Supplemental Information

Ubiquitin Chain Enrichment Middle-Down Mass Spectrometry (UbiChEM-MS) Analysis Reveals Cell-Cycle Dependent Formation of Lys11/Lys48 Branched Ubiquitin Chains

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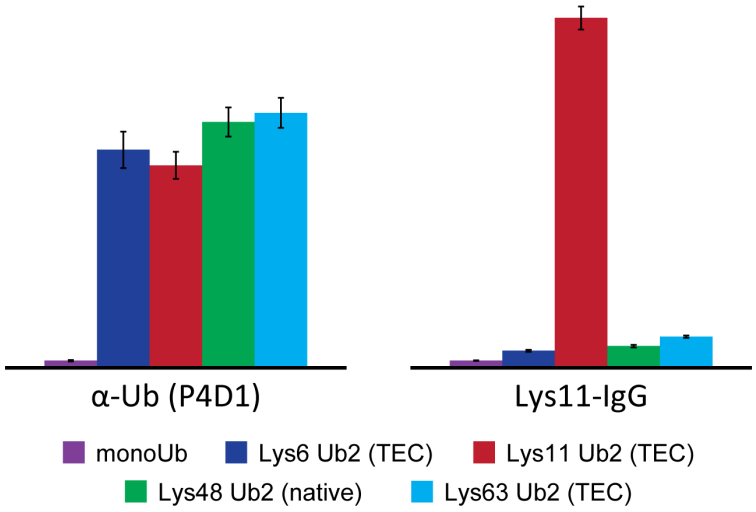

Figure S1. Selectivity of α-Lys11-IgG was assessed by ELISA. The humanized antibody was generated as described, and its specificity was tested using ubiquitin (Ub) monomer, thiolene coupling-derived (TEC) and enzymatically generated (native) Ub dimers. The signals were normalized to monoUb in each set. Bar graphs represent mean ± standard error of the mean (SEM).

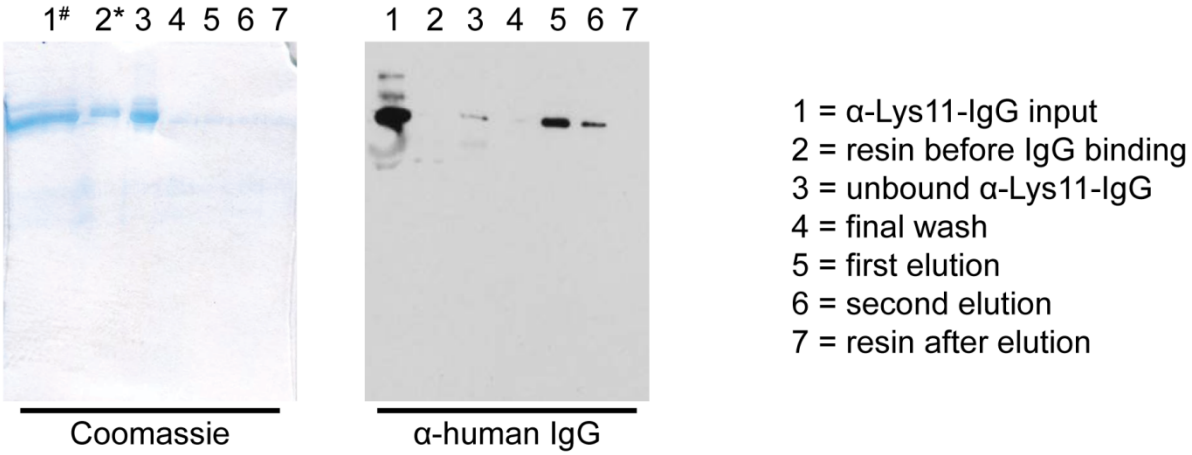

Figure S2. Qualitative assessment of α-Lys11-IgG binding to Protein A agarose resin. α-Lys11 IgG (1) generated was incubated with pre-equilibrated Protein A resin (2) for 16 hr at 4 ºC. The resin was centrifuged, the unbound supernatant (3) saved and the resin was washed rigorously. An aliquot of this α-Lys11 IgG:Protein A resin was incubated with 0.1 M glycine pH 2.7 for 5 mins and then centrifuged to separate supernatant (5) and resin. The elution step was repeated with this same resin and centrifuged to separate supernatant (6) and resin (7). Aliquots of the samples were separated on a 15% SDS-PAGE gel and then analyzed by Coomassie staining and Western blot using α-human IgG.

