Accessing deeper in-gel crosslinking with *in vivo* formaldehyde fixation and sequential reactions reveals intermolecular crosslinks for modeling the primed epichaperome.

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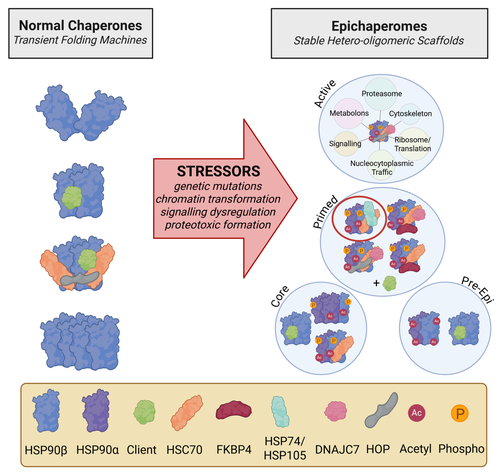
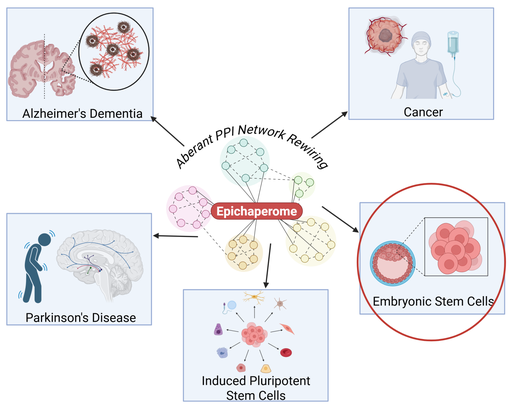
INTRODUCTION

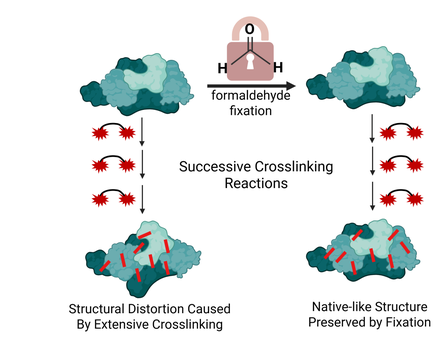


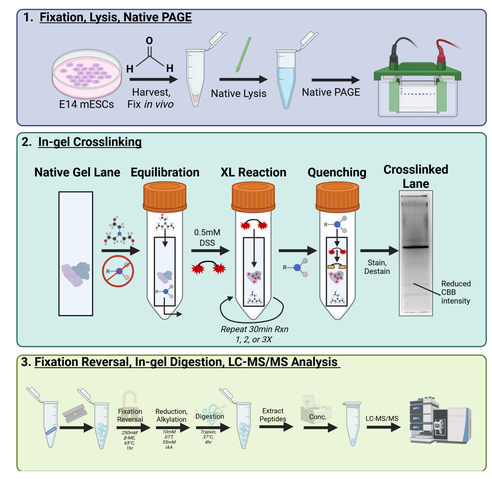


Intrinsic and conditional stressors drive epichaperome formation in stem cells, cancer, and neurodegenerative disease.

- **A)** Stressors induce stable, scaffold-like epichaperomes of HSP90, HSC70, HOP and other factors; HSP90 PTMs tune assembly by altering dimer closedness, client interactions and ATPase activity.
- **B)** Epichaperomes are abundant in cancer and stem cell lines, and found at significant levels in human tumors and neurodegenerative brain tissues.
- This study investigates a "primed" epichaperome (HSP90, HSC70 and HSP74) in mouse embryonic stem cells (mESCs).

