Visualizing endogenous Nanog behavior using a novel nanobody-based toolkit

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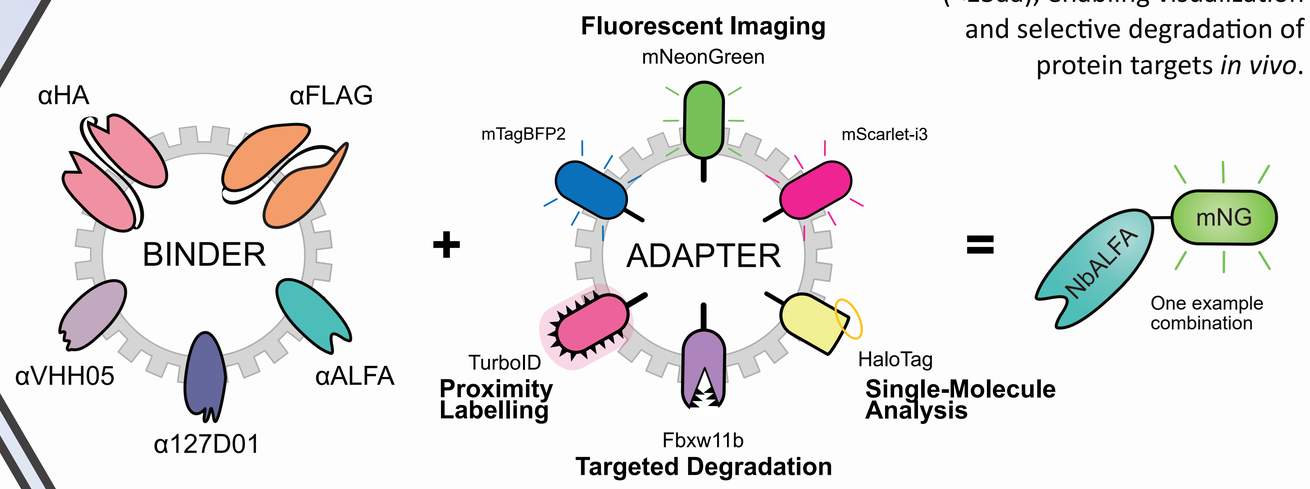
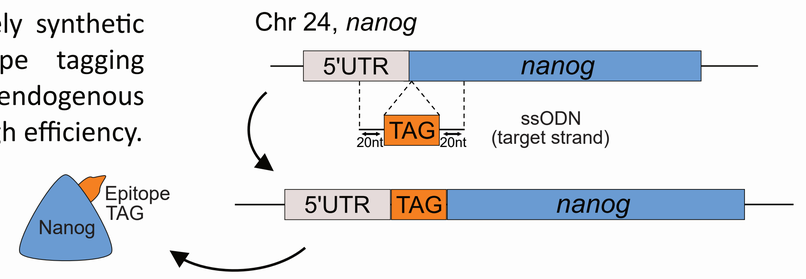
1. Genetically encoded affinity reagents (GEARs): A toolkit for visualizing and manipulating endogenous protein function in vivo.

Probing endogenous protein localization and function *in vivo* remains challenging due to laborious gene targeting and monofunctional alleles. To address this, we developed a multifunctional toolkit based on genetically encoded affinity reagents (GEARs). GEARs use nanobodies (Nb)[1-3] and single chain variable fragments (scFv)[4-5], which recognize small epitopes (<15aa), enabling visualization and selective degradation of protein targets *in vivo*.



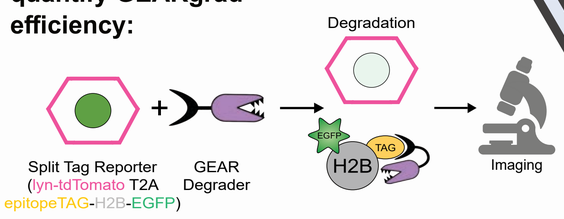
We also established a purely synthetic CRISPR/Cas9-based epitope tagging pipeline to generate endogenous knock-in alleles with high efficiency.

