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Therapeutic Opportunities and Challenges in Targeting the Orphan **G Protein-Coupled Receptor GPR35**

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opportunities in targeting this receptor in a range of conditions. GPR35 is, however, unusual in a variety of ways that challenge routes to translation. These include the following: (i) Although a substantial range and diversity of endogenous ligands have been suggested as agonist partners for this receptor, it officially remains defined as an "orphan" GPCR. (ii) Humans express two distinct protein isoform sequences, while rodents express only a single form. (iii) The pharmacologies of the human and rodent



orthologues of GPR35 are very distinct, with variation between rat and mouse GPR35 being as marked as that between either of these species and the human forms. Herein we provide perspectives on each of the topics above as well as suggesting ways to overcome the challenges currently hindering potential translation. These include a better understanding of the extent and molecular basis for species selective GPR35 pharmacology and the production of novel mouse models in which both "on-target" and "offtarget" effects of presumptive GPR35 ligands can be better defined, as well as a clear understanding of the human isoform expression profile and its significance at both tissue and individual cell levels.

KEYWORDS: orphan receptor, ulcerative colitis, pain, species selective pharmacology, single nucleotide polymorphisms, transgenic mice

1. GPR35 ISOFORMS AND ORTHOLOGUES

The human GPR35 gene can be transcribed and translated into three variants.¹ Variants 2 and 3 both encode the same longer isoform, GPR35b, which differs from GPR35a by an additional N-terminal extension of 31 amino acids, resulting in a longer extracellular domain but subsequent equivalence in sequence (Figure 1). Orthologues of GPR35 are found also in both mouse and rat. However, three transcript variants in mouse and one in rat each encode a single GPR35 species which have 73 and 72% identity with human GPR35a.² Although distribution of mRNA expression of GPR35a and GPR35b in human tissues has been established,³ potential distinctive functions of GPR35a and GPR35b are unknown as they display highly similar pharmacology⁴⁻⁶ and, where examined, signaling mechanisms. The extracellular domain of GPR35b does not appear to result in differential targeting as the isoforms localize to similar subcellular compartments when expressed in human HeLa cells and in rat neurons.⁴ Sequence comparisons highlight the potential of GPR35b to form an additional extracellular disulfide interaction compared to GPR35a (Figure 1), but this, or its consequences, has yet to be examined directly. It has been suggested that GPR35b may be associated with carcinogenesis due to detection of expression in gastric and colon cancer tissues.^{1,7} However, GPR35b appears to display lower agonist response efficacy than GPR35a.^{4,5,8}

Although little explored, orthologues of GPR35 exist in a wide range of species (Figure 2). For example, Xenopus tropicalis (amphibian) GPR35 shares ~33% identity with human GPR35a, and although GPR35-like genes are predicted in fish, the similarity to hGPR35 is low. Although the pharmacology of GPR35 is generally quite distinct between human and rat GPR35 (Table 1), given that both lodoxamide and bufrolin are potent agonists at both these orthologues⁵ and both are symmetric diacids (Table 1), researchers then⁹ and, more extensively,⁵ explored the contribution of various positively charged residues in these orthologues to attempt to define the agonist binding pocket and whether variation in such residues might help explain species selectivity of other

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ligands. Such mutagenesis and species residue swap (human to rat) studies included R6.58Q (R240Q), R7.32S (R255S), and L4.62 (R4.62). Interestingly, in hGPR35a the L4.62R alteration increased potency for the agonist zaprinast, as is the case for the full-length rat receptor compared to human (Table 1). However, in other species residue 4.62 is quite variable (Figure 2). It is phenylalanine in all other apes, old world monkeys, Artiodactyla (even-toed ungulates), Cetacea (whales and dolphins), and most Carnivora (carnivore placental mammals), while in rodents it is almost always a positively charged residue, usually arginine though sometimes histidine. Residue 6.58 is positively charged in most species except for rodents where it is predominantly glutamine. Residue 164 is serine or glycine/alanine in most mammals, except primates and nonmuridae rodents. The rat orthologue lacks positive charges at residue 161 (h164) and positions 6.58 and 7.32, all of which sit in the upper portion of the predicted binding pocket. As the more deeply located 4.62 is positively charged in this orthologue, it is possible that ligands sit further down in the pocket of the rat receptor. However, in the continuing absence of direct structural details, the insights from these studies were not definitive in defining the basis of species ligand selectivity.

2. PHARMACOLOGY OF GPR35

disulfide bond.

2.1. Potential Endogenous Activators of GPR35. As an orphan GPCR, the nature of the true endogenous activator(s) of GPR35 remains undefined. This, however, does not reflect a lack of suggestions, although these have been complicated by issues of species selectivity, whether concentrations of suggested ligands might be sufficient to activate the receptor *in situ* and the potential signaling mechanisms engaged by the receptor. GPR35 was nominally deorphanized when it was shown that the tryptophan metabolite kynurenic acid could

activate human, rat, and mouse GPR35 at micromolar concentrations.³ While these initial studies coexpressed a panoply of promiscuous and chimeric G proteins to help to overcome a lack of knowledge on potential selectivity of G protein coupling,³ researchers were able to demonstrate pertussis toxin-sensitive binding of $[^{35}S]GTP\gamma S$ in response to kynurenic acid in CHO cells transfected to express human GPR35a. This is diagnostic of activation of G_i-family G proteins, but the contribution and roles of G_i compared to G₁₃ activation by GPR35 in various cells and tissues has been a significant point of variability between studies (see later). While it is certainly possible that kynurenic acid is indeed an endogenous activator of GPR35 in some circumstances and, indeed, in certain species where potency at the receptor is relatively high, it may not act across species in an equivalent fashion. For example, kynurenic acid is at least 40- to 100-fold less potent at human than rat GPR35,¹⁰ and certain studies have indicated this ligand to be almost inactive at human GPR35, even at very high concentrations.¹¹ This led to a suggestion that 2-acyl lysophosphatidic acids could be potential endogenous agonists,¹¹ but this has not been replicated. The most recent and indeed interesting report of a potential endogenous agonist suggested the chemokine CXCL17 fulfilling this role.¹² Although CXCL17 was reported to be potent and an interesting sequence alignment was used to suggest particular relatedness of GPR35 to the atypical chemokine receptor ACKR3 (previously designated CXCR7), Maravillas-Montero and colleagues¹² were premature in suggesting GPR35 be renamed CXCR8 (CXC chemokine receptor 8). A number of subsequent publications, and also personal communications from other teams, have failed to support the CXCL17-GPR35 pairing.^{13,14} Despite this, a small number of studies have subsequently used CXCL17 in animal models to attempt to define roles for GPR35. However,

