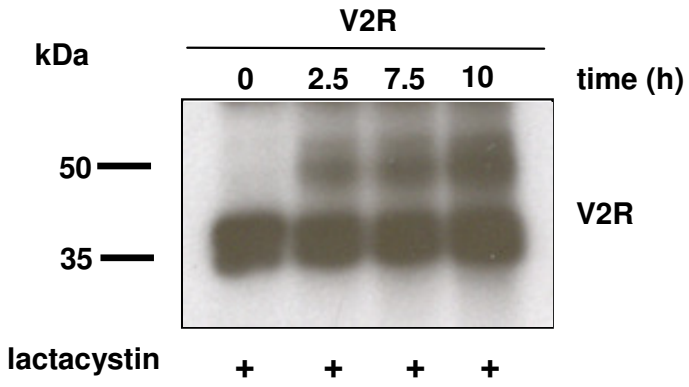
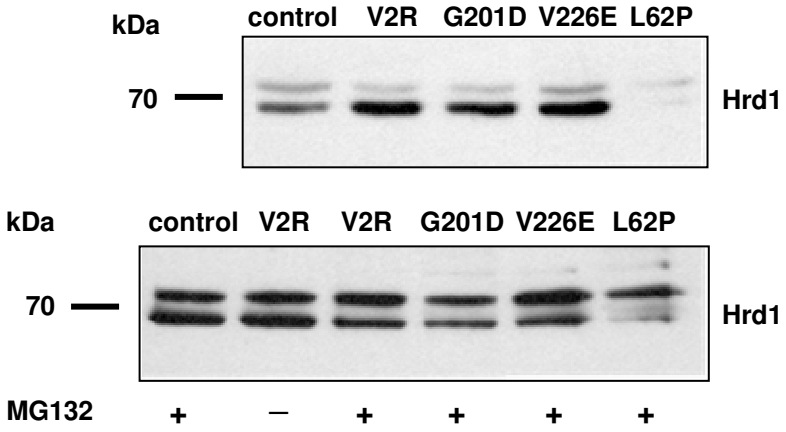
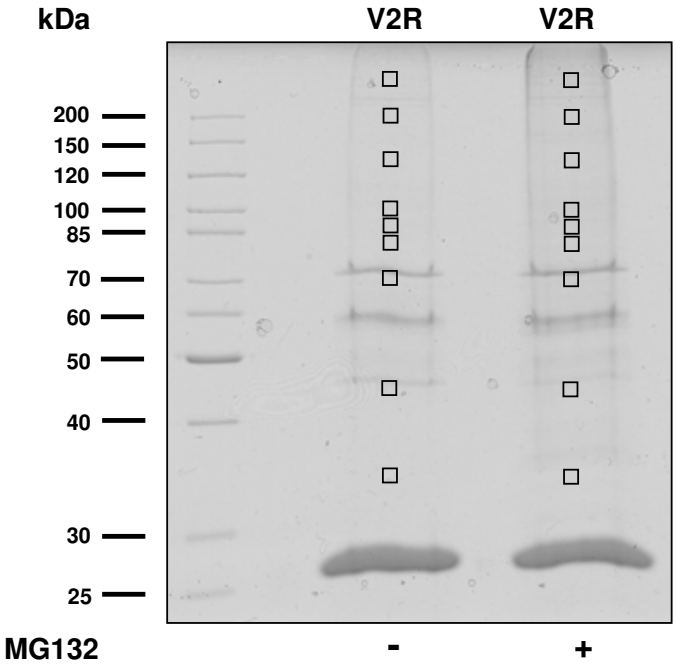
Appendix: Figures



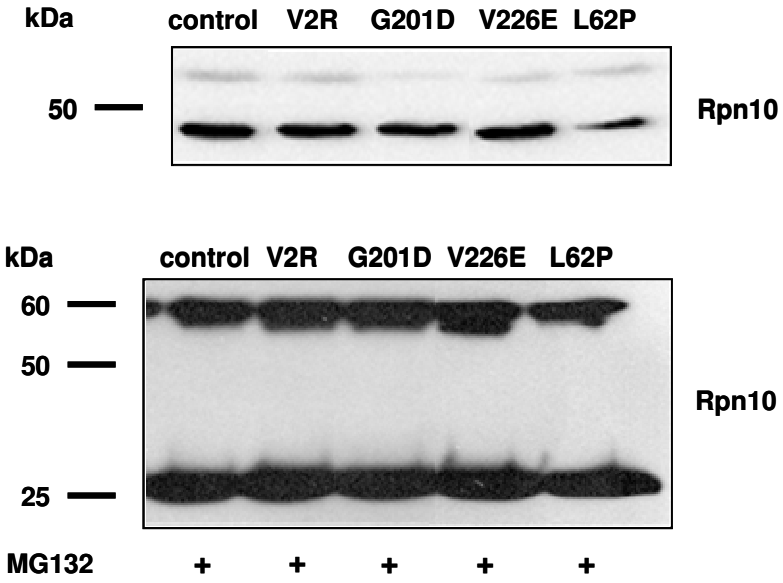
App. Fig. 1: Lactacystin-inhibited degradation of wild-type V2R in stably expressing HEK293 cells. Cells expressing the FLAG-tagged wild-type V2R were starved in serum-free DMEM without methionine and cysteine for 16 h and metabolically labeled with 220 µCi EasyTag™ EXPRESS^{35}S Protein Labeling Mix for 45 min in presence of 5 µM lactacystin. The metabolic labeling was stopped and at time points 0, 2.5, 5, and 10 h the cells were harvested, lysed and proteins were immunoprecipitated and separated by SDS-PAGE. The gels were dried and exposed to X-ray films. Molecular mass markers are shown on the left. The results are representative of three individual experiments.