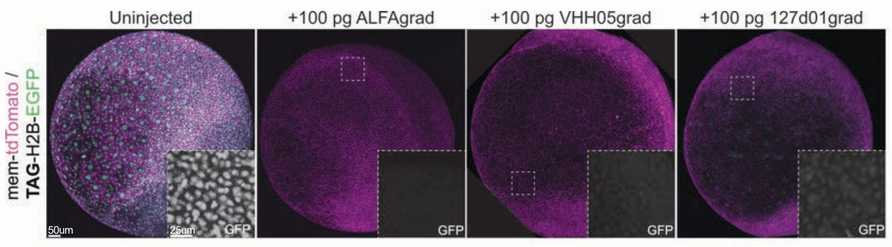


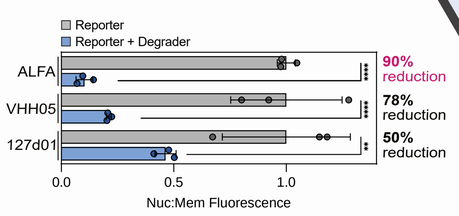
2. GEARs can bind and degrade protein targets with high efficiency and mimic loss-of-function mutation

Nanobody-based GEARs were fused to zebrafish Fbxw11b (based on zGrad system[6]) to generate degraders (GEARgrads), and tested for their performance at degrading fluorescent reporters, which were injected into 1-cell staged zebrafish embryos. ALFAgrad performed best with **90% clearance.**

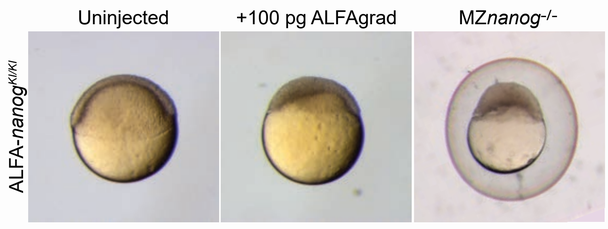
A bicystronic reporter was used to quantify GEARgrad efficiency:

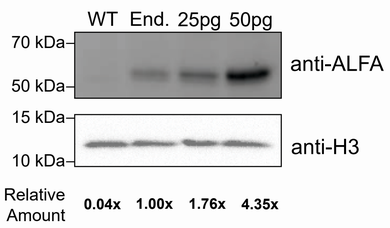






ALFAgrad injection into embryos homozygous for endogenously tagged ALFA-Nanog induced a **maternal-zygotic *nanog* loss-of-function phenotype**[7] in 100% of the embryos.



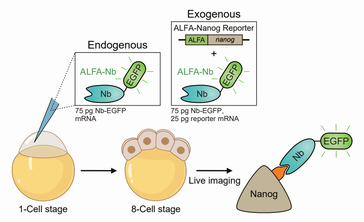


A western blot using anti ALFA antibody showed that the endogenous amount of ALFA-tagged Nanog was nearly half the amount relative to exogenous Nanog.

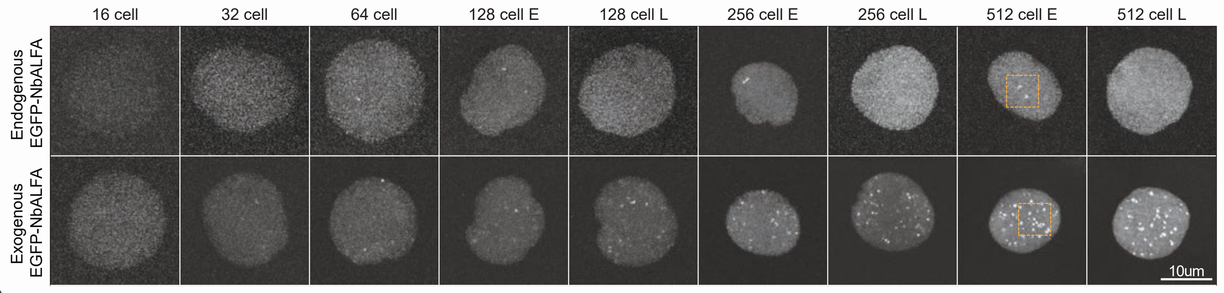
3. GEARs visualize the native behavior of Nanog during the earliest transcriptional events in zebrafish embryos