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Perspective

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Homo sapiens	1	MCTYNTOGSS-D	LTWPPAIK-LGFY	ANLOVING	CLUMNSL/MOME	CEM-OOMINET	MITNINAV/ADDCO	THE THE POWERSTRUTS	TPLOOLSO	GIMUTINISMS	SLVTANDEN	WRITERADO	AAVAAV	VINIGSIVAR	LCIORC	160
Pan paniscus	1	MAGTYNTIGSS-D	LTWPPTIK-LGFY	ANLOVINVI	GLULINSLALWVIN	CEM-OO201201ST	MINTAWADLC	NICTIN AVUES TRADES	DTPLOOLSO	GTYLTNRYMST	SLUTAIDUNDSY	WRHPURABOU	SERONAAVOAVU	VINTATOSLVAD	FLOMOR G	160
Pan troglodytes	î	MCTYNTICSS-D	TTWPPTTK-LCEY	ANTCHING	CLUDINST ALWWR	CPM-OOOMISHIPT	MINTAWADLC	INCTINDEVICEST. RDTS.		CTVTTNPVMST	SLUTATOWNEY	AUPHPHPHPAPCI	PSERONAAVOAVU	WINTGSTVARW	FLOMORCO	160
Camilla gamilla	1.6	WCTTNIEGSS-D	I TWEETIK-LGET.	ANT CURRENT	OT T TANGE AT MUSE		AUDIT AUTODIC	INGTINA VINSINDIS.	mpt out of	CTVTTDIDVNCT	ST 1/m A HALLAND	NUTRINITIAN	TOTTOTATUCAU	AUTOTOOLUNIN	IFL CHOREC	175
GOIIIIA GOIIIIA	10	SKGIINIGGSS-D	LIWPPIIK-LGPI.	ALLGVINVI	GLIDIN SLATING		IMINIAVADIC	HIGTHUS WHALENDIS.	The local sector	GINTINKINSI	SLVIAUNUN	AVIATEDRARGI	NOTA VOAV	VLUISSLVH,	F LGNQLCG	1/0
Pongo abelli	11	SNGTINIOGSS-D	LIWPPIIK-LGPI.	ALLGVIN VI	GLIDINSDALWVE		IMINLAVADIC.	INCAUST VINSURD IS	TPLOULSU	GINTINKINSI	SLVIAR	AVROLEDRARGE	RESERVINARY ON VI	VEVISSEVARW	TELGRODEG	100
Nomascus ieucogenys	1	SRGTINICGSG-D	LTWPPTIK-LGFI	THEGINAVI	GLUINSDALWVE	COM-COMPARIT	IMINILAVADIC	ULCALIFI VICHSLRDTS	- OTPLOQUES	GITTTNRIMST	SLVTANAVIORI	AVREEDRARGI	REFEROMANUAVI	VLVIGSLVASW	IFTOWORCG	100
Macaca mulatta	1	SNGTYNTOGSS-D	LTWPPTIK-LGFY.	ANLGINEVI	CLUINSDAUWF	CRM-QRANDWRID	YMTNILAVADLC	INCAMPSVIHSLODTS	DTPLOQLSO	GIMPTNRMASIIS	SLVTAROVORY	AVR: BURACCI	RSEROMAAVOAVI	MLWIGSLVARW	FLGMQEEG	160
Cebus c. imitator	1	ONGTSNTONSS-D	ITWPPPLR-LGFY.	ANSGVINIVI	GLUINGDALWVF	LORM-QQ0009081	IMSNLAVADLC	INCAMPRIVIES VIES LODIES	DTPLOQUSO	GINDTNRYMSIIS	SLVMADAVDRY	/AVREED/RARWI	RESERVAAVOVVI	*VLVVSSLVAUW	ILLGMQECG	160
Carlito syrichta	1	WNTODSR-N	LTWPSPLQ-HIFY.	AHSGMVHVH	<u>GLLIINGLAIIWV</u> FI	OCRM-QQANDINRI	YMINLAVADLC	INCAM DOVI HSMKGTS	DTPLOQLSO	GITTNRYMSI	SLIMANOVDRY	/AVRHORARWI	RSFQQAVAVOAVL	*VLVISSLVVRW	ILGIQECG	157
Propithecus coquereli	1	MIDTCSA	PDWPPPLR-YIFY.	ANLGTINIVI	GLILLNGLALWVP	REM-QQ000000000	IMINLAVADLC	ILCALPRVIESLRHTS:	S-DTPLCQLSO	GVYL TNRYMSI	SLVVAIAVDRY	AVRHPURARSE	RSEROMAAVCAIL	WVLVVSSLVARW	VLGIQECG	150
Tupaia chinensis	1	SRTOGRE	VMWPTAVT-YVTR	ANTGTINIVIL	GLILINSLALWVL	RRM-QOMPENRV	YMANLAWADLC	ILCALPOMILSLSHTS	DTPLCOLSO	AIYLANRYMSIS	SLVTAIALDRY	/ALRHPLWARGI	RSPROAAAVOATL	WVLWGSSLGGRW	IVVGPNECG	156
Castor canadensis	1	MITTOVT.	AIWPSSVN-NVFL	ANSVTILLVIL	GLILINGLALWV	CRM-QQANDINEVI	MENLAVADLC	LLCCLPRMIHSLSDTT	DTPLCOLSO	GIYLLNRYMSIS	SLVTAIAVDRY	AVRHPLRARGI	RSEROMAAVOAAU	WWWVSSLVVRW	HLGLQDCG	155
Mus musculus	1	MNSTTONST	LTWPASVN-NFFI	INSALUUVI	GLILINSVALWVF	YEM-HOMMONEI	MENLAVADLC	INCSIDEVILYSLKYSS	S-DTPVCOLSO	GITLANRYMST	SLVTAIAVDRY	AVRHEIRAREI	RSEROAAAVOVAL	WINVTSLVVRW	RLGMOECG	158
Rattus norvegicus	1	NNNTNO-SI	LPWPAAVN-HVFT	INLVLINVI	GLUINGLAIWVE	YSM-HOOMSWICK	MINLAVADVC	HIGSUPPVILYSLKYST:	S-OTPICOLSO	GIVEVNRYMST	SLVTADAVDRY	AVRHPLRAREI	ESEROAGAVOVA/	VINVTSLVL.W	RLGIOECG	157
Equus caballus	1	MNNSNESSN-G	LTWPPAVM-STFY	TOTOVNIVI	GUTINSTATWVD	CEM-00000000000000	MANLAWADLC	INCTINEEVILYSLKHST"	TERTPEOLSO	GTYDANRYMST	SLIMATAVDRY	AVRHOMBVDGI	RSEROAMAVOLAU	WTWOST VLDW	TLCMORES	160
Ochotona princepe	1	ND TOCOL		SVIT CITUM M	OT THINCT A TRAVES	PIM OCOUNTRINT	MANT AWADLC	INCOMPRESSION COLORS	DTRI OT SO	CEVIT T NIDVMOT	T TMATET DRV	7-AZPPEDIEPADCI	DEPONANUOTAU	AVT WAS STOLDN	TI CVORC	150
Hipposideros armiger	î	MPSNOSES-E	LPSSDAFT-VFTF	VALCINA	CLEINCLAIWWD	CUT - DROWNAHU	MUNTAWADISC	INCOMPOMINEL KDHSI	E-DTPEOUSO	CTVTTNPVMST	SLIVATOVORY	AUPHPLYAPCT	PSEPPAANUVU	WIMMESTVIEW	TLERFORCE	150
Galeopterus variogatus	î	AN DIGRECK	DING DAUT-UNT V	SWTCALLAR	OT TITNOT AT WWD	O-M-ORONA2NA-ZO	MINT AWADT C	TWO SUDDMINT OUDA	CUNTLE OT SO	AWYTTNIEVIGT	ST VMA TOWNEY	AVERADI CAPDI	PSPOONA AVOAVU	AUT WEWET OT DW	IFI CVORC	160
Galeoptelus valleyatus	1	DIGGEDGV	LOODONIN MAL	TURGOVIN			THE REAL PROPERTY AND LC.		COT OCT OF	OTHER					TT DWODGO	100
Dicinus ofca	-	WHSIGSSK-E	DFGFQAVR-TIDE				THE REAL PROPERTY AND INC.	In CARIFORNIA FOR AN			SLIVING		NO ROTATION	NVL DGSLVD.		100
Beninops terrarri	24	ING CHECKER D	OWNELLEN-LANC	MITGS NV	GET TANGE AT WILL	COLL-POINTSHAW	IMINIAMADIC.	TON TOTAL TOTAL	W DEPT COLOR	AINT	SLV I ADVIDIO	AV ROUGH PROPERTY	DOD OD A AVOAVI	ALVISSLVL.	TUDIODEG	131
Bos taurus	24	GNSSNOSSW-D.	ANPVYY	TYMGGULAI	GLILINGDALWVID	MIGT - DEWARDANGT	YMAN LAVADLC	INGAILSEN YFOROTS	K-OTPLOQISO	AVYLLARYASI	SLVTAUAVORY	VAN BELED RALER	RISEGRAAVOTAL	MAVVLGSLVLRW	IFTDAÖDGG	176
Orycteropus a. arer	522	SMESONHS-H	SAWPPWVS-PLTL	ANTSTILL	GLVIINSDALIWVL	CRI-PRW0900RV	INVINLAAAD LC	ULCALPSLITHSLRHRD:	S-OMLLODISC	SITUDARTASIS	SLIMA VOVDRM	AV REPUWERGI	REPROVIVAVOAVI	TLVVGGSLAARW	ALDVORGG	675
Elephantulus edwardii	1	SNISONFS-Q	KFWPPSVT-YLSM	ANTGLMUVA	GLILINAVALWVI	CET- DEWARDANGT	YNIVIN LAIAIAD DW	INCAUSS VEHTLRTET	Q-DALFOSFS0	GITLDARYMSI	SLITA WORM	AVREEDCARG	RISERQUVAVOAL	VLVGGSLVVRS	SLLGMQENR	155
Dasypus novemcinctus	23	SPWTMASCNSS-G	DIWPHPVLS.	ANVGTHISH	GLUINGDALMIF	CRM-RV@05087	YMSNLAVADIL	HICAN SIGNATION RELOOP	-LESMSCOLPO	GINDANRYMSTO	JLITADAVDRY	JAVR: DURSER	RESERVAAL CAAD	TLLVGGSLLGRW	ALDEQUCSN	186
Felis catus	1	UNGTOHSS-E	LTWPYWVK-NIVD.	ANVGLIMAN	CLUMNGLAINWF	CEV-RRAMERHI	MANLAVADLC	INCAMPAFIYSLKQRT	E-DTLFOQLSO	GVYLITNRYMSI S	SLVTAINVORY	MLRHDREPAU	KISHRQAVAVQAAL	MALVTGSLVLRW	ALGVRECG	158
Erinaceus europaeus	1	WSSNSSCPAC	WASSPPVIV	PHLGCONAI	CLPINGAALWVI	RRL-RV	YMANLALADIC	HICTHESFILISLOELG	DTAFCRLSO	GVYLANRYMST	GLTAAHALDRY	AVRHPLRARGI	RSEROALAVELGE	MALWAGSLLARG	LLASHRCG	150
Manis javanica	1	MSSNCSSS-E	LAWPRAVT-YVLY	THISLEEVE	GLAINGVALWVL	HRL-QQMMDMRV	YMANLAVS DLC	HICTHONFI HSLR-ST	V-DTPLCQLSO	GIYDANRYMSIC	SLVTAINVDRY	MLQHRURARGI	RSFRQALAVOAGL	WVLVVGSLVLRW	IVLAVQECS	158
Sarcophilus harrisii	1	MG-TEKCP	HDNSTHFQKMQT-	VIMINUU	GIMENSLALWVE	CRMRKK WIDT VI	IMINLAVADLC	ILCALF INTYSOKHIK	EKDTVLOQVSO	SITUMNRYMSI	SIITIIAVDRY	/AIRFEMQAKR	RSPGRSVGICAL	VLVIIFTVTSG	AKEIQECN	159
Phascolarctos cinereus	1	MD-TGNCT	YEDESQLQDLAMK	VHISIMUVI	GIVFNALALWVF	CRMRKK/MDUVI	YMENLAVAD FC	INCVINSIAN YSLKYSR	QEDTVLOQISO	SITUVNSTASI	SIIAMIAVDRY	MIRFEMLAKR	RSEGCSAIICAVE	VLVIISIASRL	AWQVKDGG	160
Ornithorhynchus anatinus	72	VSAKSWVMNSTVN	CSFAAPSI-TSEI	INISLUIIF	CILINAVALWV F	YKM-KROWDWGV	MANLAMADIC	IN FAILPOVIWTLKMKD:	SODTLLCRISO	SIYLMNRHMSIY	TVTLIALDRY	SVKYPIKSCI	RSPKKAALACALL	IFVISLVSILY	TVPFOEGN	234
Sorex araneus	1	MSSSOGQC	QFRGSVVHY.	ANTGLIMAN	CLPENALALWVF	CRV-PROPTOHV	YMVNLABADLC	HOTHELL LP-GELA	LLPCHISO	GCYLANRYMSI	SLVMALALDRY	MVRHPLRARGI	RSERRALVLOAAL	WVLVTGSLALRG	LLGVRSNH	152
Sturnus vulgaris	1	MNHSSCN	ITAYEVFPLVQLC	VHIPVIV-II	GIVLNVLALWVI	CKL-GKOUSINGV	YMVNLAVADCL	ILFTIPSKTLSOFOHL	KVD-GWCLVLE	GGYFLNRFMSIC	GIITINADRY	AIKYPERAKAI	RSPLKAAFASGFL	VIVIICEISLIK	SFEDRREDD	157
Aptervx rowi	1	VVNG-SOSN	DTVODGVLLFOLI	INIPUM-F	OVILLINATARWVR	CKL-KROWNWOWS	MINEMVADCE	FAMORLIYFHOODH	PTG-KLOFTIC	CINETNEEMSI	LIITIDAIDRY	AIKERDKAKTI	SSELKSASIGL	WITLLTYAYFYP	KF-EEGOEO	157
Chelonoidis abingdonii	1	-TNSTNG-NNS-	LKWSYNVRLIOVI	INATIFP-F	CATENVLADWVE	CKL-KKOWSWEV	MINEVIADOS	VVOTUPOTIYFIWNEW	HED-TLOFTIC	SIVETNMSMST	TITVISTORY	ATKYPEKAKS	RSPWKAALTOGLU	VSTITGLNI	KOERSEP	156
Pogona witticeps	1	MVNGNSS	TKTDKNTLTVELT	SSSLVTE-F	OT THNIT FRAMWAR	FKL-GRI MOMET	MISDAVADIM	ALTIN ARKMY FHNNNS	PTD-LEGRITE	CTACMAMPAST	TTTLIMEDRY	TREPUKAKVI	RSERNSVITELED	OLCCELETMSHE	HL-SKEKOE	156
Yenopus tropicalis	22	NSNOTEN TRIM	EERNATLAMFOLT	ILSLT F-F	OTLENSEATIONE	CKM-KKOWOWEW	MSULESDOG	NUT TIDOR TYATOHRW	DI PRELOTTIA	VAVEMNTVLST	TTTLUSVDEV	ATKEPURSES	SUCKKAALA ATV	FALLLCTRIFTL	TTARKSVPSPG	186
nenopuo eropicario		- Sugarange man	DDIGGITIMEN YDT					and a second second							ananonoro	100
		170 190	190	200	210	220	220 2	40 250	260	270	290	200 20	0 210	220	220	
		170 180	190	200	210	220 2	230 2	40 250	260	270	280	290 30	10 310	320	330	
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Homo sapiens	161	170 <u>180</u> . .♠ . 505RST-RHNF-N	190 	200	210	220 2 VGQAEATRKAARM	230 2 WANLLVEVVC	40 250	260 . NACALL	270 • .	280	290 30	0 310	320 . AKAHKSODSL	330 	309