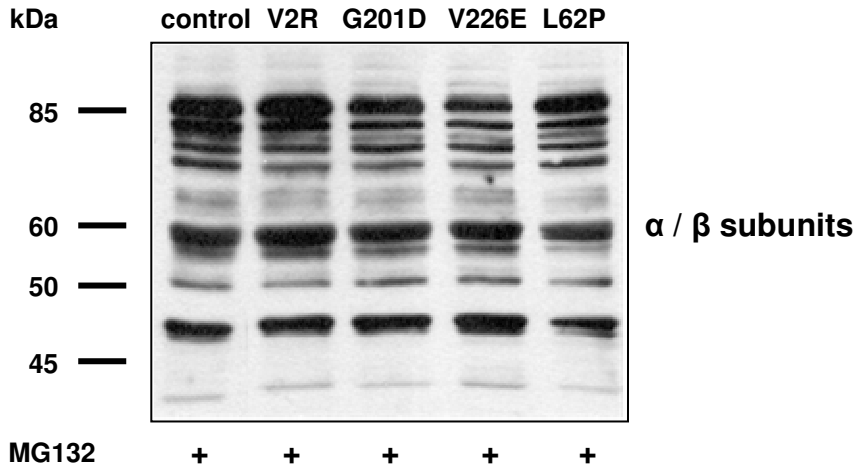
App. Fig. 2: Interaction of V2Rs with Hrd1. Whole cell lysates of HEK293 cells stably expressing wild-type and mutant V2Rs were probed for Hrd1 expression levels by western blotting (top panel). Immunoprecipitation of FLAG-tagged V2R wild-type and mutant receptors in presence (+) or absence (-) of MG132 was performed and analyzed by immunoblotting in the lower panel. Detection of the co-precipitated ubiquitin ligase Hrd1 was done with a polyclonal rabbit α-Hrd1 antibody and a POD-conjugated α-rabbit antibody. Controls were non-transfected HEK293 cells.



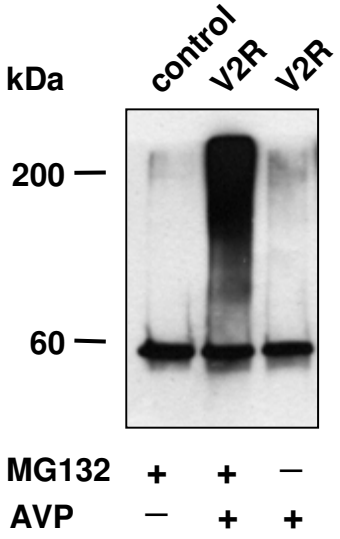
App. Fig. 3: Representative gel for V2R-associated proteins subjected to mass spectrometry. V2Rs in presence (+) or absence (-) of the proteasome inhibitor MG132 were immunoprecipitated via their FLAG tag and separated by SDS-PAGE. Co-precipitated proteins were stained with Coomassie blue, excised from the gel (size is indicated by □) and subjected to in-gel digestion by trypsin. NanoLC-MS/MS experiments were performed and peptides identified in a data-dependent mode (survey scanning) using one MS scan followed by MS/MS scans of the most abundant peak.



App. Fig. 4: Interaction of V2Rs with the Rpn10 subunit of the 19S regulatory particle. Whole cell lysates of HEK293 cells stably expressing wild-type and mutant V2Rs were analyzed for Rpn10 expression levels by western blotting. Immunoprecipitation of FLAG-tagged wild-type V2R and mutant receptors pretreated with MG132 was performed and proteins were subjected to western blot analysis. Detection of co-precipitated subunits of the 19S regulatory particle was done with a monoclonal mouse α-Rpn10 antibody and a POD-conjugated α-mouse antibody. Controls were non-transfected HEK293 cells. Data are representative of three individual experiments. Two additional mutants tested were excised from the blot.



App. Fig. 5: Test of an antibody directed to α and β subunits of the 20S proteasome. Total cell lysates of HEK293 cells stably expressing wild-type and mutant V2Rs pretreated with MG132 were analyzed for expression levels of α and β subunits of the catalytic particle by immunoblotting. Detection of the 26S proteasome was done with a polyclonal rabbit α–20S proteasome α / β subunit IgG and a POD-conjugated α-rabbit antibody. Controls were non-transfected HEK293 cells. Similar data have been obtained in three independent experiments.