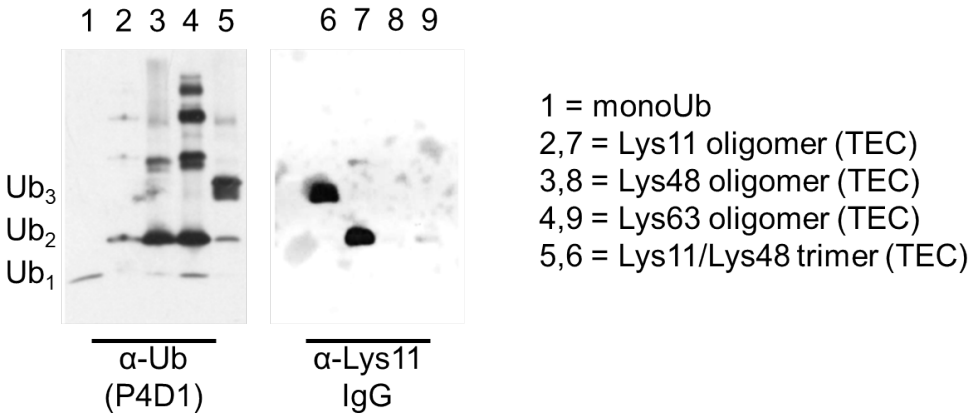

Figure S3. Assessing the ability of α-Lys11-IgG to recognize Lys11/Lys48-linked branched Ub oligomers. Aliquots of Ub monomer (monoUb) and thiolene (TEC)-derived oligomers were separated on a 15% SDS-PAGE gel and then analyzed by western blot using α-Ub (P4D1, *left*) and α-Lys11 IgG (*right*).

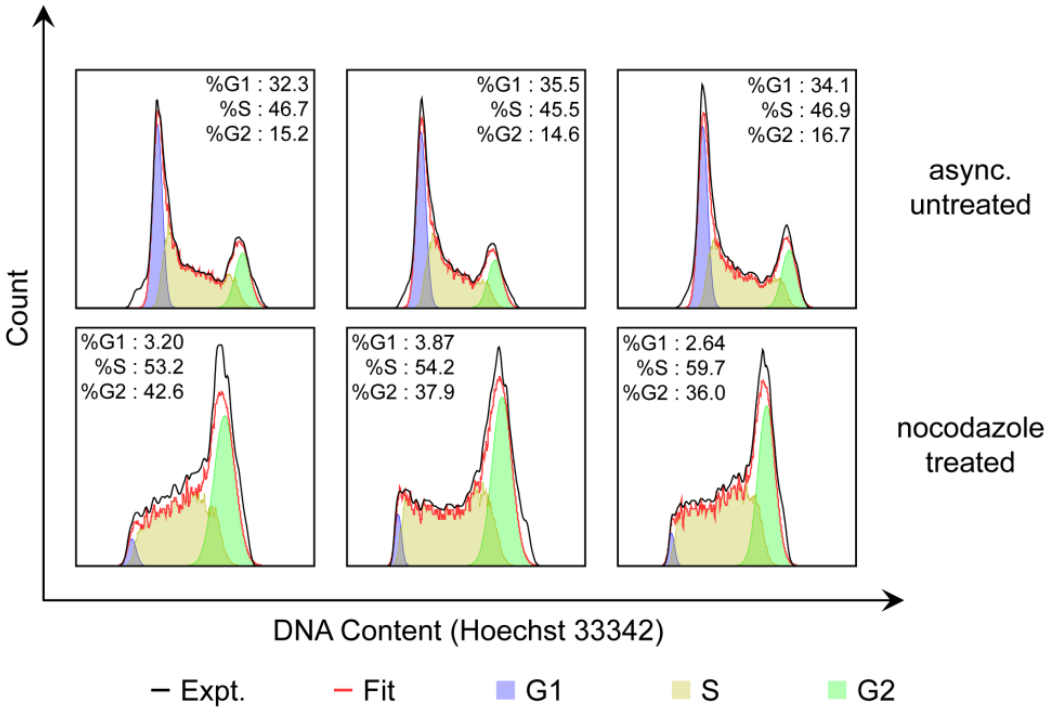

Figure S4. Analysis of cell cycle distribution by flow cytometry. Cell cycle profiles are calculated using area and width parameters on the Hoechst 450/50nm channel. The population distribution was analyzed in FlowJo using Watson Pragmatic model.