Homo sapiens Pan paniscus	161 161	170 180 .	190 SMARPIIGFYLPL SMARPIIGFYLPL	200 AVVVFCSLK AVVVFCSLK	210 VVTALAQRPPTD	220 VGQAEATRKAARM	230 2 	40 250	260 . NACALL NACALL	270 • -ETIRRALYIA -ETIRRALYIA	280 SKLSDANCCLD	290 30 ATCYYYMAKE	0 310 	320 . AKAHKSQDSL	330 CVTLA	309
Homo sapiens Pan paniscus Pan troglodytes	161 161 161	170 180 	190 SMASPHIGEYIPL SMASPHIGEYIPL SMASPHIGEYIPL	200 AVVVFCSLK AVVVFCSLK AVVVFCSLK	210 VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD	220 VGQAEATRAARM VGQAEATRAARM VGQAEATRAARM	230 2 WANLIVEVVC	40 250	260 NACALL NACALL NACALL	270 ETIRRALYIA -ETIRRALYIA -ETIRRALYIA	280 SKLSDANCCHD SKLSDANCCLD SKLSDANCCLD	290 30 AICYYYMAKE AICYYYMAKE AICYYYMAKE	0 310 	320 AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL	330 .CVTLA .CVTLA	309 309 309
Homo sapiens Pan paniscus Pan troglodytes Gorilla gorilla	161 161 161 176	170 180 	190 SMASPIIGFYIPL SMASPIIGFYIPL SMASPIIGFYIPL SMASPIIGFYIPL	200 II. AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK	210 VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD	220 VGQAEATRAARM VGQAEATRAARM VGQAEATRAARM VGQAEATRAARM	230 2 WANLIVEVVC WANLVVEVVC WANLVVEVVC	40 250 SAPHYG TVRLAVGW SAPHYG TVRLAVGW SAPHYG TVRLAVGW SAPHYG TVRLAVGW	260 NACALL NACALL NACALL NACALL	270 -ETIRRALYIN -ETIRRALYIN -ETVRRALYIN -ETVRRALYIN	280 SKISDANCCID SKISDANCCID SKISDANCCID SKISDANCCID	290 30 AI CYYYMAKE AI CYYYMAKE AI CYYYMAKE AI CYYYMAKE	0 310 	320 AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL	330 CVTLA CVTLA CVTLA	309 309 309 309
Homo sapiens Pan paniscus Pan troglodytes Gorilla Pongo abelii	161 161 176 171	170 180 CFRST-RHNF-N FCFRST-RHNF-N FCFRST-RHNF-N FCFRST-RHNF-N FCFRST-RHNF-N	190 SMAEPIIGEYLPL SMAEPIIGEYLPL SMAEPIIGEYLPL SMAEPIIGEYLPL SMAEPIIGEYLPL	200 AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK	210 VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD	220 VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM	230 2 VWANLEVEVVC VWANLEVEVVC VWANLEVEVVC VWANLEVEVVC	40 250 J.J. V. J. V. J. J. J.J. J.	260 	270 -ETIRRALYI -ETIRRALYI -ETVRRALYI -ETIRRALYI -ETIRRALYI	280 SKLSDANCCHD SKLSDANCCHD SKLSDANCCHD SKLSDANCCHD SKLSDANCCHD	30 31	0 310 	320 AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL	330 	309 309 309 309 324
Homo sapiens Pan paniscus Pan troglodytes Gorilla gorilla Pongo abelii Nomascus leucogenys	161 161 176 176 171	170 180 	190 SMASSITESYIPI SMASSITESYIPI SMASSITESYIPI SMASSITESYIPI SMASSITESYIPI SMASSITESYIPI	200 AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK	210 VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD	220 VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM	230 2 WANELVEVVC WANEVVEVVC WANEVVEVVC WANEVVEVVC WANEVVEVVC	40 250 SIDDHVGTVRLAVGWI FIDDHVGTVRLAVGWI FIDDHVGTVRLAVGWI FIDDHVGTVRLAVGWI FIDDHVGTVRLAVGWI FIDDHVGTVRLAVGWI	260 AGALL NAGALL NAGALL NAGALL NAGALL NAGALL	270 -ETIRRALYI -ETIRRALYI -ETIRRALYI -ETIRRALYI -ETIRRALYI -ETIRRALYI	280 SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD	290 30 AI CYYYMAKE AI CYYYMAKE AI CYYYMAKE AI CYYYMAKE AI CYYYMAKE AI CYYYMAKE	10 310 	320 AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL	330 	309 309 309 309 324 319
Homo sapiens Pan paniscus Pan troglodytes Gorilla gorilla Pongo abelii Nomascus leucogenys Macaca mulatta	161 161 176 171 161 161	170 180 FGRST-RHNF-N FGRST-RHNF-N FGRST-RHNF-N FGRST-RHNF-N FGRST-RHNF-N FGRST-RHNF-N FGRST-RHNF-S	190 SMASPITGSYIPI SMASPITGSYIPI SMASPITGSYIPI SMASPITGSYIPI SMASPITGSYIPI SMASPITGSYIPI SMASPITGSYIPI	200 AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK	210 VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD	220 VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM	230 2 WANILVEVVC WANILVEVVC WANILVEVVC WANILVEVVC WANILVEVVC WANILVEVVC	40 250 SUPING TVRLAVGM SUPING TVRLAVGM SUPING TVRLAVGM SUPING TVRLAVGM SUPING TVRLAVGM SUPING TVRLAVGM SUPING TVRLAVGM	260 NAGALL NAGALL NAGALL NAGALL NAGALL	270 -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN	280 SKUSDANCCLD SKUSDANCCLD SKUSDANCCLD SKUSDANCCLD SKUSDANCCLD SKUSDANCCLD SKUSDANCCLD SKUSDANCCLD	30 AI CYYWARE AI CYYWARE AI CYYWARE AI CYYWARE AI CYYWARE AI CYYWARE AI CYYWARE AI CYYWARE	0 310 	320 AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSOJSL AKAHKSOJSL AKAHKSOJSL	330 	309 309 309 324 319 309
Homo sapiens Pan paniscus Pan troglodytes Gorilla gorilla Pongo abelii Nomascus leucogenys Macaca mulatta Cebus c. imitator	161 161 176 171 161 161	170 180 CORST-RINF-NN FCORST-RINF-NN FCORST-RINF-NN FCORST-RINF-NN FCORST-RINF-NN FCORST-RINF-NN FCORST-RINF-SN FCORSS-RINF-NN	190 SMAFPLIGFYIPL SMAFPLIGFYIPL SMAFPLIGFYIPL SMAFPLIGFYIPL SMAFPLIGFYIPL SMAFPLIGFYIPL SMAFPLIGFYIPL SVAISLIGFYIPL	200 II AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK	210 VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VITALAQRPPID	220 VGQAEATRKVARM VGQAEATRKVARM VGQAEATRKVARM VGQAEATRKVARM VGQAEATRKVARM VGQAEATRKVARM	230 2 WANILVEVVC WANILVEVVC WANILVEVVC WANILVEVVC WANILVEVVC WANILVEVVC WANILVEVVC	40 250 FIP DING TYRLAVGW FIP DING TYRLAVGW FIP DING TYRLAVGW FIP DING TYRLAVGW FIP DING TYRLAVGW FIP DING TYRLAVGW FIP DING TYRLAVGW	260 	270 -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM	280 SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD	290 30 AI CYYWAKE AI CYYWAKE AI CYYWAKE AI CYYWAKE AI CYYWAKE AI CYYWAKE AI CYYWAKE AI CYYWAKE AI CYYWAKE	0 310 	320 AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL	330 	309 309 309 324 319 309 309
Homo sapiens Pan paniscus Pan troglodytes Gorilla Pongo abelii Nomascus leucogenys Macaca mulatta Cebus c. imitator Carlito syrichta	161 161 176 171 161 161 158	170 180 	190 SMAPPLIGFYIPL SMAPPLIGFYIPL SMAPPLIGFYIPL SMAPPLIGFYIPL SMAPPLIGFYIPL SMAPPLIGFYIPL SMAPPLIGFYIPL TVIFSLAGFYIPL	200 II.S. AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK	210 VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPSTD	220 VGQAEATRKARM VGQAEATRKARM VGQAEATRKARM VGQAEATRKARM VGQAEATRKARM VGQAEATRKARM VGQAEATRKARM VGQAEGTRKATRM	230 2 WENTLUVEVVC WANLVVEVVC WANLVVEVVC WANLVVEVVC WANLVVEVVC WANLVVEVVC WANLVVEVVC WANLVVEVVC	40 250 Internet of the second	260 NAGALL NAGALL NAGALL NAGALL NAGALL NAGALL NAGALL HAGGLL HAGGLL	270 	280 SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD	290 30 II	0 310 FORASLAVAPS- FORASLAVAPS- FORASLAVAPS- FORASLAVAPS- FORASLAVAPS- FORASLAVAPS- FORASLAVPS- FORASLAVPS- FORASLAVPS-	320 AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL AKAHKSQDSL TKAHKSQDSL TKAHKSQDSL AKAHKSQDSL	330 	309 309 309 324 319 309 309 309
Homo sapiens Pan paniscus Pan troglodytes Gorilla Pongo abelii Nomascus leucogenys Macaca mulatta Cebus c. mitator Carlito syrichta Propithecus coquereli	161 161 176 171 161 161 158 157	170 180 FGFRST-RHNF-N FGFRST-RHNF-N FGFRST-RHNF-N FGFRST-RHNF-N FGFRST-RHNF-N FGFRST-RHNF-N FGFRST-RHNF-N FGFRSS-RHNF-N FGFRSS-RHNF-N FGFRSS-RHNF-N FGFRSS-RHNF-N FGFRSS-RHNF-N	190 SMASPLIGFTIPL SMASPLIGFTIPL SMASPLIGFTIPL SMASPLIGFTIPL SMASPLIGFTIPL SMASPLIGFTIPL TVISSUGFTIPL TVISSUGFTIPL	200 AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLQ AVLVFCSLQ	210 VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPDD VVTALAQRPPD VVTALAQRPSTD VVTALAQRPSTD	220 VGQAEATRKJARM VGQAEATRKJARM VGQAEATRKJARM VGQAEATRKJARM VGQAEATRKJARM VGQAEATSKJARM MGQAEGTRKJARM	230 2 WANE VEVC WANE VEVC WANE VEVC WANE VEVC WANE VEVC WANE VEVC WANE VEVC WANE VEVC WANE VEVC	40 250 PUPUNGTVRLAVGW FUPINGTVRLAVGW FUPINGTVRLAVGW FUPINGTVRLAVGW FUPINGTVRLAVGW FUPINGTVRLAVGW FUPINGTVRLAVGW FUPINGTVRLAVGW FUPINGTVRLAVGW FUPINGTVRLAVGU FUPINGTVRVAVGL FUPINGTVRVAVGL	260 	270 -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -KTINHVLYIM -NILRRALYIM -STIRHALFIM	280 SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD	290 30 AI CYYYMAKE AI CYYYMAKE AI CYYYMAKE AI CYYYMAKE AI CYYYMAKE AI CYYMAKE AI CYYMAKE AI CYYMAKE AI CYYMAKE AI CYYMAKE AI CYYMAKE	0 310 TFOSRALAVAPS- FOSRALAVAPS- FOSRALAVAPS- FOSRALAVAPS- FOSRALAVAPS- FOSRALAVPS- FOSRALAVPS- FOSRALAVPS- FOSRALAPPS- FOSRALATPS-	320 - AKAHKSQDSL - AKAHKSQDSL - AKAHKSQDSL - AKAHKSQDSL - AKAHKSQDSL - AKAHKSQDSL - AKAHKSQDSL - AKAHKSQDSL - AKAHKSQDSL	330 	309 309 309 324 319 309 309 309 309
