIAFSRSLHS-LGVDVPAFACLSALVLITDRHGLQD DTMTFSD-GLTLNRTQMINA-GFGPLTDLVFAFAGQLLE-LEMIDTETGLSAIVLITDRHGLQD DGILLAT-GLHVHRNSAHSÅ-GVGAIFDRVLTELVSKMNDMQMIKTELGCLRAIVLFNPDRKGLSN QQLFLNQ-SFSYHRNSAIKA-GVSAIFDRILSELSVKMKRLNLDRELSCLKAIIFYNPDAKGLSD QQLFLNQ-SFSYHRNSAIKA-GVSAIFDRILSELSVKMKRLNLDRELSCLKAIILYNPDIRGIKS APLLAAA-GLHASPMSADRV-VAFMIHRIFQEQQVKKLKALHVISAEYSCLKAIVLFTS	383 393 438 338 527 486 346 385 297 427 337
hTRβ1 hPPARα hROR1 hVDR dE75A rNGFI-B hRARγ hRARα rHNF-4 dUSP hCOUP-TFI hER	321 330 377 284 464 425 284 322 232 362 274	E h h Lh h ETLTLING-EMAVTRQQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLIMSSDRPGLAC MLVAYGNGFITREFLKSLRK-PFCDIMEPKFDFAMKFNA-LELDDBDISLFVAATICGDRPGLAC MTVYFDN-GKXASDPUFKSL-GCEDFISVFFERGKSLG-MHLTEDEIALFSAFVLMSADRSWLQE MSWTCGNQDYKYRVSDVTKA-GHSLELIEPLIKFQVGLKKLNLHEEEHVLLMAICIVSPDRPGVQD NSIICLN-GQVMRRDAIQNG-ANARFLVDSTRNFARMNSMNLTDAEIGLFCAIVLTPDRPGLRN GKLIFCS-GUVLRLQCARGFGDUIDNILAFSRSLHG-LGVUPAFACLSALVIITDRPGLRD DGILLAT-GLHVHRNSAHSÅ-GVGAIFDRVLTELVSKMRDMQMDKTELGCLRAIVLFNPDŠKGLSN UVLLGN-DYIVFRICFELA-EMSRVSIRILDELVLPFGELQIDDEYACLKAITFFPPDRGLRSD QQLFING-SFSYRNSALKA-GVSAIFDRILSESVKMRLMLDRRELSCLKAITLFNPDAKGLSD GLLAAA-GLHASPMSADRV-VAFMDHIRIFQEQVEKLKALHVDSAEYSCLKAIVLFTSDAKGLSD GKLLFAP-NLLDRNQGKCVEGMVEIFDMLLÅTSSRFRM-MNLGBEFVCLKSIILLNSGVYŤFLSSTLKSLÉE	383 393 438 527 486 346 385 297 427 337
hTRβ1 hPPARα hROR1 hVDR dE75A rNGFI-B hRARγ hRARγ hRARα rHNF-4 dUSP hCOUP-TFI hER hPR	321 330 377 284 464 425 284 322 232 362 274 400 774	E h h Lh h ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLIDTEVALLQAVLLMSSDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMERKFDFAMKFNA-LELDDBJISLFVAAICGDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMERKFDFAMKFNA-LELDDBJISLFVAAICGDRPGLAC MSWTCGNQDYKRYSDVTKA-GHSLELIEPLIKFQVGLKKLNLHEEHVLLMATCIVSPDRPGVQD MSWTCGNQDYKRYSDVTKA-GHSLELIEPLIKFQVGLKKLNLHEEHVLLMATCIVSPDRPGVQD MSWTCGNQUMRRDAING-ANARFLVDSTFMFARMNSMINITDAEIGLFCAIVLTPDRPGLRN GKLIFCS-GLVLHRLQCARGFGDWIDMILAFSRSLHS-LGVDVPAFACLSALVLTTDRHGLQD DTMTFSD-GLTLNRTQMINA-GFGELTDLVFAFAGQLE-LEMIDTETGLSAICLICGDRMDLEE IGILLAT-GLHVHRNSAHSÅ-GVGAIFDRVİTELVSKMDMQMIKTELGCLRAIVLIPNPDSKGLSN IVLLGM-DJIVFRICPELA-EMSVSIRILDELVLPFGEQUIDMEYACLKAIIFFDPDAKGLSD QLFINQ-SFSYHRNSAIKA-GVSAIFDRLISELSVLMRKNLNLDRELSCLKAIIFFDPDAKGLSD GKLLFAP-NLLĎRNGKCVEGNVEIFDMLLÅTSSRFRM-MNLGGEEFVCLKSITLNSGVYŤFLSSTLKSLĚE GKLLFAP-DLLLĎROGKCVEGNVEIFDMLLÅTSSRFRM-MNLGGEEFVCLKSITLNSGVYŤFLSSTLKSLĚE	383 393 438 527 486 346 346 385 297 427 337 471 837
hTRβ1 hPPARα hPOR1 hVDR dE75A rNGFI-B hRARγ hRXRα rHNF-4 dUSP hCOUP-TFI hER hPR hAR	321 330 377 284 464 425 284 322 232 362 274 400 774 760	E h h Lh h ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLÜMSSDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMEPKFDFAMKFNA-LELDDSDISLFVAAIICGDRPGLAC MIVYZDN-GKXASDVFKSL-GCEDFISFVFFFGKSLCS-MLITEDEILAFSAFVLMSADRSMLQE MSWTGGNQDYKKNVSDVTKA-GHSLELIEPLIKFQVGLKKLNLHEEHVLLMAICIVSPDRPGLQN MSIICLN-GQVMRRDAIQNG-ANARFLVDSTFNFAENNSMLIDDEIGLFCAIVLTPDRPGLQN DSIICLN-GUVLRRLQCAR-OFFSDUIDNILFSSLHG-LGVUVPAFACLSALVLTPDRHGLQD DTMTFSD-GLTLNRTQMHA-GFGPLTDLVFAFAGQLLI-LEMIDTETGLSAIVLTPDRHGLQD DILLAT-GLHVHRNSAHSÅ-GVGAIFDRVLTELVSKMRDMQMDKTELGCLRAIVLFNPDSKGLSN UVLLGN-DYIVFRICPELA-EMSRVSINILDELVLFFGELQIDDNEYACLKAITEPPPDSKGLSD QUFFING-SFSYHRSAIKA-GVSAIFDRILSESVKMRLNLDRELSCKAIVLFNPDIKGLSD GKLLFAP-NLLDRNQGKCVEGMVEIFDMLLÅTSSRFRM-MNLQGEEFVCLKSIILLNSGVYFFLSSTLKSLĚE MLYFAP-DLILDRQGMKES-SFYSLCITMWQIPQEFVK-LQVSQEEFLCMKVLLLENTIPLGGLRS	383 393 438 338 527 486 346 346 385 297 427 337 471 837 823
hTRβ1 hFPARα hFOR1 hVDR dE75A rNGFI-B hRARγ hRARα rHNF-4 dUSP hCOUP-TFI hER hPR hAR hAR hAR	321 330 377 284 464 425 284 322 232 362 274 400 774 760 619	E h h Lh h ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLLMSSDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMERKFDFAMKFNA-LELDDBJISLFVAAICGDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMERKFDFAMKFNA-LELDDBJISLFVAAICGDRPGLAC MSWTCGNQDYKTHVSDVTKA-GHSEHIEPLIKFQVGLKKLNLHEEHVLLMATCIVSPDRPGVQD MSWTCGNQDYKTHVSDVTKA-GHSEHIEPLIKFQVGLKKLNLHEEHVLLMATCIVSPDRPGVQD MSWTCGNQOVMRRDAINGA-AHSLELIEPLIKFQVGLKKLNLHEEHVLLMATCIVSPDRPGVQD DTMTFSD-GLTLNRTQMHA-GFGPLTDLVFAFAGQLLF-LEMDDTETGLSAICLICGDRMDLEE IGILLAT-GLHVHRNSAHSÅ-GVGAIFDRVİTELVSKMRDMQMIKTELGCLKAIUTFDRHGLQD ITMTFSD-GLTLNRTQMHA-GFGPLTDLVFAFAGQLLF-LEMDDTETGLSAICLICGDRMDLEE IGILLAT-GLHVHRNSAHSÅ-GVGAIFDRVİTELVSKMRDMQMIKTELGCLKAIIFPDPDSKGLSN IVLLGAD-JTVFRICPELA-EMSVSIRILDELVLFFGEQUIDMEYACLKAIUFPDDAKGLSD QUEFINQ-SFSYHRNSAIKA-GVSAIFDRILSELSVKMRRNLNLDRELSCLKAIUFPDDAKGLSD GKLLFAP-NLLĎRNGKCVEGNVEIFDMLLÅTSSRFRM-MNLIGGEEFVCLKSIILNSGVYFFLSSTLKSLĚE GMLYFAP-DLILNGCRMKES-SFYSICLTMWQIPQEFVK-LQV3QEEFLCMKVLLLNSIFSIFSVDGLKN NLLCFAP-DLINNGCRMES-SFYSICLTMWQIPQEFVK-LQV3DEFLCMKVLLLNSIFSIFS	383 393 438 338 527 486 346 346 385 297 427 337 471 8337 471 823 682