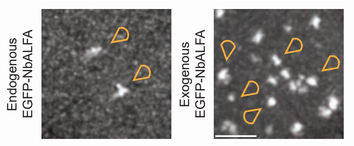
Using GEARs we investigated the spatiotemporal dynamics of endogenous Nanog protein. Nanog is a pioneer transcription factor that has intrinsically disordered regions that engage in concentration dependent interactions and exogenous fluorescently tagged Nanog localizes to subnuclear puncta during the maternal-to-zygotic transition[8-9]. While live imaging of exogenous Nanog has revealed important molecular behaviors[8-9], the localization of endogenous Nanog has not been studied due to the lack of appropriate tools.

Live imaging setup:

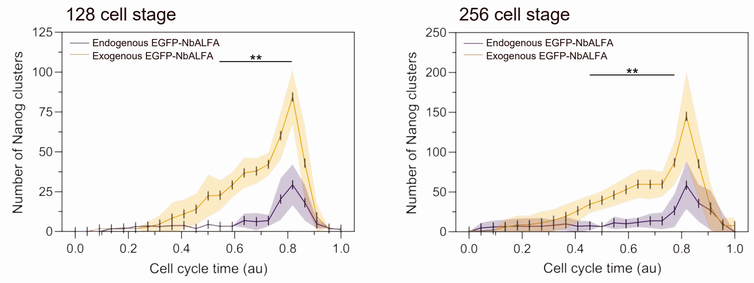


Endogenous vs exogenous Nanog visualization





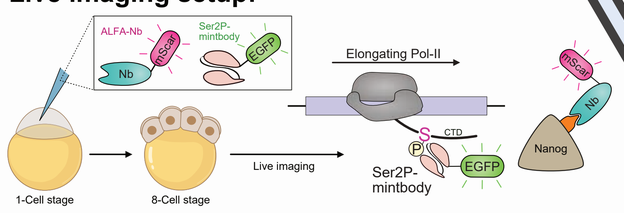
Endogenous Nanog protein forms significantly fewer foci then overexpressed Nanog.