Homo sapiens Pan paniscus Pan iroglodytas Dongo abelila Nomascus leucogenys Macaca mulatia Cebus c. imitator Carlito syrichta Propithecus coquereli Tupaia chinensis	161 161 176 171 161 161 158 157 157	170 <u>180</u> 	190 SMASPLIGFYIPL SMASPLIGFYIPL SMASPLIGFYIPL SMASPLIGFYIPL SMASPLIGFYIPL SMASPLIGFYIPL SMASPLIGFYIPL TVISSLIGFYIPL TTASSLIGFYIPL TTASSLIGFYIPL	200 AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLQ AVVVFCSLQ AVVVFCSLQ	210 VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPTD VVTALAQRPPDD VVTALAQRPSD VVTALAQRPSD VVTALAQRPAD	220 VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM MGQAEGTRKATRM MGQAEATRRAARM VGQAEATRKAARM	230 2 WANEJUSVC WANEJUSVC WANEJUSVC WANEJUSVC WANEJUSVC WANEJUSVC WANEJUSVC WANEJUSVC WANEJUSVC WANEJUSVC WANEJUSVC WANEJUSVC WANEJUSVC		260 	270 -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN -ETIRRALYIN -ETIRRTLFIN -KTINHVLYIN -NILRRALYIN -STIRHALFIN -TIALEAN	280 SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD SKLSDANCCLD	290 30 1 CYYMARE AI CYYMARE	0 310 FOSASLAVAPS- FOSASLAVAPS- FOSASLAVAPS- FOSASLAVAPS- FOSASLAVAPS- FOSASLAVPS- FOSASLAPSS- FOSASLAPPS- FOSASLAPPS- FOSASLTSAPS-	320 AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL TKAHKSODSL TKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL	330 	309 309 309 324 319 309 309 309 306 306
Homo sapiens Pan paniscus Pan troglodytes Gorilla Pongo abelii Nomascus leucogenys Macaca mulatta Cebus c. imitator Carlito syrichta Propithecus coquereli Tupaia chinensis Castor candensis	161 161 176 171 161 161 158 157 157	170 180 FGFRST-RHNF-N FGFRST-RHNF-N FGFRST-RHNF-N FGFRST-RHNF-N FGFRST-RHNF-N FGFRST-RHNF-S FGFRSS-RHNF-S FGFRSS-RHNS-S FGFRSSRQHS-S	190 SMASPIGFYPI SMASPIGFYPI SMASPIGFYPI SMASPIGFYPI SMASPIGFYPI SMASPIGFYPI TYJSIGFYPI TYJSIGFYPI TYJSIGFYPI	200 AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLQ AVVVFCSLQ AVVVFCSLQ AVVVFCSLQ	210 VTALAGREPTD VVTALAGREPTD VVTALAGREPTD VVTALAGREPTD VVTALAGREPTD VVTALAGREPTD VVTALAGREPTD VVTALAGREPTD VVTALAGRESTD VVTALAGRESTD VVTALAGRESTD	220 VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM VGQAEATRKAARM	230 2 WANE2 VEV/C WANE2 VEV/C		260 	270 -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -ETIRRALYIM -NILRRALYIM -NILRRALYIM -STLARALFIM 	280 SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD	30 31 CYY MAKE 31 CYY	0 310 FORASLAVAPS- FORASLAVAPS- FORASLAVAPS- FORASLAVAPS- FORASLAVAPS- FORASLAVAPS- FORASLAVPS- FORASLAVPS- FORASLAVPS- FORASLAVPS- FORASLAVPS- FORASLAVPS- FORASLAVPS- FORASLAVPS-	320 AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL AKAHKSODSL	330 	309 309 309 324 319 309 309 309 306 306 306
Homo sapiens Pan paniscus Pan troglodytes Gorilla gorilla Page abeli Page abeli Maccas mulatis Cebus c. imitator Carlito syrichta Propithecus coquereli Tupaia chinensis Castor canadensis Mus musculus	161 161 176 171 161 161 158 157 157 156 159	170 <u>180</u> 	190 SMASPIJGFYIPJ SMASPIJGFYIPJ SMASPIJGFYIPJ SMASPIJGFYIPJ SMASPIJGFYIPJ SMASPIJGFYIPJ TVISSIJGFYIPJ ATISSIJGFYIPJ SIVSIJGFYIPJ SIVSIJGFYIPJ SIVSIJGFYIPJ	200 AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLK AVVVFCSLQ AVLVFCSLQ AVLVFCSLQ AVLVFCSLQ AVIVFCSLQ	210 WTALAGREPTD WTALAGREPTD WTALAGREPTD WTALAGREPTD WTALAGREPTD WTALAGREPTD WTALAGREPTD WTALAGREPTD WTALAGREPTD WTALAGREPAD WTALAGREPAD WTALAGREPAD WTALAGREPAD	220 VQQAEATRXARM VQQAEATRXARM VQQAEATRXARM VQQAEATRXARM VQQAEATRXARM VQQAEATRXARM VQQAEATRXARM VQQAEATRXARM VQQUEASRXAGM VQQUEASRXAGM VQQAEATRXAQM VQQAEATRXAQM	230 2 WAND VSVC WAND VSVC	40 250 xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx	260 	270 — TIRRALYID — ETIRRALYID — ETIRRALYID — ETIRRALYID — ETIRRALYID — ETIRRALYID — ETIRRALYID — KTINHULYID — NILRRALYID — STIRRALFID — IALEAD — ITFGQALSUD — TFFSRALSID	280 SRISDANCCLD SRISDANCCLD SRISDANCCLD SRISDANCCLD SRISDANCCLD SRISDANCCLD SRISDANCCLD SRISDANCCLD SRISDANCCLD SRISDANCCLD SRISDANCCLD SRISDANCCLD SRISDANCCLD	30 31 - CYY WARK 31 - CYY WARK	0 310 FORMALAVARS- FORMALAVARS- FORMALAVARS- FORMALAVARS- FORMALAVARS- FORMALAVARS- FORMALAVARS- FORMALAVARS- FORMALAPSS- FORMALAPSS- FORMALATSSN-	320 	330 	309 309 309 324 319 309 309 309 309 309 309 309 309 309 30
Homo sapiens Pan paniscus Pan troglodytes Gorilla Pongo abelii Nomascus leucogenys Macaca mulatta Cebus c. imitator Carlito syrichta Propithecus coquereli Tupaia chinensis Castor candensis Mus musculus Rattus norvegicus	161 161 176 171 161 161 158 157 157 156 159	170 <u>180</u> CFRST-RHNF-N FCFRST-RHNF-N FCFRST-RHNF-N FCFRST-RHNF-N FCFRST-RHNF-N FCFRST-RHNF-N FCFRST-RHNF-N FCFRSS-RHNF-S FCFSSQHRNS-S FCFSSQHRNS-S FCFSSQHRNF-S	190 SMASPIGYIPI SMASPIGYIPI SMASPIGYIPI SMASPIGYIPI SMASPIGYIPI SMASPIGYIPI TASIGYIPI TASIGYIPI TASIGYIPI SIVUSIGYIPI SIVUSIGYIPI TASIGGYIPI	200 AVVVECSLK AVVVECSLK AVVVECSLK AVVVECSLK AVVVECSLK AVVVECSLK AVVVECSLK AVVVECSLQ AVVVECSLQ AVVVECSLQ AIVVECSLQ AIVVECSLQ	210 WTADAQRPTD' WTADAQRPTD' WTADAQRPTD' WTADAQRPTD' WTADAQRPTD' WTADAQRPTD' WTADAQRPTD' WTADAQRPTD' WTADAQRPSTD' WTADAQRPSTD' WTADAQRPATD' WTADAQRPATD' WTADAQRPATD' WTADAQRPATD' WTADAQRPATD'	220 VGQAEATR VARM VGQAEATR VARM VGQAEATR VARM VGQAEATR VARM VGQAEATR VARM VGQAEATR VARM VGQAEATR VARM VGQAEATR VARM VGQAEATR VARM VGQAEATR VARM VGQAEATQ VATM VGQAEATQ VARM	230 2 WANEY VEVVC WANEY VEVCC WANEY VEFUCC WANEY VEFUCC WEFUCC WANEY VEFUCC WANEY VEFUCC WEFUCC WEFUCC WEFUCC WEFUCC		260 	270 ETIRRALYIT ETIRRALYIT ETIRRALYIT ETIRRALYIT ETIRRALYIT ETIRRALYIT ETIRRALYIT STIRRALYIT STIRRALYIT STIRRALYIT OTFGQALSVO DTFGRALSIT	280 SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD	290 30 1	0 310 FTOERSALAVAPS- FTOERSALAVAPS- FTOERSALAVAPS- FTOERSALAVAPS- FTOERSALAVAPS- FTOERSALAVAPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVPS- FTOERSALAVAPS- SALAVAP	320 	330 	309 309 309 309 309 309 309 309 309 309
Homo sapiens Fan paniscus Fan troglodytes Gorilla gorilla Fongo abelii Nomascus leucogenys Mebus au leucogenys Autor au leucogenys Mebus au leucogen	161 161 176 171 161 161 158 157 157 157 156 159 158 161	170 <u>180</u> 	190 SMASPIJGYVPJ SMASPIJGYVPJ SMASPIJGYVPJ SMASPIJGYVPJ SMASPIJGYVPJ SMASPIJGYVPJ SMASPIJGYVPJ SMASPIJGYVPJ SMASJIGYVPJ SIVSJIJGYVPJ SIVSJIGYVPJ TASSIJGYVPJ TASSIJGYVPJ	200 AVVVJCSLK AVVVJCSLK AVVVJCSLK AVVVJCSLK AVVVJCSLK AVVVJCSLK AVVVJCSLQ AVVVJCSLQ AVVVJCSLQ AVVVJCSLQ AIVVJCSLQ AIVVJCSLQ AIVVJCSLQ	210 WTADAGRPTD' WTADAGRPTD' WTADAGRPTD' WTADAGRPTD' WTADAGRPTD' WTADAGRPTD' WTADAGRPTD' WTADAGRPAD' WTADAGRPAD' WTADAGRAD' WTADAGRAD' WTADAGRPAD' WTADAGRPAD' WTADAGRPAD' WTADAGRPAD'	220 GOAEATRE AREA GOAEATRE GARM GOAEATRE GARM GOAEATRE GARM GOAEATRE GARM JGOAEATRE GARM JGOAEATRE GARM JGOAEATRE GARM JGOAEATRE GARM JGOAEATRE GARM JGOAEATRE GARM JGOAEATRE GARM JGOAEATRE GARM JGOAEATRE GARM	230 2 WANL VEVUC WANL VEVUC		260 avall N=avall N=avall N=avall N=avall N=avall N=avall Q=svarr N=svarr N=tvarr N=tvarr	270 TIRALYID ETIRRALYID ETIRRALYID ETIRRALYID ETIRRALYID ETIRRALYID ETIRRALYID ETIRRALYID STIRHALFID STIRHALFID STIRHALFID UTFSRALSID UTFSRALSID MIFSRALTID TFSRALSID	280 SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD SKISDANCCLD	290 30 1	0 310 FFOEASALAVAPS- FFOEASALAVAPS- FFOEASALAVAPS- FFOEASALAVAPS- FFOEASALAVAPS- FFOEASALAVAPS- FFOEASALAVAPS- FFOEASALAVPS- FFOEASALAPSS- FFOEASALAPSS- FFOEASALAPSS- FFOEASALAPSS- FFOEASALAPSS- FFOEASALAPSS- FFOEASALAPSS- FFOEASALAPSS- FFOEASALAPSS- FFOEASALAPSS- FOEASALAPSS-	320 AKAHKGOBL AKAHKGOBL AKAHKGOBL AKAHKGOBL AKAHKGOBL AKAHKGOBL AKAHKGOBL AKAHKGOBL AKAHKGOBL AKAHKGOBL AKAHKGOBL AKAHKGOBL T- PHKGODG T- PHKGOBGL AKYHKGOBL	330 	305 305 305 305 305 306 306 306 306 306 306 306 306 306 306
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Figure 2. GPR35 orthologues are present in a wide range of species. GPR35 orthologues from the indicated species are aligned. Start number indicates trimmed N-terminal extension to correspond to hGPR35a. Residues from hGPR35a noted in the text are marked as follows: spade, L153^{4,62}; three-leaved clover, R164; heart, R240^{6,58}; diamond, R255^{7,32}; \checkmark , T108^{3,44}; \blacktriangle , S294. The solid black line indicates the predicted transmembrane domains for hGPR35 (https://gpcrdb.org).