hTRβ1 hPPARα hROR1 hVDR dE75A rNGFI-B hRARγ hRXRα rNNF-4 dUSP hCOUP-TFI hER hPR hAR hAR hAR hAR	321 330 377 284 464 425 284 322 232 362 274 400 774 760 619 825	E h h Lh h ETLTLNG-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLIDTEVALLQAVLUNSSDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMERKFDFAMKFNA-LELDDBJSLFVAAICGDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMERKFDFAMKFNA-LELDDBJSLFVAAICGDRPGLAC MSWTCGNQDYKTNVSDVTKA-GHSLHLIPPLIKFQVGLKKLNLHEEHVLLMAICTVSPDRPGLQD MSIICLN-GQVMRDAIQNG-ANARFLVDSTFNFAERMNSMNLTDAEIGLFSAFVINSADRPGVQD NSIICLN-GQVMRDAIQNG-ANARFLVDSTFNFAERMNSMNLTDAEIGLFSAFVINSADRPGLQD DTMTFSD-GLTLNRTQMINA-GFGPUTDLVFFFAGLLI-LGVUVPAFACLSALVLTFDRPGLQD UTMTFSD-GLTLNRTQMINA-GFGPUTDLVFFFAGLLI-LENDDTETGLSAICIICGDRPGLSN QULFLNG-SFSYHRNSAHSA-GVGAIFDRUTELVSKMNDMQMIKTELGCLRAIVIPPDSKGLSN UVLLGN-DYIVFRHCPELA-EMSRVSIRILDELVLPFGEQUIDDNEYACLKAIIFPDPDAKGLSD QULFLNG-SFSYHRNSAIKA-GVSAIFDRILEELSVMMKINLNIRRELSCLKAIVIFTSDAKGLSD GKLLFAP-NLLIDRNQGKCVEGMVEIFDMLLÅTSSRFN-MNLGGEFVCLKSILLNNGVYŤFLSSTLKSLEE GMLYFAP-DLILNGQRMTUFONLLÅTSSRFN-MNLGGEFVCLKSILLNSGYTFLSSTLKSLEE GMLYFAP-DLILNGQRMTHS-RNYSQCVMMRLSQFGM-LQTPQSFLCMKVLLLSSTP	383 393 438 338 527 486 346 385 297 427 337 471 837 823 682 888
hTRβ1 hPPARα hPOR1 hVDR dE75A rN0FI-B hRARγ hRARγ hHR-4 dUSP hCOUP-TFI hER hPR hAR hGR hMR	321 330 377 284 464 425 284 322 232 2362 274 400 774 760 619 825	E b bLh b ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLDDTEVALLQAVLLMSSDRPGLAC MLVAYGNGFITREFLKSLRK-PFCDIMEPKFPAKKINA-LELDBDISLFVAAICGDRFGLAC MTVYFDN-GKYASPDVFKSL-GCEDFISVFFFGKSLCS-MHLTEDEIALFSAFVLMSADRSWLQE MSWTCGNQDYKTVSDVTKA-GHSELLIEPLIKFQVGLKKLNLHEEHVLLMAICIVSPDRFGVQD MSWTCGNQDYKTVSDVTKA-GHSELLIEPLIKFQVGLKKLNLHEEHVLLMAICIVSPDRFGVQD MSITCLN-GQVMRFDAING-ANSHLVDSTFMFARMNSMNLTDAEIGLFCATVLTPDRFGLRN GKLIFCS-GLVLHRLQCARGFGDWIDMILAFSRSLHS-LGUDVFAFACLSALVLTDRHGLQD DGILLAT-GLHVHRNSAHSÅ-GVGAIFDRVLTELVSKNRDMQMDKTELGCLRATVLFNPDSKGLSN UVLLGN-DYIVFRICPELA-EMSRVSINILDELVLPFGELQIDDNEYACLKAIIFFDPDSKGLSN QUEFINQ-SFSYHRNSAIKA-GVSAIFDRLISELSVKMRRLNLDRELSCLKAIIFFDPDAKGLSD GKLLFAP-NLLLDRNQGKCVEGMVEIFDMLLÅTSSRFRM-MNLGGEEFVCLKSIILNNGVYFLSSTLKSLEE GKLLFAP-NLLLDRNMKSS-SFYSLCLTMWQLPQEFVK-LQVSLKSIILLNNGVYFLSSTLKSLEE RMLYFAP-DLIINEQRMKSS-SFYSLCLTMWQLPQEFVK-LQVSLEFLCMKALLLNTIPLEGLRS RMLYFAP-DLIINEQRMTHS-SMYSLCQUMHQISLQFVK-LQVSYEFLCMKATLLIKSIFP	383 393 438 338 527 486 346 385 297 427 337 471 837 682 888
hTRβ1 hPPARα hROR1 hVDR dE75A rNOFI-B hRARγ hRXRα rHNF-4 dUSP hCOUP-TFI hER hPR hAR hGR hMR	321 330 377 284 464 425 284 322 232 362 274 400 774 760 619 825	E h h Lh h ETLTLING-EMAVTRGQLKNG-GLGVVSDAIFDLGMSLSS-FNLIDTEVALLQVLLMSSDRPGLAC MLVAYGNGFITREFLKSLRK-FFCDIMERKFDFAMKFNA-LELDDBJSLFVAAICGDRPGLAC MIVYFDN-GKXASPDVFKSL-GCEDFISVFFEGKSLC3MHITEDEIALFSAFVLMSADRSMLQE MSWTCGNQDYKYRVSDVFKA-GHSLELIEPLIKFQVGLKKLNLHEEHVLLMATCTVSPDRPGLQD MSITCLN-GQVMRRDAIQNG-ANARFLVDSTFMFARMNSMILTDAREIGFCATVLTFPDRPGLQD DTMTFSD-GLTLNRTQMINA-GFGPLTDLVFAFAGQLE-LEMIDTETGLSAICLICGDRHGLQD DTMTFSD-GLTLNRTQMINA-GFGPLTDLVFAFAGQLE-LEMIDTETGLSAICLICGDRHGLQD DTMTFSD-GLTLNRTQMINA-GFGPLTDLVFAFAGQLE-LEMIDTETGLSAICLICGDRHGLQD DTULLGD-DYTVFRICPELA-EMSVSIRILDUVFGFUGLIDNEYACLKAIIFPDP	383 393 438 338 527 486 346 346 385 297 427 337 427 337 421 823 682 888
hTRβ1 hPPARα hPOR1 hVDR dE75A rNGFI-B hRARγ hRARγ hRARγ hCOUP-TFI hER hCOUP-TFI hER hAR hGR hMR	321 330 377 284 464 425 284 322 232 362 274 400 774 760 619 825	$\label{eq:linear} \begin{array}{c c c c c c c c c c c c c c c c c c c $	383 393 438 338 527 486 346 346 385 297 427 337 427 337 471 837 823 888
