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	1	1 0	2 Q	зņ	4 Q	5 Q	6 0	7 Q	8 Q	9 Q	100
hGsdmD	MGSAF	RVVRRVVQEI	D.HGGEFIPV	TSLQSSTGE	PYCLVVRKP	SSSWFWKPR	YKCVNLSIKDI	LEP DAAEP DV	QRGRSFH <mark>F</mark> YD	AMDGQIQGSVE	LAAPGQ
mGsdmD mGsdma3	MPSAFE . MPVF	CKVVKNVIKEV DVTRALVREI	SGSRGDLIPV	DSLRNSTSFE	RPYCLLNRKF RPFCLVLRKR	SSSRFWKPR KSTLFWGAR	Y S C V N L S I K D I Y V R T D Y T L L D L	LEPSAPEPEP LEPGSSPSDL	ECFGSFKVSD TDSGNFS F KN	VVDGNIQGRVM. MLDVOVOGLVE	LSGMGE VPKT
mGsdma	. MTMF	NVTRALARQI	N.PRGDLT <mark>P</mark> L	DSLIDFKRF	IPFCLVLRKR	KSTLFWGAR	YVHTDYTLLDV	LEPGSSPSDP	TDSGNFS <mark>F</mark> KN	MLDARVÊGDVD	VPKT
mGsdma2 mGsdmC	. MSMFE	DVTRALARQI	N. PRGDLTPL	DSLIDFKRFF	IPFCLVLRKR	KSTLFWGAR SSRLALKTE	YVRTDYTLLDV Y I PVGFTLLHL	LEPGSSPSDP LEPNTPVPEP	T L L G N F S F K N E V S A P T P L K H	MLDVRVEGDVE	VPTM VETT
mGsdc4	MGYSE	RASKDVVKKI	QGRDLRPV	ECLSDATKF	LFHI.LQET	P.RSGWETE	DIPVGFTLLDL	LEPNFPVPEP	EVSAPKPFIH	VQSTDLEANLN	VAD.I
mGsdc2	MGYSEL	RASKDVVKKI	Q GRDLRPV	ECLSDATKER	RLFHI.LQET	P.RSGWETE	DIPVGFTLLDL	LEPNFPVPEP LEPNFPVPEP	EVSAPKPFIH EVSAPKPFIH	VQSTDLEANLN	VADI VADT
mosaco	1010	INA DADAY ANAL			CDENT. DVC1	r.koomara			LUDATRI	VQ 5 I DELENIEN	AD1
		110	120	130	140	150	160	170	180	190	200
hGsdmD mGsdmD	AKIAGO	AAVSDSSSTS	MNVYSLSVDP	NTWQTLLHE	RHLRQPEHKV	LQQLRSRGD	NVYVVTEVLQT	QKEVEVTRTH KEEVOTTEVH	KREGSGRFSL SOFGSGOFTL	PGATCLQGEGQ	GHLSQK
mGsdma3	VKVKGI	AGLSQSST	LEVQTLSVAP	SALENLKKE	KLS.ADHSF	LNEMRYHEK	NLY <mark>VV</mark> MEAVEA	KQEVTVEQTG	NANAIFSLPS	LALLGLQGS	LNNN
mGsdma mGsdma2	VKVKGI	AGLS RSST	LEVOTLSVAP	TALENLHKE	KLS.ADHPF	LKEMRERGE	N L Y VV MEVVET N L Y VV MEVVEA	LOEVTLERAG	KAEGCFSLPF NAISKFSLN	FAPLGLQGS	VNHK
mGsdmC	AGG	AGFVKSCGYD	IEVQSKSIPN	PKLESLQNR	LLD.QLPTF	MKTCWKDGK	NLY <mark>VV</mark> TEAYEV	TKDTVLEGTS	NSKFAIKGII	NQLVKVGGSGQ	WQTEKT
mGsdc4	AR.G	VGYVGYGGYN	IEVQSTSIPN	PKLEILQNR	LLD.KLPTF	MKFCRMERK	N L Y VV TEAYEV	SKDTMLTGLS	SVNLLVKGFF	KQLFKVRGKAG	RSEK
mGsdc3	AR G	VGYVGYGGYN	IEVOSTSIPN	PKLEILQNR	LD.KLPTF	MKFCRMERK	NLYVVTEAYEV	SKDTMLTGLS	SVNLLVKGFF	KQLFKVRGKAG	RSEK
		Fragm	ent Required F	or Cell Death	mNeon-G	reen Insert	с	aspase-1/11 Cle	avage Site		
	21	.₀ < 22	0	230	240	250	260	270	280	290	
hGsdmD	KTVTI	SGSTLAFRVA	Q <mark>L</mark> VIDSDL	DVLLFPDKK(QR <mark>TF</mark> QPPATG	HKRSTSEGA	WPQLPSGLSMM	RCLHNFLTDG	VPAEGA	FTE DF QG L RA E	VETISK