for example, as kynurenic acid and zaprinast (see below) reduced rather than mimicked the effect of CXCL17 in neuropathic pain in mice,¹⁵ these results are not consistent with CXCL17 acting via GPR35. Other endogenously produced or potentially produced molecules including 5,6-dihydroxyindole-2-carboxylic acid, reverse T3 (3,3,5-triiodo-thyronine), and cyclic guanosine 3'-5' monophosphate (cGMP) have also been reported to display modest potency at GPR35 in scattered and yet to be confirmed reports.^{16,17}

2.2. Synthetic Agonists of GPR35. Significant effort has been expended to identify tool compounds with which to explore the biology of GPR35. Although better known as a blocker of cGMP phosphodiesterases, zaprinast has remained one of the most widely used activators of GPR35, and in many cases has been used as a reference by which to compare potency and efficacy of other ligands. This reflects the fact that despite at best moderate potency and unlike many other compounds the potency of zaprinast is relatively similar at human, rat, and mouse orthologues.¹⁸ The general use of human GPR35a expressed in heterologous cells for most screening assays^{9,19,20} or the use of HT-29 human colorectal adenocarcinoma cells that express the receptor endogenously in label-free screens^{21,22} means that many of the most potent reported synthetic agonists either have not been assessed at rodent orthologues or show substantially lower potency at

either or both rat and mouse GPR35. This is true of 4-{(Z)-[(2*Z*)-2-(2-fluorobenzylidene)-4-oxo-1,3-thiazolidin-5-ylidene] methyl} benzoic acid ("compound 1" in ref 19) and the 8benzamidochromen-4-one-2-carboxylic acids described by Muller and colleagues²⁰ (Table 1), which implies that the radiolabel 6-bromo-8-(4-[(3)H]methoxybenzamido)-4-oxo-4H-chromene-2-carboxylic acid ([(3)H]PSB-13253) from this series²³ is unlikely to be of use in studies on rodent orthologues of GPR35. Even between rat and mouse GPR35 there can be substantial variation in the potency of agonists. For example, the mast cell stabilizer lodoxamide, which is administered topically for the treatment of allergic keratoconjunctivitis, is a potent agonist of human GPR35 and is essentially equipotent at rat GPR35.5 However, it has greater than 100-fold lower potency at mouse GPR35.²⁴ Although Kim et al.²⁵ used lodoxamide to assess a potential role for GPR35 in a mouse model of hepatic fibrosis, they specifically noted the low potency of this compound at mouse GPR35, which would seem to negate its use. Although Kim et al.²⁵ claim that Mackenzie et al.5 reported a high potency of lodoxamide at mouse GPR35, this is incorrect. Mackenzie et al. did not examine mouse GPR35 in this study,⁵ but rather, as noted above, the rat orthologue. In addition, Kim et al.²⁵ reported that the effects of lodoxamide in mouse were prevented by treatment with 1-(2,4-difluorophenyl)-5-[[2-