hTRβ1 hFPARα hROR1 hVDR dE75A rNGFI-B hRARγ hRARα rHNF-4 dUSP hCOUP-TFI hER hPR hAR hAR hAR hMR	321 330 377 284 464 425 284 322 232 362 274 400 774 760 619 825	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{h} \\ $	383 393 438 338 527 486 346 346 346 385 297 427 337 427 337 471 837 823 682 888
hTRβ1 hPPARα hOOR1 hVDR dE75A rNGFI-B hRARγ hRXRα rNF-4 dUSP hCOUP-TFI hER hPR hAR hGR hMR	321 330 377 284 462 284 322 232 362 274 322 274 400 774 760 9825	$\label{eq:linear} \begin{array}{c c c c c c c c c c c c c c c c c c c $	383 393 438 338 527 486 346 346 385 297 427 337 471 837 823 682 888
hTR β 1 hPPAR α hPOR1 hVDR dE75A rNOFI-B hRAR γ hRAR γ hRAR γ hRAR α rHNF-4 dUSP hCOUP-TFI hER hPR hAR hMR hMR	321 330 377 284 464 425 284 322 232 362 274 400 774 760 619 825	$\label{eq:constraint} \begin{array}{cccc} & & & & & & & & & & & & & & & & & $	383 393 438 527 486 346 346 346 347 337 427 337 427 337 471 837 823 888
$\label{eq:hardenergy} \begin{split} & h TR\beta 1 \\ h PPAR\alpha \\ h PPAR\alpha \\ h CR1 \\ h VDR \\ dE75A \\ r M GFI-B \\ h RAR \\ \gamma \\ h RAR \\ \gamma \\ h RAR \\ \gamma \\ h RAR \\ h COUP-TFI \\ h ER \\ h PR \\ h AR \\ h GR \\ h MR \\ h MR \\ h TR\beta 1 \\ h PPAR \\ \alpha \\ \end{split}$	321 330 377 284 465 284 322 232 232 232 274 400 774 760 619 825	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{H}} & \underline{\mathbf{A}} & \underline{\mathbf{G}} & \underline{\mathbf{H}} & \underline{\mathbf{F}} & \underline{\mathbf{C}} & \underline{\mathbf{H}} & \mathbf{$	383 393 438 527 486 346 385 297 427 337 427 337 427 337 421 823 682 888
hTR β 1 hPPAR α hPOR1 hVDR dE75A rNOFI-B hRAR γ hRAR γ hHRR α rHNF-4 dUSP hCOUP-TFI hER hPR hAR hGR hMR hMR hTR β 1 hPPAR α hROR1	321 330 377 284 464 425 284 322 232 362 274 400 774 760 619 825 384 394 439	$\begin{array}{cccc} & \underline{\mathbf{E}} & \underline{\mathbf{h}} & \mathbf{$	383 393 438 527 486 346 385 297 427 337 471 823 682 888 888 456 466 511
hTR β 1 hPPAR α hVDR dE75A rNGFI-B hRAR γ hRXR α rNGFI-B hRXR α rNF-4 dUSP hCOUP-TFI hER hPR hAR hGR hMR hMR hTR β 1 hPPAR α hOPAR α hVDR	321 330 377 284 464 425 284 322 232 262 274 400 774 760 825	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{H}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{H}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{H}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{H}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{H}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{h}} & \mathbf{$	383 393 438 527 486 385 297 427 337 427 337 427 337 427 337 427 427 337 427 588 888 456 466 511
$\label{eq:hardborndensity} \begin{split} & h TR\beta 1 \\ h PFPAR\alpha \\ h PFPAR\alpha \\ h CR 1 \\ h VDR \\ dE75A \\ rNoF1-B \\ h RRAF \\ h MRAF \\ h MRAF \\ h COUP-TFI \\ h ER \\ h COUP-TFI \\ h ER \\ h AR \\ h GR \\ h M \\ h M $	321 330 377 284 464 425 284 322 232 362 274 400 7760 619 825 384 394 439 3394 328	$\label{eq:construction} \begin{split} & \underline{\mathbf{k}} \underline{\mathbf{h}} \mathbf{b} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \mathbf{b} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \underline{\mathbf{h}} \\ & \underline{\mathbf{h}} $	383 393 438 527 426 346 346 346 346 486 346 486 346 483 488 888 456 466 511 424 426 602
$hTR\beta 1hPPAR\alphahROR1hVDRdE75ArNGFI-BhRAR\gammahRXRArNNF-4dUSPhCOUP-TFIhERhPRhARhGRhMRhTR\beta 1hPPAR\alphahOR1hVDRdZ75ArNOFI-B$	321 330 377 284 464 425 284 322 232 262 274 400 774 760 825 384 394 4394 4394 4399 528	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}}$	383 393 393 385 527 486 346 346 346 346 346 427 337 471 823 682 888 426 511 424 602 563
hTR β 1 hPPAR α hPOR1 hVDR dE75A rNGFI-B hRAR γ hRXR α rNF-4 dUSP hCOUP-TFI hER hPR hAR hGR hMR hMR hPPAR α hROR1 hVDR dE75A rNGFI-B hRAR γ	321 330 377 284 464 425 284 322 232 362 274 3 22 274 4 00 774 776 619 825	$\label{eq:product} \begin{split} & \underline{\mathbf{k}} \underline{\mathbf{h}} \mathbf{k} \\ & \underline{\mathbf{k}} \underline{\mathbf{k}} \underline{\mathbf{k}} \underline{\mathbf{k}} \underline{\mathbf{k}} \underline{\mathbf{k}} \underline{\mathbf{k}} \\ & \underline{\mathbf{k}} $	383 393 527 486 346 346 346 346 427 337 427 823 337 471 837 823 868 888 456 456 511 424 602 553