mGsdmD mGsdma3	KAVTI	AGSILAFRVA	QLLIGSKW	DILLVSDEK	QRTFEPSSGD	RKAVGQRHH	GLNVLAALCSI AFT	GKQLSLLSDG	IDEEELI	EAADFQGLYAE	VKACSS
mGsdma	EAVTIE	KGCVLAYRVR	QLMVNGKDEW	GIPHICNDSM	4Q TF PPGEKP	GEG		.LIQ	ASDVGE	MHEDFKTLKEE	VQRETQ
mGsdma2	EAVTIE	KGCVLAYRVR	QLIIYGKDEW	GIPYICTDNM	4PTFNPLCVL	QRQ	GS	TV	QMISGE	MHEDFKTLKKE	VQQETQ
mGsdc4	YSIPI	KGSVLAYKKČ	QLVIENNTCV	ILPSAT.KKH	(M <mark>TF</mark> PDRPLK	LYDLPVTLR	YQEEVI	.ETGSWID.D	IDPIGTIEEP	ANLNFMCLQHE	VSEQTQ
mGsdc2	YSIPIE	KGSVLAYKKO	QLVIENNTCV	ILPSAT.KKH	(MTEPGTPKY	ASASEPTEI	YRTE	. LQGLWIN.D	IVPIGRIQEP	AHLDFMCLQNE	YKQTE
mosues		NGOVERIN'S	UNITER RECO	IDF ORI . KKI	e o re kr	NUNULLILI			THIIGKIYHI	AUTOR CODAL	0 L Q L K
3 bCodeD		310	320	330	340	350	360 NUTROTUL COC	370	380	390 	TROTT
mGsdmD	ELESLE	MELRQQILVN	IGKI <mark>L</mark> QDQ	PSMEALEASI	GQGLCSGGQ	VEPLDGPAG	CILECLVLDSG	ELVPELAAPI	FYLLGALAVL	SETQQQLLAKA	LETTVL
mGsdma3	EVEKLS	PVGRSSLLTS	LSHLLGKK	KELQDLEQKI	E	. GALDKGQK	VTLEALPKDVL	L.SKDAMDAI	LYFLGALTEL	TEEQLKILVKS	
mGsdma mGsdma2	EVEKLS	PVGRSSLLIS	LSHLLGKK	KELQDLEQM	E	.GALDKGHE	V T L E A L P K D V L	L.LKDAMDAI	LYFLGALTEL	SEEQLKILVKS	LENKVL
mGsdmC	LLAELS	KDVQEVVFSS	FLHMLCDR	DVLYDLMKMI	ELNQ	LGHMDGPGG	K I LD E L R K D S S	LSWINLKDLI	LYLLQALMVL	SDTQLCLLALS'	VEMRLL
mGsdc2	QLAELS	KGVQEVVLSS	ILSMLYEGDR	KVLYDLMNMI	ELNQ	LGHMDGPGG	KILDELRKDSS	NPCVDLKDLI	LYLLQALMVL	SDSQLNLLAQS	VEMGIL
mGsdc3	LLPELS	KDVQEV <mark>VLSS</mark>	FLSMLYEGDR	NVLHDLMKMI	ELSQ	LGHMDGPGG	K I <mark>LD</mark> E <mark>L</mark> RKDSS	NPCVDLKDLI	LYLLQALMVL	SDSQLNLLARS	VEMRLL
	4 O Ọ	410	4 2 Ņ	430	440	4 5 Q	460	47 Q	4 8 Q		
hGsdmD	LGPLEI	VGSLLEQSAP	WQERSTMSLP	PGLLGNSWG	G.APAWVLL	DEC <mark>GL</mark> ELGE	DTPHVCWEPQA	QGRMCALYAS	LALLSGLSQE	PH	
mGsdmD mGsdma3	PVQLKI	VESTLEQNFL	QDKEGVFPLQ	PDLLSSLGE	ELTLTEALV	GLS GL EVQR	SGPQYAWDPDT	RHNLCALYAS	LSLLHLLSRK	SNALTYCALS.	
mGsdma	PVQLKI	VESTMEKNFL	QDKEGVFPLQ	PDLLSSLGE	ELILTEALV	GLS <mark>GL</mark> EVÕR	SGPQYTWDPDT	LPHLCALYAG	LSLLQLLSKN	S	
mGsdma2 mGsdmC	PHQVEI	VKSILQPNFK	YPWNIPFTLO	POLLAPLOGE	GLAITYELL	GLSGLEVQR EECGLKMEL	NNPRSTWDLEA	KMPLSALYGS	LSFLQQLSEA	ö	
mGsdc4	PHQVEI	VKSILQTNFK	YSSNTPFTLQ	PQLLAPLQG	GLAITYELL	EECGLKMEL	NNPRSTWDLEA	KMPLSALYGS	LSFLQQLQKA	NSSSKPSLSPG	ΥI
mGsdc2 mGsdc3	PHQVEI	VTSILQPNFK	YPWNIPFTVQ	PQLLAPLQG	GLAITYELL	EECGLKMEL	NNPRSIWDLEA	KMPLSALYGS KMPLSALYGS	LSFLOQLOKA	NSSFKPSLRPG	YI