Table 1. GPR35 Ligands and their species Selectivity^a

Name	Structure	Selectivity	References
Agonists			
6-Bromo-8-(4- methoxybenzamido)-4-oxo-4H- chromene-2-carboxylic acid		h>>r	20
Amlexanox		r>m>>h	24
Bufrolin		r=h>m	24
Compound 1		h>>r>m	24, 19
Cromolyn disodium		h>r>m	24, 26
Kynurenic acid		r>m>h	3
Lodoxamide	но у Ц Ц Ц Ц Ц Ц Ц Ц Ц Ц Ц Ц Ц Ц Ц Ц Ц Ц	h=r>>m	24
Pamoic acid	C C C C C C C C C C C C C C C C C C C	h>>r	24, 26
Zaprinast		r>m>h	24, 26
Antagonists			
ML-145	HO G G G H	h	26
CID2745687		h	26

"Potency variation between human (h), rat (r), and mouse (m) orthologues is shown for a number of compounds with activity at GPR35. Where a species is not shown, the relative activity has either not been reported or is very low. For the antagonists, affinity at human is markedly greater than that for either mouse or rat.

[[(1,1-dimethylehyl)amino]thioxomethyl]hydrazinylidene]methyl]-1H-pyrazole-4-carboxylic acid methyl ester (CID2745687) which (see later) is a high-affinity antagonist of human GPR35 but lacks any significant affinity at the mouse orthologue.^{26,27} While potential "off-target" effects of many poorly studied synthetic ligands should be anticipated, particularly *in vivo*, in this example a potentially "off-target" effect of a compound possessing *in vitro* GPR35 agonist activity was blocked by what must also be an "off-target" effect of a reported (human) GPR35 antagonist. There is no obvious reason either that lodoxamide should be an agonist or that CID2745687 is an antagonist of a protein other than GPR35, so the observations of Kim et al.²⁵ are difficult to explain at a molecular level. This provides a clear illustration that careful pharmacological profiling of ligands at appropriate species orthologues of a poorly characterized receptor such as GPR35 is required to allow validation of potential therapeutic opportunities. Serendipity can be a useful ally in efforts to identify agonists with high potency at GPR35 orthologues beyond human: For example, amlexanox (Table 1) was identified initially in a screen as a low-potency activator of human GPR35,⁵ but subsequent studies showed it to have substantially higher potency at rat GPR35.⁵ However, more direct efforts to identify potent agonists at preclinical orthologues of GPR35 could potentially involve direct screening of compound libraries against a series of receptor

orthologues if the aim is then to assess receptor function in suitable animal models of disease.

2.3. Assays Measuring Activation of GPR35. Most studies designed to identify ligands with agonist potency at GPR35 have employed assays that report recruitment of an arrestin to the agonist-occupied human receptor.^{6,19,20} There are many benefits in using assays based on arrestin recruitment, not the least of which is the ease of employing these in a highthroughput format and because GPR35 provides a particularly robust signal in such assays. However, leaving aside the topic of whether arrestin "recruitment" equates to "signaling",²⁸ it has been challenging to develop assays suitable for highthroughput screening of GPR35 that report G protein activation. This may reflect that where G_i-mediated links of GPR35 have been recorded the assays have focused on either the binding of $[{}^{35}S]GTP\gamma S^1$ or downstream end points where the signal window is small, e.g., phosphorylation of the extracellular signal regulated kinases 1 and 2,6 or for other G protein-mediated but hard-to-automate end points that reflect activation of Rho-kinases. This likely reflects that quantitative comparison between different G proteins indicates that GPR35 couples most effectively with G_{13}^{27} and until recently, assays that record this interaction have not been compatible with screening. However, by using a bioluminescence resonance energy transfer (BRET)-based sensor in which human GPR35a is linked to the C-terminal 27 amino acids of various G protein α subunits, not only was the selectivity of the receptor for G₁₃ confirmed but also equivalence was shown in rank-order of potency of various agonists with those recorded in GPR35aarrestin interaction assays.²⁷ Such sensors have been developed for both human GPR35a and GPR35b⁸ as well as mouse GPR35²⁷ and should allow screening for both isoform selective activators and potentially ligands with high potency at specified species orthologues. In support of the conclusion that GPR35 couples selectively with G_{13} , expression of human GPR35a in HEK293 cells gene-edited to lack expression of different G protein subsets showed a requirement for G_{12}/G_{13} to allow GPR35 agonists to promote shedding of an alkaline phosphatase fusion protein of TGF- α .²⁷ An entirely different approach to the identification of activators of GPR35 has been to combine the use of human HT-29 adenocarcinoma cells that express GPR35 endogenously with a label-free assay system that measures alterations in "dynamic mass redistribution" over time.^{16,21} This has the distinct advantage of assessing activation of GPR35 at endogenous levels of expression but does require specialized equipment, and to date, this has only been reported for human GPR35.

2.4. Antagonists of GPR35. Although antagonist compounds are vital for characterization of GPCRs, identification and use of such tools has been fraught with challenges for GPR35. The only well-characterized GPR35 antagonist is methyl-5-[(*tert*-butylcarbamothioylhydrazinylidene)methyl]-1-(2,4-difluorophenyl)pyrazole-4-carboxylate (CID2745687).⁶ This compound was originally shown to block effects of both of the agonists pamoic acid and zaprinast in cells expressing either human GPR35a or GPR35b, with K_i estimated in the region 10–20 nM, and to potentially function in a competitive manner (a single concentration of CID2745687 was shown to shift the concentration—response curve for pamoic acid in an apparently surmountable manner).⁶ An interesting aside in these studies was that CID2745687 was also noted to block effects of pamoic acid and zaprinast at mouse GPR35.⁶ By contrast, Jenkins et al.²⁶ subsequently reported that

CID2745687 was ineffective at both mouse and rat GPR35, while confirming its ability to block effects of both zaprinast and pamoic acid at human GPR35a. However, while more extensive pharmacological analysis was consistent with CID2745687 acting in a competitive manner with both zaprinast and cromolyn disodium at this isoform with estimated pA_2 7.7-7.8, it functioned instead as a noncompetitive blocker of pamoic acid.²⁶ Subsequent studies using the GPR35-G α_{13} BRET sensors described above have confirmed the inability of CID2745687 to block the function of zaprinast at mouse GPR35, but they also showed that it does so at human GPR35a.²⁷ As there are no atomic level structures of any isoform or orthologue of GPR35 available to date, neither the basis of high-affinity binding of CID2745687 to human isoforms of GPR35 nor its observed lack of affinity at mouse GPR35 currently have structural underpinning. Moreover, no cell-based in vitro studies since Zhao et al.⁶ have provided evidence for CID2745687 being able to effectively antagonize mouse or rat GPR35. Despite this, CID2745687 has been reported to prevent kynurenic acid mediated inhibition of forskolin-induced cAMP production in cultured mouse astrocytes²⁹ and to prevent wound repair induced by a GPR35 agonist in young adult mouse colon epithelium cells.³⁰ Importantly, in a number of cases, associated siRNA-mediated knockdown studies have been performed to support the contribution of GPR35 to such end points. Furthermore, a compound described as CID2745678 (but likely also CID2745687 where the last two numbers have been incorrectly inverted) was recently indicated to limit anoxiainduced mitochondrial injury and apoptotic cell death in neonatal murine ventricular myocytes.¹^rAs anticipated from the species selective pharmacology of CID2745687 defined above, this ligand does effectively block human vascular smooth muscle cell migration in response to either pamoic acid or zaprinast.³² There is clearly an ongoing disconnect between the underpinning pharmacological studies in transfected cells and the use of this compound in some physiological preparations that is hard to understand because the in vitro data indicate clearly that CID2745687 should not function in rodent tissues, at least not in a GPR35-dependent manner.

A second described GPR35 antagonist 2-hydroxy-4-[4-(5Z)-5-[(E)-2-methyl-3-phenylprop-2-enylidene]-4-oxo-2-sulfanylidene-1,3-thiazolidin-3-yl]butanoylamino]benzoic acid (ML-145, CID2286812)³³ also has high affinity for human GPR35²⁶ but is little used in practice. It also lacks appreciable affinity for the mouse and rat orthologues,²⁶ and as a compound with many chemical liabilities it is useful, if at all, only for *in vitro* studies.³² A single publication³⁴ reports the use of the compound ML-194 (1-(2,4-difluorophenyl)-5-[[2-[[(1,1-dimethylehyl)amino]thioxomethyl]hydrazinylidene]methyl]-1H-pyrazole-4-carboxylic acid methyl ester) as a potential GPR35 antagonist, but since its initial description in a screen for GPR35 antagonists,³⁵ it has hardly been used and nothing is known about its activity across species.

3. POTENTIAL THERAPEUTIC OPPORTUNITIES

Although sodium cromoglycate (as RVT-1601 or PA101) is the only GPR35 activator that has recently been part of clinical trials,³⁶ there is considerable interest in targeting this receptor.