$\label{eq:hardbarrendom} \begin{split} & h \mbox{tr} \beta \mbox{1} \\ & h \mbox{tr} p \mbox{2} \\ & h \mbox{cn} \mbox{1} \\ & h \mbox{cn} \mbox{cn} \mbox{1} \\ & h \mbox{cn} \mbox{cn} \mbox{1} \\ & h \mbox{cn} \mbo$	321 330 377 284 464 425 284 322 232 362 274 400 774 760 619 825 384 394 439 339 528 487 347	$\label{eq:product} \begin{array}{cccc} & \underline{\mathbf{b}} & \mathbf{b$	383 393 438 338 527 486 346 346 346 427 337 471 833 682 888 426 426 426 426 426 426 426 426 426 426
hTR β 1 hPPAR α hPOR1 hVDR dE75A rNGFI-B hRAR γ hRXR α rNHF-4 dUSP hCOUP-TFI hER hPR hAR hGR hMR hMR hMR hTR β 1 hPPAR α hROR1 hVDR dE75A rNGFI-B hRAR γ hRXR α	321 330 377 284 464 425 232 232 232 232 274 400 774 619 825 384 394 439 339 439 339 487 339 528 487 384	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{H} & \underline{\mathbf{h}}$	383 393 393 385 527 427 336 297 427 337 427 337 427 823 682 888 888 456 456 511 424 602 563 418 457
$\label{eq:hardensity} \begin{split} & h TR\beta 1 \\ h PFPAR\alpha \\ h PFPAR\alpha \\ h CR1 \\ h VDR \\ dE75A \\ rMOFI-B \\ h RAR\gamma \\ h RAR\gamma \\ h RAR\gamma \\ h COUP-TFI \\ h COUP-TFI \\ h COUP-TFI \\ h COUP-TFI \\ h RAR \\ h M \\ h MR \\ h M \\ h$	321 330 377 284 464 425 222 284 222 232 362 274 400 774 60 619 825 384 394 439 339 528 487 347 386	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \mathbf{E} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \mathbf{E} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \mathbf{E} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \mathbf{E} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \mathbf{E} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \mathbf{E} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \mathbf{E} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \mathbf{E} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \mathbf{E} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \mathbf{E} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \mathbf{E} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \mathbf{E} & \underline{\mathbf{c}} & \mathbf{$	383 393 438 338 527 486 346 346 346 427 427 337 337 427 823 888 888 456 466 662 888 888 456 418 424 602 563 418
$hTR\beta 1hPPAR\alphahPPAR\alphahPPARahPPARahPPARahARAhTRSThRXRarHNF-4dUSPhCOUP-TFIhERhPRhARhGRhMRhMRhMRhTR\beta 1hPPARahROR1hVDRdE75ArNOFI-BhRARYhRXRarHNF-4dUSP$	321 330 377 284 464 425 232 232 232 232 232 232 232 274 400 774 619 825 384 439 339 439 339 487 339 487 386 296	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{E} & \underline{\mathbf{h}}$	383 393 393 385 527 427 337 427 337 427 337 427 823 682 888 837 823 682 563 456 511 424 602 563 418 457 367 367
$\label{eq:hardensity} \begin{split} & hTR\beta 1 \\ hPPAR\alpha \\ hPPAR\alpha \\ hPPAR\alpha \\ hPPAR\alpha \\ rMOFI-B \\ hRAR\gamma \\ hRAR\gamma \\ hHRR\alpha \\ rHNF-4 \\ dUSP \\ hCOUP-TFI \\ hER \\ hPR \\ hAR \\ hGR \\ hMR \\ hMR \\ \end{split}$	321 330 377 284 464 425 222 284 222 284 222 274 400 774 60 619 825 384 394 439 339 528 487 347 386 426 296 426 296	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} & \underline{\mathbf{b}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}} \\ \\ \underline{\mathbf{E}} & \underline{\mathbf{c}}	383 393 438 338 527 486 346 346 385 297 427 337 337 427 823 888 888 456 466 662 888 888 45511 424 4602 563 418 457 497 497
hTRβ1 hPPARα hPPARα hVDR dE75A rNGFI-B hRARγ hRXRα rNNF-4 dUSP hCOUP-TFI hER hPR hAR hGR hAR hGR hMR hMR hTRβ1 hPPARα hVDR dE75A rNGFI-B hRARγ hRXRα rNF-4 dUSP hRXRα rNF-4 dUSP hRXRα rNF-4 dUSP hRXRα	321 330 377 284 464 425 232 232 232 232 274 400 774 400 619 825 384 439 528 487 339 528 487 3347 386 296 338	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \\ \underline{\mathbf{h}} & \mathbf{$	383 393 438 338 527 486 385 297 427 427 427 427 427 427 427 427 427 42
