Dysregulation of mutant ALK2: More than just loss of binding of inhibitory factors

Eleanor Williams[1], Rod Chalk[1], Alex N. Bullock[1]

[1] SGC, University of Oxford, Old Road Campus Research Building, Roosevelt Drive, Oxford, OX3 7DQ, UK

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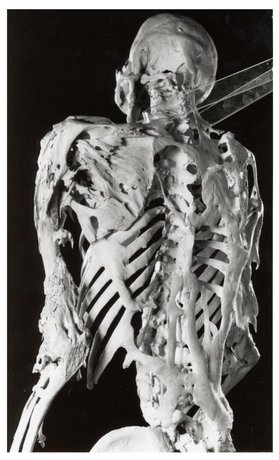
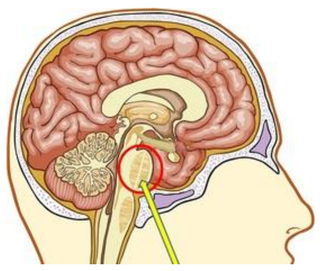
1. Gain of Function Mutations in ALK2

Gain of function (GOF) mutations in the intracellular domain of ALK2 are linked to two rare genetic conditions. Fybrodisplasia Ossificans Progressiva (FOP) and Diffuse Intrinsic Pontine Glioma (DIPG).

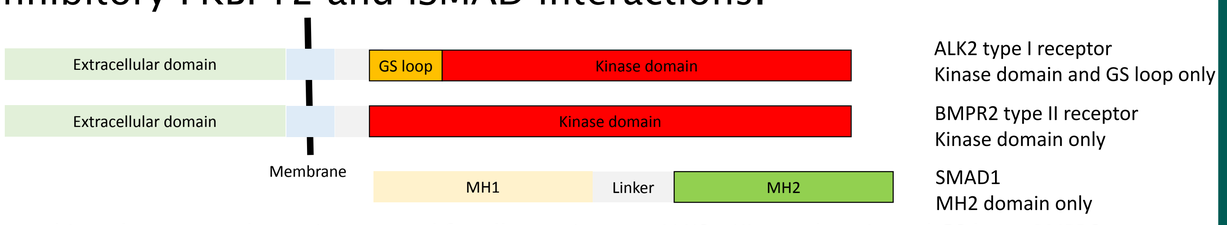


FOP is caused by a germline mutation leading to uncontrolled bone formation in muscle. This ossification occurs in 'flair up episodes' which are often in response to an inflammatory response from physical trauma or infection.



DIPG is driven by a histone H3K27M mutation but 25% of cases also show a GOF mutation in Alk2.

GOF mutations in ALK2 show activin neofunction, however the mechanism is unclear given the intracellular location of the mutations. It has been theorised that this was due to loss of inhibitory FKBP12 and iSMAD interactions.

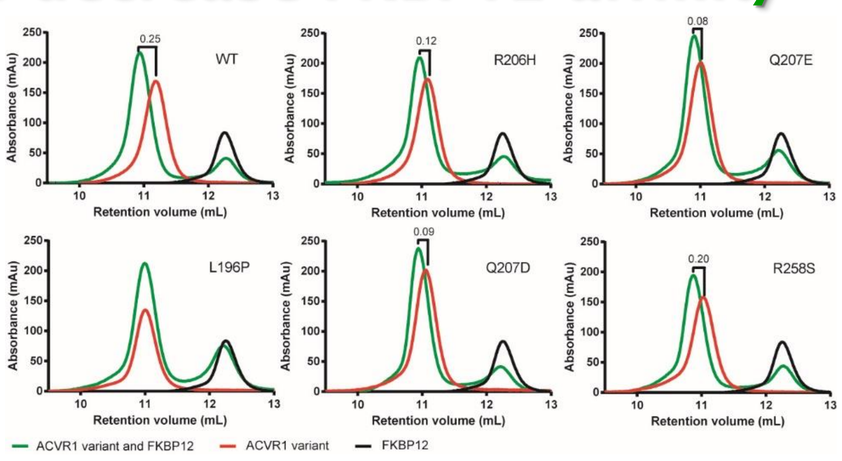


Recombinantly expressed protein was used for all experiments: ALK2 - Kinase domain and GS loop. BMPR2 - Kinase domain. SMAD1 - MH2 domain. Proteins were incubated together under optimised conditions in the presence of 0.5mM ATP, 5mM Mn^{2+} and 2mM Mg^{2+} to measure activity.