3.1. Immune Cell Expression and Inflammation. As highlighted earlier, GPR35 is expressed by human immune cells including monocytes (CD14⁺), T-cells (CD3⁺), neutrophils, and various dendritic cells and natural killer T cells

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Table 2. Single Nucleotide Polymorphisms in GPR35 That Are Associated with Dise

	-				
variant	loci	change	MAF	disease	refs
rs4676410 ^a	chr2:240624322	G > A	0.272	ankylosing spondylitis eosinophil count inflammatory bowel disease primary sclerosing cholongitis	80-85
rs34236350 ^b	chr2:240628909	C > T	0.261	systemic lupus erythematosus monocyte count	86
		C > T		ankylosing spondylitis psoriasis	
rs3749171 ^c	chr2:240630275	T108 ^{3.44} M	0.151	inflammatory bowel disease Crohn's disease	85,87—89
rs3749172 ^c	chr2:240630832	C > A S294R	0.484	ulcerative colitis Crohn's disease coronary artery calcification	79,90,91
rs4676408 ^d	chr2:240634984	A > G	0.4	inflammatory bowel disease ulcerative colitis	92
4				ulcerative colitis Crohn's disease	
rs4676406"	chr2:240639691	T > G	0.374	ankylosing spondylitis psoriasis primary sclerosing cholangitis	87,93

^aIntron variant. ^b5' UTR variant. ^cMissense variant. ^dIntergenic variant. Positions reference Human Genome Assembly GRCh38.p13.

(CD56⁺) and has a broadly similar expression pattern in mouse.^{1,10,37} More recently, single-cell RNA-seq analysis of immune cells from the lamina propria and Peyer's patch cells from mouse small intestine found GPR35 expression to be most common in dendritic cells (CD103+CD11b-) and macrophages clusters, as well as small, 100-cell "unresolved" clusters.³⁸ Wang et al.¹ showed that sustained treatment with kynurenic acid could inhibit LPS-induced TNF- α secretion from peripheral blood mononuclear cells. In contrast, however, Zheng et al.³⁹ have reported that kynurenic acid inhibits LPS +ATP-induced IL-1 β secretion, but not TNF- α secretion, in mouse bone marrow-derived macrophages and human THP-1 cells. In fact, in these studies the TNF- α level was slightly increased. It is worth noting that at the 4 h point Zheng et al.³ removed the LPS and kynurenic acid, along with any already secreted IL-1 β or TNF- α , before subsequent stimulation with ATP. If kynurenic acid inhibited LPS-induced TNF- α secretion before the wash, then this could have left more available for secretion after ATP.

On the basis of this expression profile, perhaps unsurprisingly, several SNPs located in the GPR35 gene have been reported to be associated with immune and inflammationrelated diseases, including inflammatory bowel diseases (ulcerative colitis and Crohn's disease), ankylosing spondylitis, and primary sclerosing cholangitis (Table 2). One of the most common of these variants, (rs3749171), results in replacement of a threonine by methionine in transmembrane domain III (T108M) (Ballesteros and Weinstein position 3.44) and has a minor allele frequency (MAF) of approximately 0.15 (Table 2). T108 M exhibits strong linkage disequilibrium to several other variants, at least two of which have been reported to be associated with disease. These, rs4676410 ($r^2 = 0.457$, D' =0.98 LD to T108M) and rs34236350 ($r^2 = 0.500$, D' = 0.998LD to T108M), lie outside the coding region; however, they may increase expression and/or affect the splicing of GPR35 in certain tissues (GTEx Analysis, release V8). rs4676410 and rs34236350 are strongly linked to each other ($r^2 = 0.912$, D' =0.981) and have similar MAFs (0.27 and 0.26 respectively),

indicating the two are almost always present on the same allele (Table 3). As noted, T108M is less common but is tightly

Table 3. Prevalence of Human GPR35 Haplotypes^a

Variant	Haplotypes										
rs4676410	G=0.728	A=0.272	G	G	G	А	А	G	G	G	А
rs34236350	C=0.739	T=0.261	С	С	С	т	т	С	С	С	т
rs3749171	C=0.849	T=0.151	С	С	С	т	С	С	С	С	т
rs3749172	C=0.516	A=0.484	С	А	С	А	А	С	А	С	А
rs4676408	A=0.6	G=0.4	А	G	G	А	А	G	А	А	А
rs4676406	T=0.626	G=0.374	т	G	Т	т	Т	G	G	G	G
Haplotype Count			1190	754	665	636	529	464	265	241	89
Haplotype Frequency			0.238	0.151	0.133	0.13	0.106	0.093	0.053	0.048	0.018
^{<i>a</i>} Generated using LDHap Tool (https://ldlink.nci.nih.gov).											

linked to both, meaning that almost every allele of T108 M also contains rs4676410 and rs34236350, making it difficult to distinguish disease associations between each individual variant. While *in vitro* studies showed the T108M variation to have little effect on potency of agonist ligands,⁵ Schneditz et al.⁴⁰ reported the T108M variation to differentially regulate baseline Ca²⁺ levels and Src activation in human induced pluripotent-stem-cell-derived macrophages, consistent with a gain of function effect for Met at this position. By contrast, a further variant, V76M (rs13387859), which did show reduction in agonist potency⁵ has not been linked to disease in GWAS studies, although present at some 2% allele frequency.

In vivo studies have suggested GPR35 function has a protective effect on dextran-sulfate sodium (DSS)-induced colitis.^{30,41,42} Tsukahara et al.³⁰ demonstrated that treatment with the GPR35 agonist pamoic acid reduced the severity of DSS-induced colitis. As such, there appears an opportunity for GPR35 agonists to be effective in inflammatory bowel diseases. As noted earlier, various reports have suggested pamoic acid to have relatively low potency at mouse GPR35 and to function as a partial agonist;^{27,43} therefore, it is unclear if agonists of higher efficacy might be more effective. As discussed earlier the reported effect of CID2745687 in these studies³⁰ is surprising

given its lack of affinity for mouse GPR35 when tested using *in vitro* assays. In a related study, GPR35 knockout mice were shown to have a slightly greater reduction in body weight compared to wild-type mice in response to DSS treatment, and this was coupled with earlier and more severe stool and bleeding symptoms and a larger overall disease score.⁴¹ Together, while these studies suggest a protective role for GPR35 in colitis they seem inconsistent with SNP association studies linking such lower gut disorders to a gain of GPR35 function and/or expression. However, another study that examined DSS-induced colitis in social-deficit-stressed mice found kynurenic acid treatment to result in increased bleeding and histology scores, while showing no effect on body weight or colon length.³⁹

This highlights the need for further investigations to better elucidate the precise role GPR35 plays here and to better define whether agonist or antagonist treatment might be more effective. Clearly this may also be a species-related difference, and indeed, other well-appreciated confounding influences, including cage effects, were not excluded. As mouse colitis models are very gender-specific, male and female mice should both be tested for GPR35 effects. Farooq et al.⁴¹ used only male mice. Given that the endogenous ligand for GPR35 is yet to be defined, it may also be that observed gains or losses of function may reflect action of the endogenous ligand(s) at the receptor.

3.2. Pain and the Nervous System. Several studies have shown GPR35 expression in sensory-neuron-containing dorsal root ganglia (DRG), both at transcript^{29,44–47} and potentially protein level.^{44,47} Early studies indicated GPR35 expression in rat DRG was highest in small- and medium-diameter neurons, though still present in large neurons,44 while more recent analysis of isolated mouse DRG cells by single-cell RNA-seq identified 11 subpopulations of neurons in such DRGs⁴⁶ in which expression of GPR35 was predominately in unmyelinated neuron subtypes known to express the voltage-gated sodium channel Nav1.8, most frequently in the tyrosine hydroxylase subtype, Th, and three nonpeptidergic subtypes, NP1-3, along with one myelinated, non-Nav1.8-expressing, neurofilament subtype, NF1. Analysis of expression of all GPCRs in Nav1.8-expressing vagal afferent neurons isolated from mouse nodose ganglia showed high GPR35 expression and that GPR35 expression was 100-fold lower following diphtheria toxin ablation of Nav1.8 expressing cells.⁴⁸ GPR35 is also regularly coexpressed with CCK1R and GPR65, two GPCR-encoding genes known to be associated with gastrointestinal afferents.48

We have already commented on the observed pharmacology of GPR35 in various neurons from rodents to highlight some inconsistencies in the action of the potential antagonist CID2745687 (section 2.4). However, co-staining of an anti-GPR35 antiserum with NeuN, but not GFAP, was consistent with GPR35 being expressed in neurons in the CA1 region of rat hippocampus, but not glial cells.⁴⁹ Of course, a knockout control for anti-GPR35 specificity is not currently available for rat; thus, efforts to define specific roles of potentially GPR35 active agonists made use of the blocker ML-145. As highlighted earlier, this ligand has been employed infrequently and again, like CID2745687, has been shown unable to block rat GPR35 in transfected cell studies.²⁶ As there have been broad concerns over the specificity of many available anti-GPCR antisera, we recently generated a transgenic knock-in mouse line in which we introduced the hemeagglutinin (HA)

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epitope tag sequence in-frame with the C-terminal tail of GPR35. This provides exquisite detection of GPR35 expression (Figure 3), in these animals for which wild-type mouse tissue provides the necessary specificity controls.



Figure 3. Detection of GPR35 expression in the colon of a transgenic mouse. A knock-in transgenic mouse line was produced in which mouse GPR35 has in-frame addition of the HA-epitope tag sequence at the C-terminus of the receptor. Longitudinal (A, B) and cross sections (C, D) show anti-HA staining throughout the colonic crypts in the transgenic line (mGPR35-HA) tissue (A, C) compared to wild-type tissue (B, D) as control. Scale bar = 10 μ m.

Chronic treatment of rats with kynurenic acid has also been shown to lead to elevated functional CB1 receptor in rat brain, particularly in the hippocampus.⁵⁰ However, the broad spectrum of targets of kynurenic acid, and indeed its metabolites,⁵¹⁻⁵³ make it all but impossible to attribute such an effect directly to activation of GPR35.