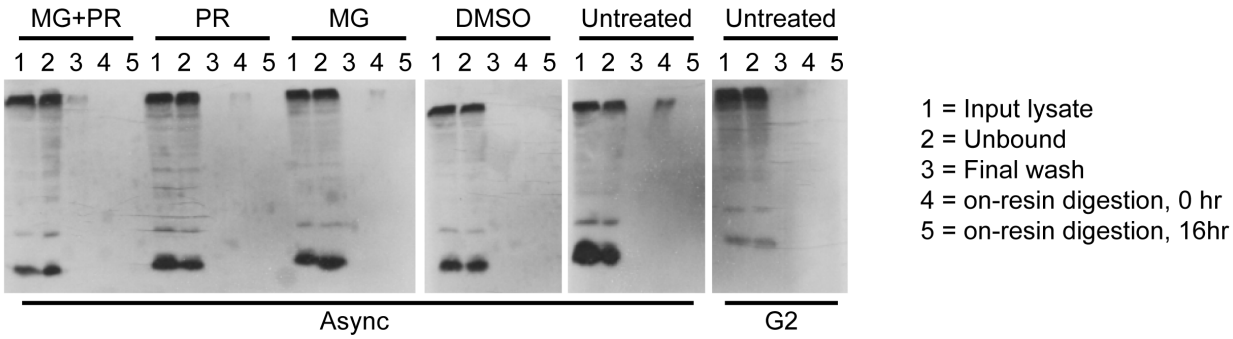

Figure S5. Ub chains enriched from cells subjected to various treatments in different cell cycle phases. The treatments were either inhibition of proteasome by 10 μM MG132, inhibition of DUBs by 30 μM PR619, or co-inhibition of DUBs and proteasome for 4 hr. DMSO treatments served as vehicle control. Batches of cells were synchronized and not further treated, for which asynchronous cells served as control. Aliquots of the samples were separated on a 15% SDS-PAGE gel and then analyzed by western blot using anti-Ub antibody (P4D1).

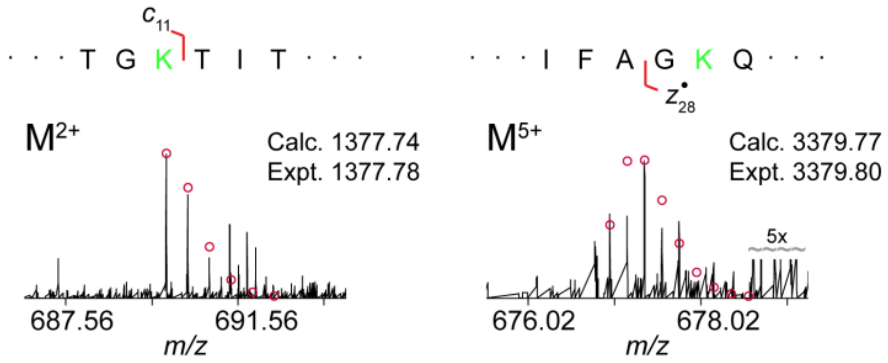

Figure S6. ETD analysis of $^{2xGG}Ub_{1-74}$ species isolated from asynchronous cells treated with MG132 and PR619. ETD fragments used to deduce the site for the two diGly modifications to be at Lys11 and Lys48. The position of modified lysine is highlighted in green.

Figure S7. ETD analysis of $^{2xGG}Ub_{1-74}$ species isolated from asynchronous cells treated with MG132 and PR619. ETD fragments mapped onto sequence of Ub highlighting all seven possibilities for Lys11-linked branched chains. The position of modified lysine is highlighted in green.

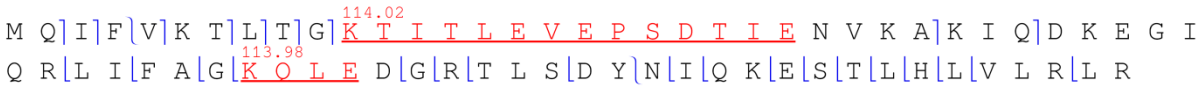

Figure S8. Result from MSAlign search using ETD spectra collected from $^{2xGG}Ub_{1-74}$ species isolated from asynchronous cells treated with MG132 and PR619. Spectral matches are mapped onto the sequence of Ub, and sites for 114 Da modifications are highlighted in red. The analysis also computed E-value of only 6.9E-26 and P-value of 6.4E-26, indicating good confidence for the match.