App. Fig. 6: Ubiquitination of immunoprecipitated FLAG-tagged V2Rs. HEK293 cells stably expressing wild-type V2Rs were treated with 20 µM MG132 for 16 h (+) or left untreated (-) and stimulated by 1 µM AVP for 90 min (+). Controls were non-transfected HEK293 cells, which were treated with MG132 for 16 h. Proteins eluted from anti-FLAG affinity gel were analyzed by SDS-PAGE and western blot analysis with a monoclonal mouse α-polyubiquitin antibody and a POD-conjugated α-mouse antibody. Data are representative of three independent experiments.

V2R-associated proteins identified by NanoLC-ESI-MS/MS (with MG132 treatment)

Protein	SwissProt	Molecular mass (*Da*)	Score	Peptides (MS/MS)	Band excised (*kDa*)
V2R (human)	P30518	40253	426	11	>200
Alpha-2-macroglobulin precursor	P01023	163175	357	7	>200
Hemoglobin subunit beta (human)	P68871	15857	224	4	>200
DNA-dependent protein kinase catalytic subunit (human)	P78527	468788	175	5	>200
Dynein heavy chain (human)	Q14204	532072	154	5	>200
Hemoglobin subunit alpha (human)	P69905	15117	119	3	>200
Apolipoprotein A-I-precursor (human)	P02647	30759	77	4	>200
Desmoplakin (human)	P15924	331569	146	4	>200
S100 Ca^{2+}-binding protein A9	P06702	13234	57	1	>200
ADP/ATP translocase 1 (human)	P12235	32912	49	1	>200
Desmocollin-1 precursor (human)	Q08554	99982	48	1	>200
V2R (human)	P30518	40253	246	5	200
Ig gamma-1 chain C region (mouse)	P01869	43359	206	4	200 #
Desmoplakin (human)	P15924	331569	79	3	200
Structural maintenance of Chromosomes 4-like 1 protein	Q9NTJ3	147091	49	1	200
Serine/threonine-protein kinase ULK1 (human)	O75385	112530	49	1	200
Large proline-rich protein BAT3 (human)	P46379	119334	102	1	130
V2R (human)	P30518	40253	82	1	130

Ig gamma-1 chain C region (mouse)	P01869	43359	46	1	130
Ig gamma-1 chain C region (mouse)	P01869	43359	130	2	100
M-phase inducer phosphatase 2 (rat)	P48966	64246	46	1	100
26S proteasome non-ATPase regulatory subunit 2 (Rpn1)	Q13200	100136	447	9	90
Heat shock protein HSP 90-β (HSP 84)	P08238	83081	390	8	90
DNA replication licensing factor	P33993	81257	384	7	90
Heat schock protein HSP 90-α (HSP 86)	P07900	84476	253	5	90
V2R (human)	P30518	40253	120	3	90
Ig gamma-1 chain C region (mouse)	P01869	43359	89	2	90
Delta 1-pyrroline-5-carboxylate synthetase	P54886	87248	55	1	90
V2R (human)	P30518	40253	96	1	80
Immunoglobulin heavy chain-binding protein (GRP78/BiP)	P11021	72288	1399	23	70
Heat shock 70 kDa protein cognate 3 precur. (drosophila)	P29844	72216	223	4	70
Heat shock 70 kDa protein C precursor (HSP7C) (Caebr)	P19208	72901	170	4	70
Heat shock 70 kDa protein cognate 4 (drosophila)	P11147	71087	152	3	70
Heat shock protein 70 A1 (anoal)	P41825	70208	117	2	70
V2R (human)	P30518	40253	89	1	70
Heat shock 70 kDa protein 6 (human)	P48741	26890	74	1	70
Elongation factor 1-a1 (eEF1A-1)	P68104	50109	62	13	45
26S proteasome ATPase regulatory subunit 7 (Rpt1/S7)	P35998	48472	352	5	45

Ig gamma-1 chain C region (mouse)	P01869	43359	121	2	45
V2R (human)	P30518	40253	86	1	45
Elongation factor Tu, mitochondrial precursor (EF-Tu)	P49411	49510	80	1	45
Eukaryotic initiation factor 4A-I (human)	P60842	46125	52	1	45
L-lactate dehydrogenase A chain (LDH-A)	P00338	36534	252	5	35
V2R (human)	P30518	40253	129	3	35
L-lactate dehydrogenase B chain (LDH-B)	P07195	36484	92	2	35
Ig gamma-1 chain C region (mouse)	P01869	43359	43	1	35

Table 2b: V2R-associated proteins identified by NanoLC-MS/MS. V2R samples (incubated for 16 h in presence of MG132) were immunoprecipitated *via* their FLAG-tag and separated by SDS-PAGE. Co-precipitated proteins were stained with Coomassie blue, excised from the gel and subjected to in-gel digestion by trypsin. NanoLC-MS/MS experiments were performed and peptides identified in a data-dependent mode (survey scanning) using one MS scan followed by MS/MS scans of the most abundant peak. The complete list of proteins identified is shown excluding ubiquitin and different types of keratin.

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