METHODS



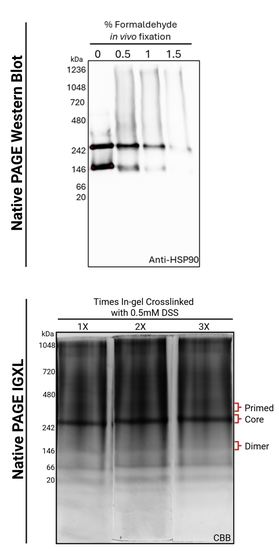


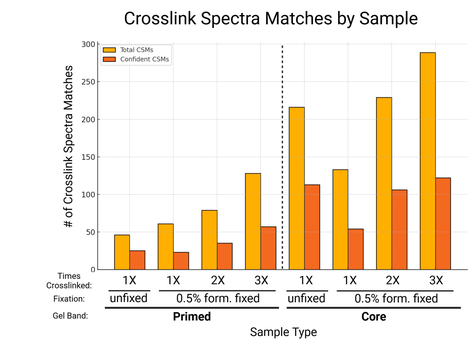
In vivo fixation stabilizes native-like structure throughout lysis, native electrophoretic separation, and in-gel crosslinking.

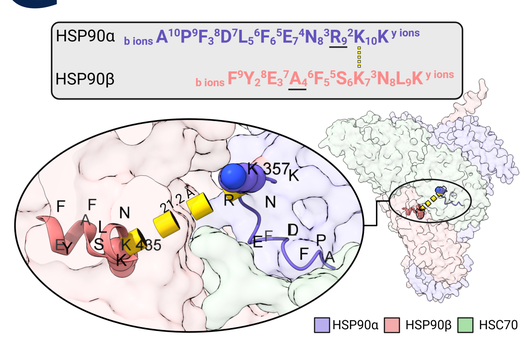
- **C)** Diagram of concept driving this investigation, pioneered by Schriemer et al.
- **D)** Experimental workflow:

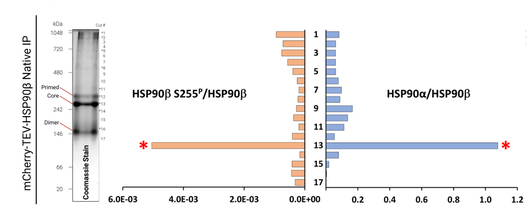
1. E14 mESCs were fixed *in vivo* with 0.5% formaldehyde, then cytoplasmic protein was extracted and separated on Native PAGE.
2. Gel lanes were equilibrated with XL buffer, then incubated in 0.5mM DSS in XL buffer/5% DMSO for 30/60/90 minutes, exchanging XL solution every 30 min.
3. The reaction was quenched, then lanes were stained. Bands from regions of interest were cut and diced, fixation was reversed, then samples were prepared for bottom-up analysis.