$\label{eq:hardenergy} \begin{split} & hTR\beta 1 \\ hPPAR\alpha \\ hPPAR\alpha \\ hPPAR\alpha \\ dE75A \\ rNoFI-B \\ hRAR\gamma \\ hRAR\gamma \\ hRAR\gamma \\ hRAR\gamma \\ hCOUP-TFI \\ hER \\ hPR \\ hAR \\ hGR \\ hMR \\ hGR \\ hMR \\ hGR \\ hMR \\ hGR \\ hMR \\ hGR \\ hAR \\ hGR \\ hAR \\ hGR \\ hAR \\ hGR \\ hAR \\ hGR \\ hAR \\ hGR \\ hAR \\ hGR \\ hAR \\ hGR \\ hAR \\ hGR \\ hMR \\ hGR \\ $	321 330 377 284 464 425 362 232 232 232 232 232 232 232 232 232	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{h} \\ \mathbf{E} \\ \mathbf{L} \\ \mathbf{L} \\ \mathbf{L} \\ \mathbf{A} \\ \mathbf{X} \\ \mathbf{G} \\ \mathbf{K} \\ \mathbf{M} \\ M$	383 393 438 338 527 426 336 297 427 427 427 423 337 427 427 427 427 427 427 427 427 427 42
hTRβ1 hPPARα hPPARα hVDR dE75A rNGFI-B hRARγ hRXRα rNNF-4 dUSP hCOUP-TFI hER hPR hAR hGR hMR hTRβ1 hPPARα hVDR dE75A rNGFI-B hRARγ hRXRα tRVF-4 dUSP hCOUP-TFI hRXRα rNF-4 dUSP hCOUP-TFI hRXRα	321 330 377 284 464 425 232 232 232 232 274 400 774 619 825 384 439 439 528 825 339 447 339 528 487 339 447 339 447 338 426 338 472 272	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{h} \\ h$	383 393 438 338 338 527 427 337 427 337 427 337 427 337 427 337 427 427 337 427 427 337 427 427 337 427 429 429 429 429 429 429 429 429 429 429
hTR β 1 hPPAR α hPOR1 hVDR dE75A rNGFI-B hRAR γ hRXR α rNNF-4 dUSP hCOUP-TFI hER hPR hAR hGR hMR hMR hMR hVDR dE75A rNGFI-B hRAR γ hRXR α rHNF-4 dUSP hCOUP-TFI hRXR α rHNF-4 dUSP hCOUP-TFI hER	321 330 377 284 464 425 362 232 232 232 232 232 232 232 232 232	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{h} \\ h$	383 393 438 338 527 486 346 346 4297 427 823 682 888 456 456 466 466 466 466 466 466 466 466
$\label{eq:hardenergy} \begin{split} & hTR\beta 1 \\ hPPAR\alpha \\ hPPAR\alpha \\ hPPAR\alpha \\ hPPAR\alpha \\ hTRS 1 \\ hRAR\gamma \\ hRAR\gamma \\ hRAR\gamma \\ hRAR\gamma \\ hRAR\gamma \\ hRC0UP-TFI \\ hER \\ hPR \\ hAR \\ hGR \\ hMR \\ hGR \\ hMR \\ hTR\beta 1 \\ hPPAR\alpha \\ hRC01 \\ hVDR \\ dZ75A \\ rMOFI-B \\ hRAR\gamma \\ hRXR\alpha \\ rHNF-4 \\ dUSP \\ hCOUP-TFI \\ hER \\ hPR \\ hAR \\ hAR \\ hRC $	321 330 377 284 425 284 425 232 232 232 232 274 400 774 619 825 384 394 439 439 439 439 439 439 439 439 43	$ \begin{array}{c} \underline{\mathbf{E}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} & \underline{\mathbf{h}} \\ \mathbf{h} \\ \mathbf{E} \\ \mathbf{L} \\ \mathbf{L} \\ \mathbf{L} \\ \mathbf{L} \\ \mathbf{A} \\ \mathbf{X} \\ \mathbf{C} \\ \mathbf{M} \\ M$	383 393 438 338 338 338 427 427 427 427 337 337 427 823 888 888 456 466 662 8888 456 418 457 409 409 546 915 901
$\label{eq:hardenergy} \begin{split} & hTR\beta 1 \\ hPPAR\alpha \\ hPPAR\alpha \\ hPPAR\alpha \\ hPPAR\alpha \\ hTRSR\alpha \\ rNGFI-B \\ hRAR\gamma \\ hRAR\gamma \\ hRAR\gamma \\ hCOUP-TFI \\ hER \\ hPR \\ hAR \\ hGR \\ hMR \\ hRCR1 \\ hPPAR\alpha \\ hROR1 \\ hPPAR\alpha \\ hROR1 \\ hPOR1 \\ hROR1 $	321 330 377 284 464 425 362 232 362 232 362 274 400 774 619 825 384 394 4339 339 439 339 487 347 347 347 347 386 228 426 426 426 338 447 425 426 426 426 426 426 426 426 426 426 426	$ \begin{array}{c} & \underline{\mathbf{b}} \ \underline{\mathbf{b}$	383 393 438 338 527 420 4297 422 337 427 427 427 427 427 427 427 427 427 42
$\label{eq:hardenergy} \begin{split} & hTR\beta 1 \\ hPPAR\alpha \\ hPPAR\alpha \\ hPPAR\alpha \\ hPPAR\alpha \\ rMOFI-B \\ hRAR\gamma \\ hHRR \\ rHNF-4 \\ dUSP \\ hCOUP-TFI \\ hER \\ hPR \\ hAR \\ hGR \\ hMR \\ \end{split}$	321 330 377 284 464 425 222 284 522 284 400 774 60 619 825 825 84 394 439 339 528 487 347 347 386 426 426 338 472 888 824 463 889	E h h h E h h h h MLVAYGNOFTTREPLKSLRK-PCDIMEPKDPAMKFNA-LELDDDDISLFVALLCQDRPGLN MSWTCGNQDYKYKSL-GCBUFTSVFEFGKSLCG-MLITEDEIALFSAFULMSA	383 393 438 338 527 426 486 346 385 2297 427 337 337 423 888 888 456 466 662 8888 418 455 418 456 446 407 407 409 559 5915 906
hTRβ1 hPPARα hPPARα hPPARα hPPARα hPPARα hTPPA hRXRα rINF-4 dUSP hCOUP-TFI hER hPR hAR hGR hMR hMR hTRβ1 hPPARα hRCR1 hVDR dE75A rNGFI-B hRARγ hCOUP-TFI hRXRα rINF-4 dUSP hRARγ hCOUP-TFI hRXRα rINF-4 dUSP hRARγ hCOUP-TFI hER hPR hAR hCOUP-TFI hER hPR hAR hCOUP-TFI	321 330 377 284 464 425 272 272 274 400 774 400 774 619 825 384 394 439 339 528 487 386 296 438 487 386 296 426 338 447 388 487 824 464 426 426 428 487 426 426 426 428 427 428 428 428 428 428 428 428 428 428 428	$ \begin{array}{c} \underline{E} & \underline{h} & \underline$	383 393 438 3393 448 336 527 427 427 427 427 427 427 427 427 427 4