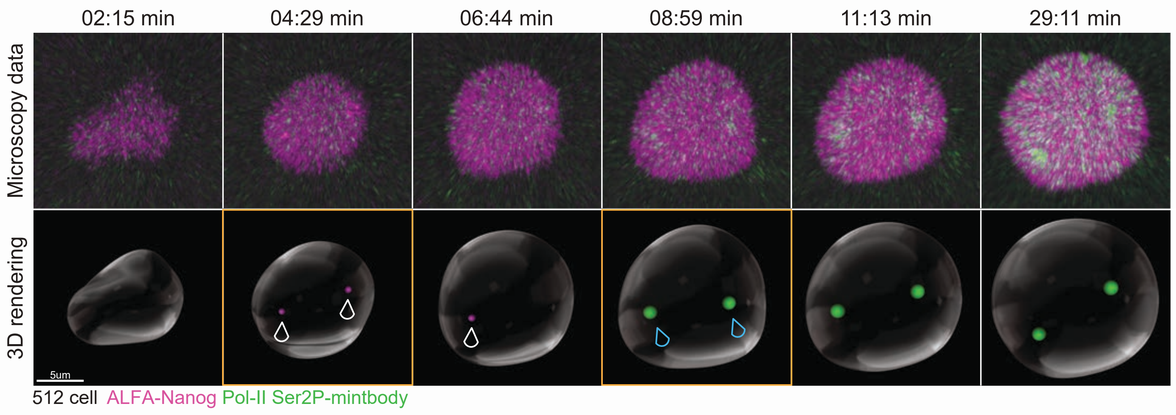


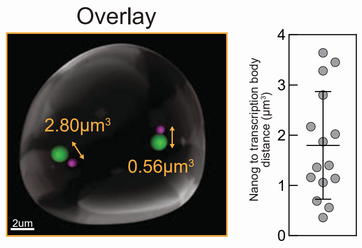
4. *Endogenous Nanog foci prime the formation of large transcription bodies*

Foci formation of both TFs and active transcription, in the form of transcription bodies, have been observed in a number of systems[8-12]. In zebrafish, the first transcription bodies are seeded at the miR430 locus[8-10].

Live imaging setup:







We used an mScarlet-I3-ALFA GEAR to detect Nanog protein, as well as a Pol-II-Ser2P[6] EGFP reporter (Mintbody detecting RNA Pol II phosphorylated on Serine 2)[13] to visualize transcriptional elongation.

Like exogenous studies, we observed that Nanog foci precede transcription bodies in close proximity but are rapidly dissolved while transcription bodies are long lived

5. Conclusions

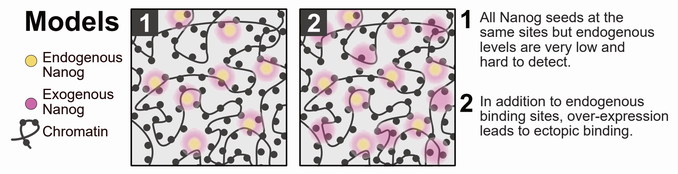
- GEARs are powerful and versatile tools to interrogate endogenous proteins. The short epitope tags required can be introduced into the genome with high efficiency.

- GEARs can be used to efficiently degrade proteins and furthermore target the pool of maternal proteins, enabling robust clearance of proteins that establish the earliest events of embryogenesis.

- ALFA-*nanog* knock-in fish report the physiological concentration of endogenously produced Nanog protein, which is found at much lower levels than previously appreciated.

- Large difference in Nanog foci number suggests that overexpressed Nanog can either seed new foci or enlarge existing foci , most likely aided by its IDRs.

Models



A little bit extra: GEARs function also in other organsims

ALFA-tag reporter mRNA was injected into one-cell stage mouse zygotes and the ALFAgrad in one of the two cells at the 2 cell stage, such that the uninjected cell acts as no degradation control. We saw robust clearance of nuclear EGFP signal in the degron injected side, with a clearance efficiency of 96%.

The GEARs toolkit provides an easy plug-and-play approach allowing users to generate endogenously tagged alleles with limitless versatility and custom control over protein function in vivo.

References

1 Götzke *et al.,* 2019 *Nat Comms.*; 2 Ling *et al.*, 2019 *Mol Immunol.*; 3 Bradley *et al.,* 2015 *Mol Pharmacol.*; 4 Zhao *et al.,* 2019 *Nat Comms.*; 5 Liu *et al.,* 2021 *Genes Cells.*; 6 Yamaguchi *et al.* 2019 *Elife*; 7 Gagnon *et al.* 2018 *Dev.*; 8 Kuznetsova *et al.,* 2023 Curr Biol.; 9 Pownall *et al.,* 2023 *Science*; 10 Hadzhiev *et al.,* 2023 Dev. Cell.; 11 Cissé *et al.,* 2013 *Science*; 12 Mir *et al.,* 2017 Genes Dev.; 13 Shibuta *et al.*, 2021 *Commun Biol.*

This work is supported by

EMBO HFSP International Human Frontier Science Program Organization

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Live imaging movies