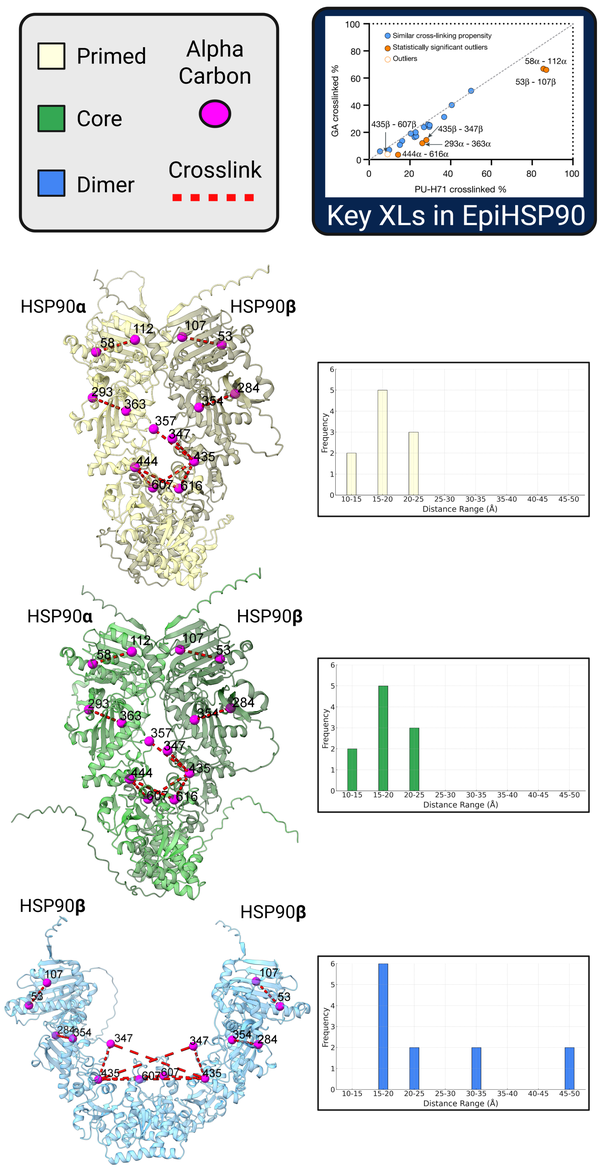
RESULTS

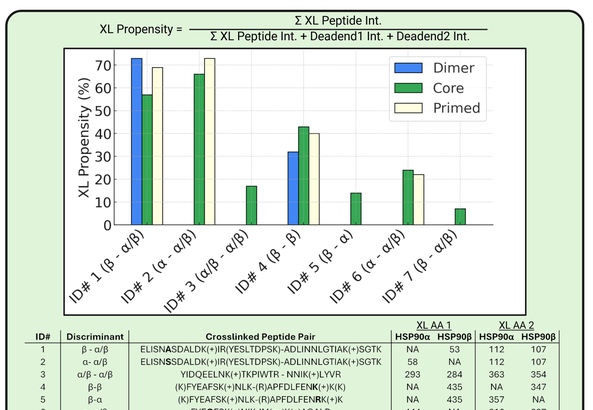




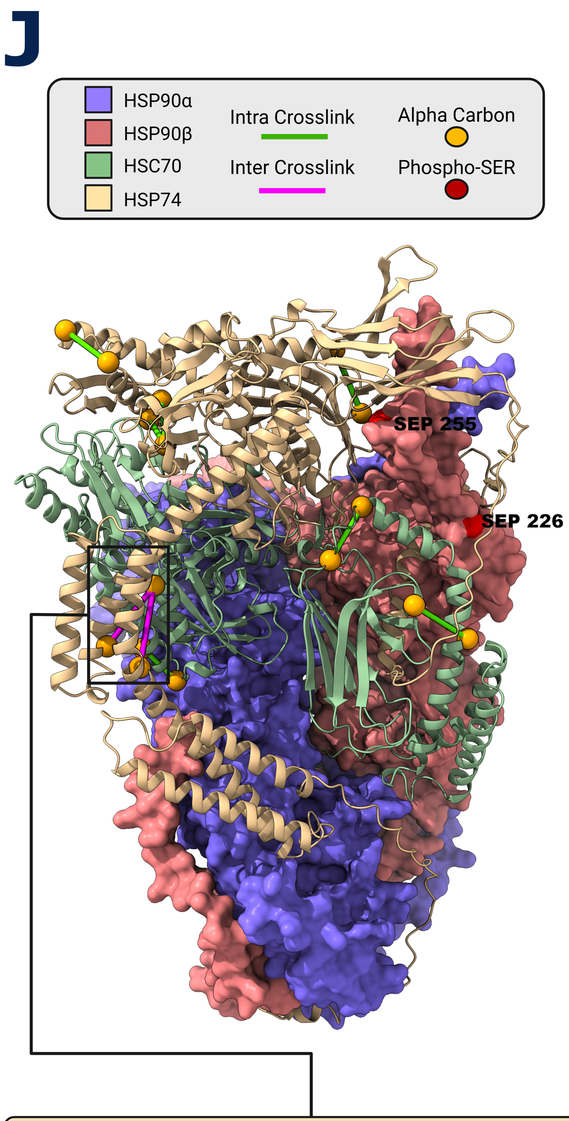


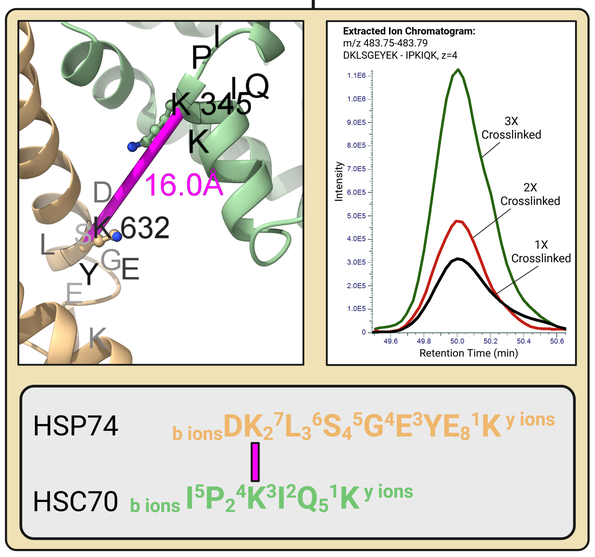






ID#	Discriminant	Crosslinked Peptide Pair	XL AA 1 HSP90α	XL AA 1 HSP90β	XL AA 2 HSP90α	XL AA 2 HSP90β
1	β - α/β	ELISN**A**SDALDK(+)IR(YESLTDPSK)-ADLINNLGTIAK(+)SGTK	NA	53	112	107
2	α- α/β	ELISN**S**SDALDK(+)IR(YESLTDPSK)-ADLINNLGTIAK(+)SGTK	58	NA	112	107
3	α/β - α/β	YIDQEELNK(+)TKPIWTR - NNIK(+)LYVR	293	284	363	354
4	β-β	(K)FYEAFSK(+)NLK-(R)APFDLFEN**K**(+)K(K)	NA	435	NA	347
5	β-α	(K)FYEAFSK(+)NLK-(R)APFDLFEN**R**K(+)K	NA	435	357	NA
6	α- α/β	FYE**Q**FSK(+)NIK-IM(ox)K(+)AQALR	444	NA	616	607
7	β - α/β	FYE**A**FSK(+)NLK-IM(ox)K(+)AQALR	NA	435	616	607





In vivo fixation and sequential in-gel crosslinking enables identification of novel intermolecular crosslinks that fit experimentally-informed AlphaFold3 model of a primed epichaperome.

- **E)** HSP90 immunoblot screening shows 0.5% formaldehyde as optimal condition (top), which was used for 1, 2, 3X IGXL experiment (bottom).
- **F)** Comparison of XL identification counts from unfixed and fixed IGXL of core and primed epichaperome bands of mESC lysate.
- **G)** HSP90α - HSP90β specific intermolecular crosslink identified in core band region of fixed and unfixed mESC lysate DSS IGXL.
- **H)** Label-free quantitation of mCherry-TEV-HSP90β native immunoprecipitate S255^{P} and HSP90 isoform ratio with discriminant isobaric peptide.
- **I)** Key epi-enriched HSP90 XLs (top right) detected by IGXL, mapped to predicted models of dimer, core, and primed epichaperomes (center) and calculation of their XL propensity in unfixed DSS IGXL (bottom).
- **J)** HSP74 and HSC70 crosslinks identified in fixed IGXL mapped to AF3-predicted primed epichaperome structure (Top; HSP90 intralinks not shown) with novel intermolecular XL shown (bottom left) and label-free quantitation of z=4 form compared between fixed 1, 2, 3X IGXL (right).

CONCLUSIONS

1. ***In vivo* fixation stabilizes complexes throughout lysis, separation, and crosslinking.**
2. **Sequential in-gel crosslinking reactions enhance XL intensity and identify new interlinks.**
3. **Identified crosslinks fit AF3 prediction for first experimental primed epichaperome model.**

KEY REFERENCES

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