Using in vitro studies on purified recombinant protein, I show that there is an additional kinase activation independent of these factors.

2. Mutations decrease FKBP12 affinity for ALK2

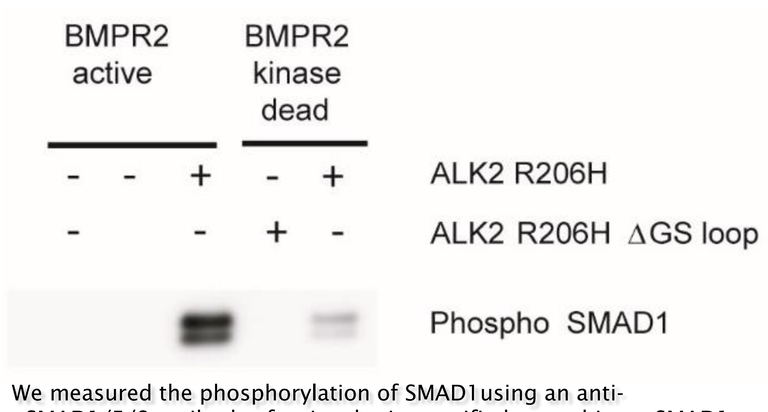
Mutations in ALK2 can disrupt FKBP12/ALK2 complex formation. L196P disrupts binding completely. R206H, Q207E, Q207D and R258S only partially disrupt complex formation



Analytical gel filtration shows the elution peaks of the ALK2/FKBP12 complex compared to the individual components

3. SMAD activation requires the GS domain in ALK2 and the presence of a type II receptor

The GS domain was shown to be essential for SMAD1 activation
The presence of a type II was essential for SMAD1 activation
Active type II receptor enhanced activity but was not essential

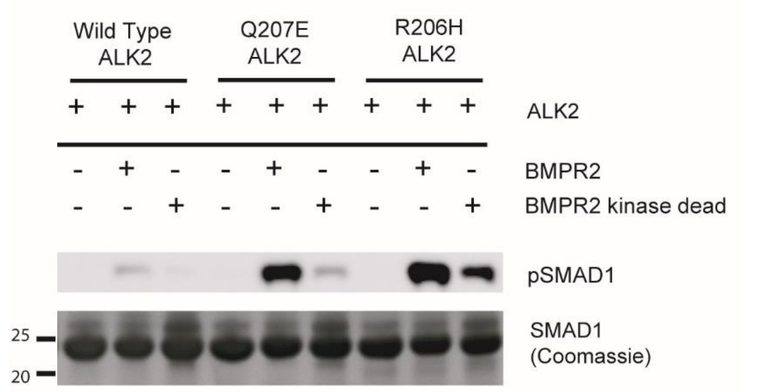


We measured the phosphorylation of SMAD1using an anti-pSMAD1/5/8 antibody after incubating purified recombinant SMAD1 with ALK2, ALK2ΔGS, and either BMPR2 or BMPR2kd

4. ALK2 mutations lead to increased SMAD1 activation

ALK2 mutants led to increased pSMAD with the $ALK2^{R206H}$ being more activating than the $ALK2^{Q207E}$

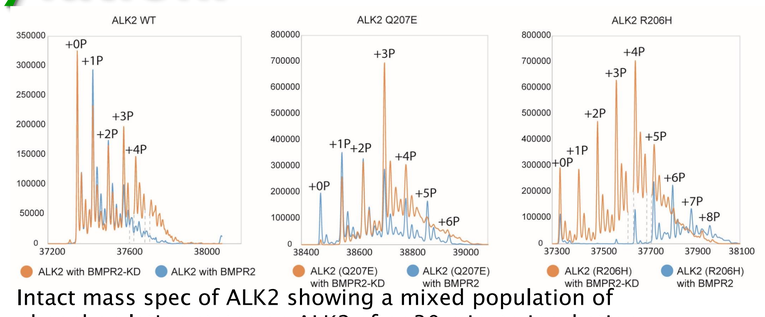
Mutations also led to increased number of SMAD1 phosphorylation sites and an increased occupation of higher phosphorylation states compared to WT, with a third site only observed in the presence of ALK2 mutants.