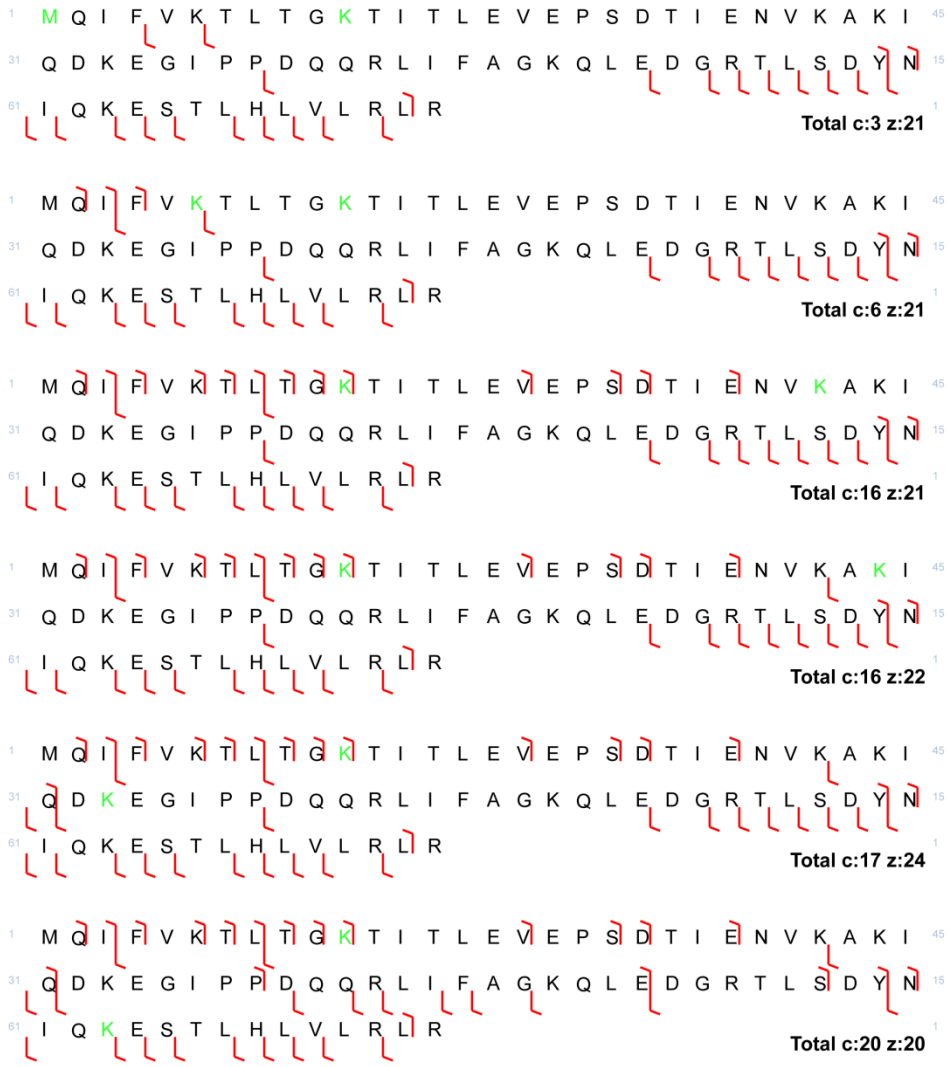

Figure S9. ETD analysis of $^{2xGG}Ub_{1-74}$ species isolated from G2 synchronized cells. ETD fragments mapped onto sequence of Ub highlighting the other six possibilities for Lys11-linked branched chains. The position of modified lysine is highlighted in green.

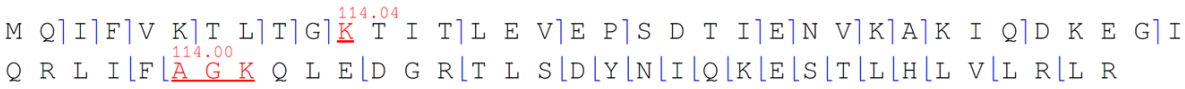

Figure S10. Result from MSAlign search using ETD spectra collected from $^{2xGG}Ub_{1-74}$ species isolated from G2 synchronized cells. Spectral matches are mapped onto the sequence of Ub, and sites for 114 Da modifications are highlighted in red. The analysis also computed E-value of 1.8E-32 and P-value of 9.9E-33, indicating high confidence for the match.