Comparaison of 3D structures from homologous proteins

Comparaison des structures 3D de protéines homologues. Relations entre la divergence en séquence et la divergence en structure 3D



Relation type exponentiel % identité / déformations

Sequence identity vs aligment lenght. Biologic / Statistical Significance







Sampling:

Simplex Conjugate Gradients Monte Carlo Brownian Dynamics Molecular Dynamics Replica Exchange Divide-and-conquer enumeration

100 455 models

5 500 good scoring

Scoring:

Density maps EM images Proteomics FRET Chemical and Cys cross-linking Homology-derived restraints SAXS H/D exchange Second harmonic generation Native mass spectrometry Genetic interactions Statistical potentials Molecular mechanics forcefields Bayesian scoring Library of functional forms (ambiguity, ...)





Analysis and assessment



Clustering solutions and generating probability densities Satisfaction of data and considerations that

were used to compute structures were not used to compute structures.





52 (87) MDa 550 copies of 30 polypeptide C8 symmetry 3082 intermolecular cross-links

Estimated resolution: 28 Å





Integrative structure and functional anatomy of a nuclear pore complex

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Albert et al. 2007, 2008 Kim et al. 2018 1/ Principles of integrative structure determination

2/ The example of the Nuclear Pore Complex

3/ Mainstream complementary experimental methods

3.1/ Immunoprecipitation assays

3.2 Two hybrid

3.3/ Analytical ultracentrifugation

3.4/ Foerster Resonnance Energy Transfer

3.5/ Fluorescence anisotropy

Immunoprecipitation assays

Popular (Flag, HA..) or specific epitopes Non-Ab pull down: Affinity tags (His, Strep....)