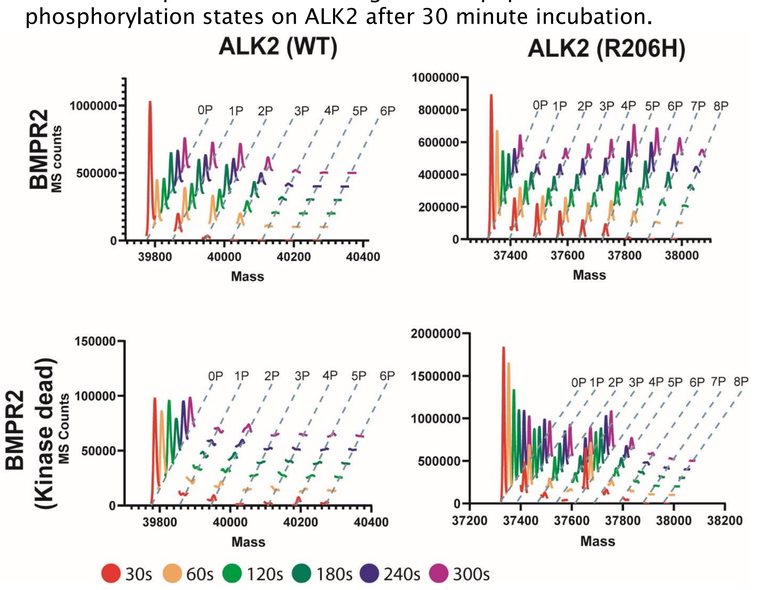
pSMAD western showing phosphorylation of SMAD1 in the presence of ALK2 (WT/Q207E/R206H) and type II receptor as shown

5. Mutations in ALK2 lead to increased ALK2 phosphorylation.

- ALK2 mutants led to increased total pALK2 vs. wild type with the $ALK2^{R206H}$ being most activating and then $ALK2^{Q207E}$
- ALK2 mutants also led to increased rate of phosphorylation when compared by mass spec over time.
- ALK2 with BMPR2 shows a higher number phosphorylation sites compared to a kinase dead BMPR2. This suggests a distinct difference between auto and trans phosphorylation mechanisms



Intact mass spec of ALK2 showing a mixed population of phosphorylation states on ALK2 after 30 minute incubation.



Intact mass spec of ALK2 timecourse experiment over 5 minutes showing increased rate of phosphorylation sites in $ALK2^{R206H}$ compared to $ALK2^{WT}$. Top: experiment contained active BMPR2. Bottom: experiment contained kinase dead BMPR2A.

6. Auto vs. trans –phosphorylation of ALK2 impacts SMAD activationBMPR2

Phosphomapping revealed patterns of phosphorylation on ALK2 dependent on whether active or kinase dead type II receptor was used

	GS Loop	SMAD tail
ALK2	LLDHSCTSGSGSGLPFLVQR	-ISSVS
ALK2 + BMPR2	LLDHSCTSGSGSGLPFLVQR	-ISSVS
ALK2 + BMPR2-KD	LLDHSCTSGSGSGLPFLVQR	-ISSVS
ALK2 (R206H) + BMPR2	LLDHSCTSGSGSGLPFLVQR	-ISSVS
ALK2 (R206H) + BMPR2-KD	LLDHSCTSGSGSGLPFLVQR	-ISSVS
ALK2 (Q207E) + BMPR2	LLDHSCTSGSGSGLPFLVQR	-ISSVS

MS/MS phosphomapping was done on incubations of ALK2 (WT and mutants) in the presence of active or kinase dead BMPR2A. Peptide coverage was around 83% using unenriched and Ti/Zr enrichment strategies and five different proteases.

- Auto-phosphorylation of Ser^{187} and Thr^{189} in DHSCT by ALK2 allowed activation of SMAD1to a lesser degree as long as the type II receptor was present suggesting a structural link.
- Transphosphorylation of the GS loop Serines in SGSGSG by the active type II receptor allows strong activation of SMAD1

7. Mutations in ALK2 cause loss of inhibition and increase basal ALK2 activity

- ALK2 mutations reduce the affinity of the inhibitor protein FKBP12 – the degree of disruption is dependent on the mutation.
- ALK2 mutations increase the rate and the number of phosphorylation of SMAD1 and of ALK2 by both trans and auto-phosphorylation
- An active type II receptor is required for full SMAD1 activation via GS loop phosphorylation.
- Kinase dead type II receptor supports only weak SMAD1 phosphorylation by ALK2, potentially arising from a scaffolding role.

FOP/DIPG mutations reduce inhibitory protein interactions, but also increase kinase activity including more rapid GS loop phosphorylation by the type II receptor.