Table S1. Data used to make bar graph in Figure 2 for Ub chains isolated from asynchronous cells.

	Relative Abundance										
	Bio Rep1			Bio Rep 2			Bio Rep 3			Mean	SEM
	TR1	TR2	TR3	TR1	TR2	TR3	TR1	TR2	TR3		
DMSO											
Ub_{1-74}	70.2%	66.3%	72.2%	73.9%	69.8%	67.8%	70.8%	70.2%	72.9%	70.4%	0.8%
$^{GG}Ub_{1-74}$	27.4%	31.0%	25.6%	23.8%	27.7%	29.2%	26.7%	26.9%	27.1%	27.3%	0.7%
$^{2xGG}Ub_{1-74}$	2.4%	2.7%	2.1%	2.4%	2.5%	3.0%	2.5%	2.9%	0%	2.3%	0.3%
MG132											
Ub_{1-74}	79.6%	79.4%	77.8%	76.5%	77.2%	77.3%	73.6%	73.0%	72.6%	76.3%	0.9%
$^{GG}Ub_{1-74}$	20.5%	20.6%	20.7%	23.5%	22.8%	22.7%	26.4%	27.0%	27.4%	23.5%	0.9%
$^{2xGG}Ub_{1-74}$	0%	0%	1.5%	0%	0%	0%	0%	0%	0%	0.2%	0.2%
PR619											
Ub_{1-74}	86.4%	83.9%	81.0%	73.3%	74.0%	73.1%	77.3%	77.5%	76.5%	78.1%	1.6%
$^{GG}Ub_{1-74}$	13.1%	15.2%	17.9%	25.1%	24.4%	25.4%	22.7%	22.5%	23.5%	21.1%	1.5%
$^{2xGG}Ub_{1-74}$	0.5%	1.0%	1.1%	1.6%	1.5%	1.6%	0%	0%	0%	0.8%	0.2%
MG132+PR619											
Ub_{1-74}	64.0%	60.3%	59.9%	65.1%	66.6%	64.4%	65.0%	65.4%	64.6%	63.9%	0.8%
$^{GG}Ub_{1-74}$	33.4%	36.2%	36.5%	32.7%	31.8%	33.4%	30.6%	30.7%	31.1%	32.9%	0.7%
$^{2xGG}Ub_{1-74}$	2.6%	3.6%	3.6%	2.2%	1.6%	2.2%	4.4%	3.9%	4.3%	3.2%	0.3%

Bio Rep – Biological Replicates, TR – Technical Replicates, Mean – average across all biological and technical replicates, SEM – standard error of the mean

Table S2. Data used to make bar graph in Figure 3 for Ub chains isolated from untreated asynchronous cells and nocodazole treated and released synchronous cells.

	Relative Abundance										
	Bio Rep1			Bio Rep 2			Bio Rep 3			Mean	SEM
	TR1	TR2	TR3	TR1	TR2	TR3	TR1	TR2	TR3		
Async.											
Ub_{1-74}	84.0%	78.4%	77.7%	78.9%	74.8%	75.0%	80.8%	79.0%	74.5%	78.1%	1.0%
$^{GG}Ub_{1-74}$	16.0%	20.5%	20.9%	21.1%	23.9%	23.3%	19.2%	21.0%	25.5%	21.3%	0.9%
$^{2xGG}Ub_{1-74}$	0%	1.1%	1.4%	0%	1.3%	1.7%	0%	0%	0%	0.6%	0.2%
Sync.											
Ub_{1-74}	59.5%	58.8%	58.0%	63.2%	63.0%	64.3%	58.5%	59.2%	58.3%	60.3%	0.8%
$^{GG}Ub_{1-74}$	36.3%	36.9%	37.4%	33.8%	33.9%	34.5%	37.1%	36.7%	37.5%	36.0%	0.5%
$^{2xGG}Ub_{1-74}$	4.2%	4.3%	4.5%	3.0%	3.0%	1.1%	4.4%	4.2%	4.2%	3.7%	0.4%

Bio Rep – Biological Replicates, TR – Technical Replicates, Mean – average across all biological and technical replicates, SEM – standard error of the mean