A nanobody based visible immunoprecipitation assays

Architectures of multisubunit complexes revealed by a visible immunoprecipitation assay using fluorescent fusion proteins Yohei Katoh*, Shohei Nozaki*, David Hartanto, Rie Miyano and Kazuhisa Nakayama[‡]









Yeast two-hybrid system

- Detecting protein-protein interactions in yeast
- Transcriptional regulator system
- "prey"-"bait" model :fusion proteins with a transcriptional activating domain (AD, prey), a DNAbinding domain (DBD, bait)
- Term "two-hybrid" derives from these two chimeric proteins.
- Most commonly used method for large scale, high-throughput identification of potential proteinprotein interactions



1/ Integrative determination of macromolecular structures

2/ The example of the Nuclear Pore Complex

3/ Mainstream complementary experimental methods

3.1/ Immunoprecipitation assays

3.2/ Two hybrid

3.3/ Analytical ultracentrifugation

3.4/ Förster Resonnance Energy Transfer

3.5/ Fluorescence anisotropy

Analytical Ultracentrifugation

There are 3 forces acting on a sedimenting particle, buoyancy, viscous drag and centrifugal force. As soon as the rotor accelerates to a constant speed, the particle reaches terminal velocity and an equilibrium between these 3 forces is established. There are several experimental conditions and sample properties that influence the sedimentation behavior:

Experimental conditions:

- 1. rotor speed
- 2. distance from the rotor center
- 3. density of the solution
- 4. viscosity of the solution
- 5. temperature

Sample properties:

- 1. molecular weight
- 2. shape
- 3. partial specific volume



Analytical ultracentrifuge

Ultracentrifuge that posseses a detection system allowing the measure of the solute concentration as a function of the distance to the rotation axis (optical density, interferrométry).

Absorbance measurement: choice of the wavelenght:

- NA and protein 260 et 280 nm
- ligand eg 380
- peptidic bond 220: low concentra





Avantage: large choice in the experimental conditions: buffer and ionic strength

Sedimentation velocity

Principle: the protein migrates towards the bottom of the tube; The speed of the particule is measured

Determine sedimentation coefficient s (SVEDBERG) 1 S = 10^{-13} s

Speed of sedimentation v = dr/dt per acceleration unit

$$s = dr/dt \cdot (1/w^2r)$$

 $dr/dt = s \cdot w^2 r$

dr/dt = speed of the particuler = distance of the particule to the rotation axisw = angular speed of the rotor



[MS⁻¹ S²M⁻¹] [S]



Sedimentation:

Forces at Equilibrium:

Fc - Fb - Fd = 0

=b (buoyancy)	=	$\omega^2 r m_0$
=d (viscous drag)	=	fv
C (centrifugal force)	=	ω²rm

Explanation:

Fb is the buoyancy force - the force required to displace the buffer surrounding the solute, and m_o is the mass of the displaced solvent.

In practice, one can measure how the sedimentation boundary moves



Diffusion impacts on the shape of sedimentation boundaries They are recorded at regular.intervals







How does the midpoint of the boundary move?



S depends on both M and f



Characterization of Monomeric Intermediates during VSV Glycoprotein Structural Transition. Albertini et al. 2012 Plos Pathogens

Equilibrium Sedimentation

At low speed, diffusion is not negligeable 0.9 0.8 The system reaches an equilibrium: centrigulation force = diffusion force Equilibrium 0.2 0.1 5.85 \$.90 5 95 0.00 6.05 6.10 Radius, cm 0.2 0.0 $Ln\left(C/C_0
ight)$ -0.2 -0.4 $Ln(C_x/C_0) = M\omega^2 (1-V\rho)(x^2-x_0^2)/2RT$ -0.6 -0,8

 $x^2 - x_0^2$

Cx macromolecule concentration

x distance to the axis

 \underline{v} (cm³/g) volume specific partial (hydrated) of the macromolecule

 ρ volumic mass of the solvant (g/cm³)

A real case



FIGURE 3

Sedimentation equilibrium data. Simulated data for a reversible monomer-dimer equilibrium: (—) total, (\cdots) monomer, (--) dimer. The concentration distribution of the dimer is steeper than that of the monomer, and the relative amounts of monomer and dimer at each radial point are determined by mass-action equilibrium.

Mixture of noninteracting solutes

$$a(r) = \sum_{n} c_{n,0} \varepsilon_n d \exp\left[\frac{M_n (1 - \overline{v}_n \rho) \omega^2}{2RT} (r^2 - r_0^2)\right] + \delta$$

Self-association

$$a(r) = \sum_{n} n\varepsilon_{1} dK_{n} (c_{1,0})^{n} \exp\left[\frac{nM_{1} (1 - \overline{\nu}\rho)\omega^{2}}{2RT} (r^{2} - r_{0}^{2})\right] + \delta \quad \text{with } K_{1} = 1$$
(10)

Hetero-association

$$a(r) = c_{A,o} \varepsilon_A d \exp\left[\frac{M_A^* \omega^2}{2RT} (r^2 - r_o^2)\right] + c_{B,o} \varepsilon_B d \exp\left[\frac{M_B^* \omega^2}{2RT} (r^2 - r_o^2)\right] + c_{A,o} c_{B,o} K_{AB} (\varepsilon_A + \varepsilon_B) d \exp\left[\frac{(M_A^* + M_B^*) \omega^2}{2RT} (r^2 - r_o^2)\right] + \delta$$

Förster Resonance Energy Transfer (FRET)

The mechanism of FRET involves a donor fluorophore (D) in an excited electronic state, which may transfer its excitation energy to a nearby acceptor chromophore (A)

Non-radiative process through long-range dipole-dipole interactions that results in the emission of light by the acceptor

The absorption spectrum of the acceptor must overlap fluorescence emission spectrum of the donor



Wavelength

FRET strongly depends on:

The relative orientation of the transition dipole moments of the Donor and the Acceptor
The distance between the fluorophores

Energy transfer studies give information about

- distance between groups
- orientation of two groups and
- the refractive index of the donor-acceptor intervening medium

The efficiency of transfer varies with the inverse sixth power of the distance.