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Β.

Merged mNeon-GSDMD mRuby-GSDMD









SUPPLEMENTAL INFORMATION

Supplementary Figure 1: Alignment of gasdermin D with gasdermin family members. Sequence alignment between GSDMD and family members was conducted using Clustal Omega and the figure generated with ESPript 3.0. The GSDMD fragment demonstrated by Shi *et al.* Nature 2015 to cause death, the mNeon-Green insertion site, and the caspase-1/11 cleavage site are indicated.

Supplementary Figure 2: mRuby-gasdermin D. (A) Epifluorescent imaging of mRuby-GSDMD expressed in HEK-293T cells using calcium phosphate transfection. (B) Epifluroescent imaging of HEK-293T cells co-expressing mNeon-GSDMD and mRuby-GSDMD. Imaging is representative of three independent transfections.

Supplementary Figure 3: Expression and pore formation of Y376H/D GSDMD in macrophages. $Gsdmd^{-/-}$ guide 1 iBMDM cells were reconstituted with untagged WT, Y38F, Y376H, and Y376D GSDMD. (A) Western blot analysis of GSDMD levels in reconstituted cell lines. (B) Pore formation measured as PI uptake in WT-GSDMD and Y376H/D-GSDMD cell lines. Data represents the mean \pm SE of three independent experiments and a total of six technical replicates.

Supplementary Figure 4: Video of live cell imaging of mNeon-GSDMD. Live cell confocal imaging of mNeon-GSDMD reconstituted iBMDM cells primed with 200ng/mL LPSand stimulated with 10µM nigericin. Video shown at 600x speed. Still images available in Figure 6. Live cell imaging is representative of three independent experiments.