3.3. GPR35 and Cancer. GPR35 expression has been linked to various cancers, including gastric, breast, and colon,^{1,7,54,55} but its role remains ambiguous, although ligands such as kynurenic acid can inhibit cell proliferation in such cancers^{56–60} and GPR35 expression in breast cancer tissues has a strong association with advanced histological grades.⁵ Furthermore, it is reported that colon cancer patients with lymph nodes expressing high levels of GPR35b have short disease-free survival times.⁷ In nonsmall cell lung cancer (NSCLC), overexpression of GPR35 is associated with poor prognosis in patients, and GPR35 knockdown significantly overcomes chemoresistance in NSCLC in vitro and in vivo.⁵ Such data imply that GPR35 may facilitate cancer growth and metastasis and, potentially, serve as a clinical tumor marker. It is, therefore, of considerable interest to investigate the significance, signaling and function of GPR35 in late stages of cancers more fully.

Mechanisms of potential relevance to cancer include that GPR35 can elevate Na/K-ATPase-mediated ion transport to subsequently activate the epidermal growth factor receptor/Src/Ras/ERK signaling pathway in colon cancer cells,⁴⁰ and

Src signaling is a well-known factor that contributes to the malignancy of many cancers, including colon cancer.⁶¹ Indeed, Schneditz et al.⁴⁰ further showed that genetic depletion of GPR35 can reduce intestinal tumorigenesis in both spontaneous and inflammation-driven colon cancer mouse models and that a lipid-coupled peptide (pepducin) able to selectively inhibit GPR35 activation also reduced tumor burden in a colitis-associated mouse model. Hypoxia-inducible factor- 1α (HIF-1 α) is known to upregulate GPR35 in certain circumstances, including in cardiac myocytes during the progression of cardiac failure,⁶² and although this feature is not likely of direct relevance to carcinogenesis, hypoxia is a major feature of solid tumors that promotes metastasis and supports tumor recurrence via stimulating cancer stem cell differentiation.^{63,64} As such, although untested, the efficacy of GPR35 antagonists in such settings could be of interest.

Although risk factors rather than direct regulators, chronic inflammation and lipid metabolism are relevant to cancer. Since GPR35 activation can modulate inflammation and enhance lipid metabolism, GPR35 agonists could also have an inhibitory effect on cancer development and progression. With the caveats discussed earlier around the likely lack of specificity of many compounds that display low potency activity at GPR35, molecules such as flavonoid glycosides have the capacity to act as agonists at GPR35.⁶⁵ Widespread in herbs and found to suppress the proliferation of oral and colon cancer cells,^{66,67} any effects are more likely akin to homeopathy, however, rather than reflecting direct GPR35 pharmacology.

3.4. GPR35 and Energy Homeostasis. Energy homeostasis encompasses biological processes regulating food intake, energy expenditure, and metabolism. Although poorly explored, some evidence indicates that GPR35 and its ligands have the potential to regulate this process. As noted earlier, alongside immune cells, expression of GPR35 is predominant in organs of the gastrointestinal (GI) tract including stomach, small intestine, and colon.¹⁸ Concentrations of kynurenic acid are also significant in the GI tract, especially in the small intestine,⁶⁸ although as highlighted earlier the potency of kynurenic acid varies markedly at species orthologues of GPR35.^{10,11} The GI tract actively modulates energy balance through the secretion of peptides hormones, such as cholecystokinin (CCK) that increases the production of pancreatic enzymes by relaying signals to the CNS;⁶⁹ GPR35 is coexpressed with both the CCK1 receptor and the potential proton sensing receptor GPR65 in GI vagal afferents. This suggest that GPR35 may be part of the gut-brain signal axis that regulates energy balance.

Lipid metabolism is also vital to energy homeostasis. The major lipid metabolic organs are adipose tissue, liver, and skeletal muscle. Imbalances of fat storage (adipose tissue) and free fatty acid oxidation (liver and skeletal muscle) ultimately results in obesity. It is reported that obese adults have elevated kynurenine serum levels.⁷⁰ Although kynurenine is not itself an activator of GPR35,^{3,67} it is the precursor of kynurenic acid, the serum concentrations of which are also raised in leptin-receptor-deficient Zucker fatty rats, a widely used rat model of obesity.⁷¹ It is thus possible that kynurenine pathway metabolites, including kynurenic acid, may be associated with the burden of obesity. A recent study in mice indicated that kynurenic acid was able to increase lipid metabolism and thermogenesis in adipose tissue by upregulating expression of genes associated with lipolysis, fatty acid oxidation, and beige

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adipogenesis.⁷² This increases cellular respiration and energy expenditure leading to reduction in body weight. As genetic deletion of GPR35 leads to impaired glucose tolerance and gain of body weight in mice,⁷² these effects of kynurenic acid, if indeed mediated in a GPR35-dependent manner, could control expression of peroxisome proliferator-activated receptor- γ coactivator 1α (PGC- 1α) in adipocytes. PGC- 1α is a transcriptional coactivator for peroxisome proliferator-activated receptor (PPAR) family members. PPARs are nuclear transcription factors and control many physiological processes such as energy metabolism, inflammation, and cancer development⁷³ potentially providing a further link between GPR35 and metabolic function. Key experiments now need to be undertaken with more carefully selected GPR35-targeted pharmacological ligands to improve understanding of this potential link. In addition to the mouse GPR35-HA expressing transgenic mouse line described above (Figure 3), we have also recently generated a transgenic knock-in mouse line in which we replaced mouse GPR35 with an HA-epitope tagged form of human GPR35a. As this receptor orthologue is blocked by the antagonist CID2745687, it should now be practical to assess on-target contributions of GPR35 to metabolic phenotypes in these animals.

Hepatic steatosis is a common chronic liver disease and can transform into cirrhosis and hepatocellular carcinoma. It is defined by an accumulation of lipid in the liver promoted by energy homeostasis imbalance. The potent human and rat (but not mouse) GPR35 agonist lodoxamide has been shown to inhibit lipid accumulation in the human HepG2 cell model and, rather more surprisingly given the pharmacological properties of this ligand, mouse primary hepatocytes.⁷ Lodoxamide was shown to decrease SREBP-1c protein expression and hence inhibit SREBP-1c-induced lipid synthesis. As such, activation of GPR35 was suggested to offer therapeutic potential in the control of hepatic steatosis. However, much remains to be assessed before this could be actively championed. As noted earlier, GPR35 shows marked selection for activation of $G\alpha_{12}$ and $G\alpha_{13}$ over other G proteins (and indeed for $G\alpha_{13}$ over $G\alpha_{12}$).²⁷ Although the impact of GPR35 on hepatic steatosis remains speculative, $G\alpha_{12}$ seems to play a critical role in hepatic lipid metabolism.⁷⁵ Activation of $G\alpha_{12}$ had been shown to limit hepatic steatosis by increasing mitochondrial respiration and fatty acid oxidation in liver. Knockout of the $G\alpha_{12}$ coding gene suppresses sets of genes related to lipid catabolism, acyl-CoA metabolism, ketogenesis, and peroxisomal oxidation processes. Notably, PPAR α and PGC-1 α are core partners in this gene network. Although at this stage little more than a hint, these data also provide a potential link for GPR35 in maintaining energy homeostasis and preventing the development of hepatic steatosis. GPR35 ligands may thus be worthy of consideration among novel therapeutic strategies for obesity, glucose intolerance, and fatty liver disease.

3.5. GPR35 and the Cardiovascular System. Initial reports of a GPR35 knockout mouse line indicated a remarkable 37.5 mmHg increase in blood pressure compared to wild-type littermates,⁷⁶ which would seem to suggest a potential opportunity for GPR35 agonists to lower blood pressure. Sadly, this was not replicated in a subsequent independent study using a different knockout line.⁷⁷ Despite this, a range of studies have noted contributions of GPR35 to cardiovascular phenotypes (see ref 78 for review). Indeed, an association between a nonsynonymous GPR35 SNP in the

intracellular C-terminal tail (rs3749172, S294R) (Table 2) and coronary artery calcification⁷⁹ was an early outcome. Despite being a potential site of agonist-induced phosphorylation, replacement of this serine residue has not been reported to result in alterations in function or regulation of the receptor. Ronkainen et al.⁶² noted increases in cardiac GPR35 expression induced by hypoxia in mouse models of cardiac failure and therefore suggested that levels of GPR35 mRNA might provide an early marker of progressive cardiac failure. As GPR35 is expressed robustly in human vascular smooth muscle and endothelial cells, MacCallum et al.³² assessed effects of GPR35 activation on cell migration and proliferation to assess the potential that GPR35 ligands might be relevant to treatment of neointima formation in vein graft failure following coronary artery bypass treatment. Effects of GPR35 activation on vascular smooth muscle migration in a scratch-wound assay were mediated via the RhoA/Rho kinase signaling axis. This is actively regulated by the $G\alpha_{12}/G\alpha_{13}$ group of G proteins for which GPR35 actively selects. Overall, however, despite a number of studies exploring cardiovascular consequences of GPR35 function, a clear path to functional translation seems distant.

4. CONCLUDING REMARKS

Despite remaining an orphan GPCR, where suggestions of various endogenous activators have waxed and waned, GPR35 is attracting increasing interest as a therapeutic target. This is nevertheless the case in inflammatory bowel disease, based on both genetic association studies and high level expression in the colonic tissue and infiltrating white cells. However, the species-selective nature of the pharmacology of both agonist and antagonist compounds means that there is an additional need to fully define ligand effects in the patho-physiological setting as genuinely being mediated by GPR35 if human tissue is not available. The suitability of standard rodent preclinical models to predict function and therapeutic utility for projects centered on GPR35 are therefore amplified above those normally considered. Hopefully, the recent development of humanized and epitope-tagged GPR35 transgenic mouse lines may help to address these issues.

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Notes

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