 R_0 in this example was set to 40 Å. When the *E* is 50%, $R=R_0$

Distances can generally be measured between $\sim 0.5 R_0$ and $\sim 1.5 R_0$



$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D\cos\theta_A)^2$$



In vivo and in vitro FRET analysis

Screening for compounds that inhibit or modulate A/B interactions

Use or fluorescent proteins fused to the proteins of interest or of fluorescent probes that are chemically coupled to the donnor and to the acceptor molecules





Structural organization of the bacterial (Thermus aquaticus) RNA polymerasepromoter open complex obtained by FRET (Mekler et al., 2002) was subsequently validated by a crystal structure (Zhang et al., 2012).

Fluorescence Anisotropy

Fluorescence is measured with a linearly polarized beam

Vertical emission Horizontal emission



Figure 8.13. Mesure de la polarisation de fluorescence. Le polariseur P polarise le faisceau incident dans le plan vertical. On observe les composantes parallèles et perpendiculaires du faisceau émis à travers deux analyseurs A_{\parallel} et A_{\perp} .

The basic idea is that a **fluorophore excited by polarized light** will also **emit polarized light**. However, if a molecule is moving, it will tend to "**scramble**" the polarization of the light by radiating at a different direction from the incident light.

The "scrambling" effect is greatest with fluorophores tumbling in solution and decreases with decreased rates of tumbling ie with increased MW.

Slow rotational diffusion



Intensities of vertical and horizontal emission differ = Anisotropy
Rapid rotational diffusion



Intensities of vertical and horizontal emission almost equal



 au_F

 τ_c

(High Rotational Diffusion) 0 < Anisotropy < 1 (Low Rotational Diffusion)

• Boyer M, Poujol N, Margeat E, Rover CA. Nucleic Acids Res. 2000 Jul 1;28(13):2494-502.







temps de correlation de rotation

Titration experiments for Kd measurements

one molecule (DNA, peptide or protein) is fused to a fluorophore

measure static fluorescence anisotropy

association results in an entity which rotates more slowly than the fluorophore and results in less scrambling of polarized light





anisotropy (10

Boyer et al, 2009 NAR



(High Rotational Diffusion) 0 < Anisotropy < 1 (Low Rotational Diffusion)

Estimates of the apparent molecular volume (Perrin Equation)

$$\langle A \rangle^{-1} = A_0^{-1} \left(1 + \frac{\tau_F}{\tau_r^{(2)}} \right) = A_0^{-1} \left(1 + \tau_F \frac{k_{\rm B}T}{\eta V} \right)$$

$$\left(\frac{1}{P} - \frac{1}{3} \right) = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(1 + \frac{\tau_F}{\tau_r^{(2)}} \right) = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(1 + \tau_F \frac{k_{\rm B}T}{\eta V} \right)$$

$$\mathcal{T}_C \quad \text{Rotation correlation time}$$

$$\tau_r^{(2)} = \frac{\eta V}{k_{\rm B}T} \qquad \tau_r^{(2)} = \frac{1}{6D_{\rm r}} = \frac{f_{\rm r}}{6k_{\rm B}T}.$$

Fluorescence labelling

- Chemical synthesis of labelled peptide or oligonucleotides
- Genetically encoded tags (EGFP, mCherry,...)
- Cys-maleimide chemistry,
- Incorporation of non-natural reactive amino acids
- Protein tags such as Halo(haloalkane dehalogenase), SNAP/CLIP(O6-alkylguanine-DNA alkyltransferase),
- Peptidic tags Avi(biotin ligase recognition peptide), Sfp phosphopantetheinyl transferase(CoA), Sortase,....



Fluorescence properties that can be measured

- spectra (environmental effects)
- fluorescence life times
- polarization (orientation and dynamics)
- excitation transfer (distances -> dynamics)
- location of fluorescence

Widely used for binding analysis









Super resolution microscopy

Nobel Price 2014 (Eric Betzig, Stefan W. Hell and William E. Moerner "for the development of super-resolved fluorescence microscopy".)

Different fluorescent probes marking the sample structure are activated at different time points, allowing subsets of fluorophores to be imaged without spatial overlap and to be localized to high precision.

Details cannot be separated by focusing light (diffraction limited)

But by using 2 molecular states of the fluorophore to limit the spacial overlap between airy circles



Fluorophores too close to resolve



Stochastic activation and localization of individual molecules



Super-resolution image reconstructed from localizations

Super resolution microscopy

STED (STimulated Emission Depletion microscopy,

PALM (PhotoActivated Localization Microscopy),

STORM (stochastic optical reconstruction microscopy,

(FPALM) fluorescence photoactivation localization microscopy,

GSDim (Ground State Depletion imaging followed by Individual Molecule return).



Observe individual proteins with a resolution down to 20 nm in intact cells, and second-order statistics to study the spatial interactions of the proteins.

Huang, 2009



Standard confocal



Göttfert, Wurm et al Biophys J (2013)





Structural data used in integrative modeling

Composition

Atomic structures of parts of the system

3D maps, 2D images, components positions

Atomic and protein distances

Binding site mapping

Size and shape

Physical proximity

Solvent accessiblity

Purification from source with gel analysis or MS

X-ray and neutron crystallography, NMR, Cryo-EM/ET, Comparative modelling and molecular docking

Electron microscopy and tomography, gold labelling, Super resolution microscopy, FRET imaging

NMR, FRET, EPR, X-link/ w/o MS.....

NMR, FRET, H-D/MS, mutagenesis

AUC, SAS, atomic force microscopy, ion mobility Fluorescence correlation spectroscopy or anisotropy

Co-purification, native MS, genetic methods, sequence convariance, Chromosome conformation Capture and other data, Y2H

Footprinting methods including H-DX/MS, NMR and chemical modifications



Perrakis et al., 2011

November 19th to 23rd 2018 - IGBMC, Strasbourg-Illkirch, France

Preparation and characterization of macromolecular complexes



INSTRUCT-FRISBI course

Hand-on workshop:

- 1/ Purification of complexes from engineered cell lines
- 2/ Production of recombinant multiprotein complexes using baculoviruses
- 3/ Biophysical characterization and sample optimization

Participants will have the opportunity to work with their own samples .

END